**Effect of Pretreatments on Physicochemical Characteristics of Olive Pomace and on Production of Cellulases from *Trichoderma reesei* RUT C30 Under solid-state fermentation**

***Running Title*: *Trichoderma* cellulases production from olive pomace**

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**Highlights:**

• Olive pomace solid waste is considered as a natural medium for fungi growth.

• Pretreatments were used for improvement of cellulases production from olive pomace.

• Effects of pretreatments on chemical composition and fibers have also been studied.

• Cellulases production was improved with alkaline pretreatment (1% NaOH).

• Milling and thermal pretreatments did not increase cellulases activity.

**Abstract**

Olive pomace (OP) is a cheap and abundant agricultural by-product generated after olive oil extraction that is valorized by different biotechnological processes. The present study contributes to a better understanding of the effects of alkaline, milling and thermal pretreatments on OP for obtaining high value-added products (Cellulases). *Trichoderma reesei* RUT C-30 fungus was used for cellulases production on OP substrate under Solid-State Fermentation (SSF) process and cellulases activity was assessed by the filter paper method (*FPAse*). The effect of the three pretreatments and their combinations on physico-chemical composition and cellulases production was investigated. The results showed that untreated olive pomace, without nutrient medium addition, was a favorable environment for the growth of *Trichoderma reesei RUT C-30* and a good fermentation substrate that gave a *FPAse* activity of 0.83 UI/gds. Thechemical composition (lipids, proteins, carbohydrates and ash) was significantly *(P<0.05)* affected by the different pretreatments as well as their combinations. Regarding the fiber fraction, a remarkable decrease was recorded after only milling pretreatment. The alkaline pretreatment (1%NaOH) of olive pomace improved the cellulase activity to a value of 1.28UI/gds, while, lower yields were obtained after milling (0.2UI/gds) and thermal (0.15UI/gds) pretreatments.

**Keywords**: Olive pomace, *Trichoderma reesei*, Pretreatments, Cellulases, Solid-State Fermentation.

**INTRODUCTION**

 The world production of olive oil is valued to be more than 18 million tones/year **(Coimbra *et al.*, 2010)** and Algeria covers more than 1.5% of this production **(Stamatelatou *et al.*, 2012).** 42% of the national production of olive oil is located in the central region: Béjaia, Bouira, TiziOuzou and Jijel **(Rives, 2021)**. However, olive oil extraction process produce great amount of wastes. According to **Nefzaoui (1991)**, 100 kg of olives produced about 35kg of crude olive pomace and 100 liters of vegetation water. Depending on the extraction method, olive pomace (OP) can reach up to 30-40% of olive oil production **(Aliakbarian *et al*., 2011).** Olive pomace by-product contains fragments of skin, pulp, stones and oil (Mirabella *et al.,* 2014). OP is composed of lignin (31%), hemicellulose (24%), cellulose (14%), fat (11%), soluble sugars (6.5%), protein (6%) and many mineral salts **(Roig *et al*., 2006).** This physico-chemical composition of OP depends principally on the type and origin of olives, environmental conditions and storage time **(Nefzaoui, 1985 ; Papaioannou *et al*., 2013).**

 Several studies have proven the negative effects of this solid by-product on the microbial flora of the soil and even on the aerial environment. **(Aranda *et al.,* 2007)**. Therefore, it is important to manage these wastes in order to minimize their negative effects on the environment.In fact, olive pomace contains valuable raw material such as a great proportion of organic matter and a varied range of nutrients, which could be used for energy generation, as an animal feed or as a fermentation substrate in biotechnological means for bio-fuels or enzymes production **(Roig *et al.*, 2006; Roussos *et al.*, 2009).** Still, some obstacles are associated with effective utilization of lignocellulosic residues for enzymes production. The main constraint is the recalcitrance of the plant cell walls of the lignocellulosic fractions **(Kumar and Sharma, 2017).**

 Several methods have been used to develop effective and low cost pretreatments as potential ways of altering the structure and improving the degradability of lignocellulose biomass **(Hendriks and Zeeman, 2009).** In literature, several pretreatments were described taking into account several aspects: (1) mechanisms concerned (2) advantages and disadvantages and (3) economic valuation **(Menon and Rao, 2012).** According to the **National Research Council (1999),** an effective pretreatment must preserve the hemicellulose fraction, minimize the production of growth inhibitors of fermentation microorganisms and reduce energy costs. Different pretreatments have been investigated on olive solid wastes, including physical **(Neifar *et al*., 2013; Leite *et al.,* 2016),** chemical **(López-Linares *et al*., 2013; Pellera *et al.,* 2016; Erdocia *et al.,* 2017)**, thermal **(Fernández-Bolaños *et al*., 2001; Aliakbarian *et al*., 2011)** and biological **(Haddadin *et al*., 2009)** methods and various combinations thereof **(El-Ghonemy *et al*., 2014; Ouyang *et al.* 2018).** Physical pretreatment means include mechanical deterioration and irradiation that lead to structural disruption and reduction of the particle size , degree of polymerization and crystallinity of the raw material **(Cara *et al.*, 2007; Ravindran and Jaiswal, 2016),** which increases the enzymatic digestibility of cellulose and hemicelluloses in the lignocellulosic biomass **(Mtui, 2009).** Chemical pretreatments are performed using alkalis (NaOH, Ammonia), acids (H2SO4), oxidants (Ozone, Oxygen, H2O2), organic solvents (Alcohols, Organic acids) or ionic liquids (Organic salts) **(Gandla *et al.,* 2018).** Chemical pretreatments favors hydrolysis of lignocellulosic biomass by eliminating hemicelluloses, disrupting lignin or reducing the crystallinity of cellulose during processing **(Mosier *et al.*, 2005; Zheng *et al.,* 2009)**. Among these, alkali has been most extensively investigated**.** The use of alkali causes the degradation of ester and glycosidic side chains causing in structural modification of lignin fraction, separation of structural bonds between lignin and carbohydrates, cellulose swelling and its partial decrystallinization, in addition to a dissolution of hemicellulose **(Zheng *et al.,* 2009; Brodeur *et al.,* 2011)**. Thermal pretreatments are effectively used on an industrial scale for lignocellulosic residue processing: hydrothermal , steam-explosion and hydro-chemical pretreatments are attested to cause elimination of hemicelluloses without being hydrolyzed, re-localization of lignin and hydratation of cellulose, and at the same time, swelling the pore size of the fibers which facilitate the enzyme accessibility **(Gandla *et al.,* 2018).** In biological pretreatments, lignocellulosic degrading fungi are used to reduce the lignin barrier from the biomass prior to fermentation. Although, this pretreatment is only significant if combined with other pretreatments **(Vasco-Correa *et al.,* 2016).**

 Filamentous fungi species are recognized for their high aptitude to secrete large amount of enzymes into their environment, making them very interesting for industrial enzyme production **(Gudynaite-Savitch and White, 2016; Srivastava *et al.,* 2018).** *Trichoderma reesei* is the most used fungus in enzyme industry, particularly for cellulases **(Jun *et al*., 2011).** *Trichoderma* cellulases are presently used in many industries such as textile, food, biofuel production, agriculture, animal feed, paper and pulp industries **(Linke *et al.,* 2015 ; Imran *et al*., 2016).** Cellulases enzymes catalyze the bioconversion of cellulose into fermentable sugars. Cellulases complex are formed of three types of enzymes: endo-1,4-β-D-glucanase, exo-1,4-β-D-glucanase and β-glucosidase **(Paloheimo *et al.,* 2016).** The production of cellulolytic enzymesby *T. reesei* has been the subject of various studies using different substrates **(Belal, 2013; Pirota *et al.,* 2014; Abdullah *et al*., 2016).** Among the several mutants of *T. reesei,*

 *T. reesei RUT-C30* is known to be one of the best producing cellulolytic strain studied **(Aftab and Vermette, 2008 ; Sun *et al.*, 2008 ; Dhillon *et al*., 2011).**

 Solid-state fermentation (SSF) process has been used for the cultivation of filamentous fungi because it simulate their living conditions in their natural habitat **(Ugwuanyi *et al*., 2009; Ray and Behera, 2017).** This process includes absence or near absence of free water. The SSF is an attractive way to produce cellulases from microorganisms because of its lower capital cost investment, simpler equipment and higher productivity **(Ray and Behera, 2017; Soccol *et al*., 2017).**

 The use of olive pomace as substrate in fermentation processes for cellulases production requires pretreatments due to the heterogeneity and complexity of this lignocellulosic biomass. Within this context, several studies have been previously carried out but, to our knowledge, no study has included at the same time, three pretreatments with their combinations, and provides elements of answer to their effects on the physicochemical parameters, the fiber fraction and the kinetics of cellulases production. Our work aimed to contribute in the field of biotechnological valorization of OP biomass from Jijel region known by its high production of olive oil in Northern Algeria, as natural medium for cellulases production using *Trichoderma reesei* RUT-C30 as producing fungus. There are numerous pretreatments that can be used for lignocellulosic valorization, but among these, the pretreatments chosen in this study are the most frequent in literature. Three major pretreatments are applied, namely alkaline pretreatment using different concentrations of NaOH (1%, 3%, 5% and 7%), mechanical milling and thermal pretreatment.

**Material and methods**

**Substrate**

The vegetal material used in this study was olive pomace provided by oil factory located in Jijel region (Northern Algeria). After the pressing operation, fresh samples were immediately collected, transported to the laboratory and kept at -20°C for further analysis.

**Pretreatments**

**Mechanical pretreatment (milling**)

 Olive pomace samples were pretreated according to the method of Haddadin *et al.* (2009). Samples were dried in an oven at 65°C for 48h.The samples obtained after drying were reduced to fine particles using an industrial mill with three-phase motor, and then separated through a sieve of 1.25 mm porosity. The fine powder was recuperated and stored at -20°C for further use.

**Alkali pretreatment**

The OP was pretreated with different concentrations (1%, 3%, 5% and 7%) of NaOH at room temperature according to **Bansal *et al.* (2012).** 20g of substrate was suspended in 100mL of NaOH solution at adequate concentration in Erlenmeyer flasks, and left at room temperature for 2h. The recuperated residues were carefully washed with distillated water to neutral pH and then dried at 65°C.

**Thermal pretreatment**

 Aliquots of 20 g of each OP sample were boiled in 100mL of distilled water for 2h on hotplates in thermo resistant flasks. After cooling, the residues were recuperated and oven dried at 65°C.

**Physicochemical characterization of olive pomace**

The pH of samples was measured according to **Haddadin *et al.* (2009)** using the OP extracts obtained by mixing 5g of OP with 50mL of distilled water. OP samples were dried at 105°C to determine dry matter (DM) and moisture content. The ash content was assessed in a muffle furnace (Nabertherm GmbH, Germany) at 550°C. Total nitrogen was analyzed using the Kjeldahl method. The crude protein contents of substrates were determined as described by **(AOAC, 1990).** Lipid content of the OP was determined by using the SoxtecTM 2043 (FOSS, Sweden) apparatus. NDF, ADF, and ADL were evaluated using Fibertec 2010 (FOSS, Sweden) apparatus and **Van Soest and Robertson (1979)** method. The total carbohydrates content was determined by **Dubois *et al*. (1956)** method. Reducing sugars content was determined by **Miller (1959)** method. All measurements were done in triplicate.

**Fungal strain and spore suspension preparation**

The fungal strain *Trichoderma reesei* RUT C30 was provided by the Industrial Microbiology Laboratory of the University of Reims Champagne-Ardenne (France). Suspension of spore was prepared by incubating the cultures of fungus on PDA plates at 30° C for about 5 or 7 days. The spores were collected with 10 mL of sterile water containing 0.1% (v/v) of tween 80 and the prepared suspension was adjusted to a concentration of approximately 3 x 107 spores/mL.

**Enzymes production under solid-state fermentation**

The solid fermentation was carried out in 250 ml Erlenmeyer containing 5 g of substrates of fresh or pretreated OP and then humidified by distilled water to a proportion of 1:1 (w/v) taking into account the initial moisture of each substrate. The preparations were sterilized at 121° C for 20 min. Once cooled, the content flasks were inoculated withspore suspension previously prepared (3×107 spores/mL) then incubated at a temperature of 30°C for 6, 12, 18, 24 and 30 days under static conditions.

**Extraction of crude enzyme**

The enzymes were extracted by mixing the fermented OP with 50 mL of distilled water and homogenized by UltraTurrax (IKA, T25digital, Germany) for 1min. The homogenates were centrifuged at a speed of 85,000g for 20 min at 4°C and the supernatants of crude extracts were recovered for cellulases enzymatic assay.

**Determination of total cellulase activity using filter paper (*FPase* activity)**

Cellulase activity against Whatman N°1 filter paper (W1FP) was measured as described by Silveira ***et al.* (2014)**. *FPase* activity was determined by mixing 0.5mL of citrate-phosphate buffer (0.05M, pH 4.8) with 0.5mL of the enzyme extract. After 10min at 50°C, the W1FP strips, each weighing approximately 50mg (1.0×6.0 cm), were added to the test tubes. The assay mixture was incubated in a water bath at 50°C for 60 min. The reducing sugars released after the enzymatic reaction were assayed by 1.5 ml of DNS reagent and the absorbance was read at 540 nm by a UV/visible spectrophotometer (Agilent Technologies Cary 60 UV-Vis, Germany) after placing the tubes in a boiling water bath for 5 min and adding 10 ml of distilled water.

 A standard curve of D-glucose was used as reference. The cellulolytic activity was expressed by the international unit, corresponding to one micromole of glucose released per minute and per ml of enzymatic extract under the assay conditions.

**Soluble proteins**

The amount of soluble proteins in the crude enzymatic extract was quantified according to the method of (Bradford, 1976). The concentration of the enzymes was determined by a calibration curve, bovine serum albumin (BSA) (VWR Chemicals Prolabo, Oud-Heverlee, Belgium) was used as standard. Coomassie brilliant blue reagent (Biochem Chemopharma, Cosne/Loire, France) was used as dye.

**Statistical analysis**

 The different results of the physico-chemical analyzes obtained are the average of three repetition expressed in the form of mean ± standard deviation. They are treated by the analysis of variance (ANOVA) followed by a multiple comparison of the means. The software (STATISTICA) version 5.5 was used for this purpose.

**RESULTS AND DISCUSSION**

**Alkaline pretreatment**

**Effect of alkali pretreatment on the chemical composition of olive pomace**

 Alkaline pretreatment effects on the composition of OP are shown in Table 1. Untreated OP can be considered as a rich nutrient substrate, it presents 37.64% moisture, 7.68% lipids, 2.68% proteins, 0.86% carbohydrates and an appreciable amount of fibers which attains 48.86% including 11.67% cellulose. Significant differences *(p<0.05)* between the physicochemical parameters of untreated and pretreated OP with different concentrations of NaOH was observed. After alkaline pretreatments, pH values had increased from 6.38 to 7.75 with the increase of NaOH concentration, this could be due to the residual quantities of NaOH in the OP even after washing with distilled water. The moisture content in the pretreated samples has decreased by about 93% for 1% NaOH and by almost 97% for NaOH concentrations above 3%, this can be attributed to the drying at 65°C of the OP during the preparation process. A significant increase in ash content is noticed, this increase is due to the residual NaOH related to the increasing dosage of the alkaline pretreatment from 1% to 7%. The alkali pretreatment affects also significantly the chemical composition of OP; it has caused diminutions in lipid, protein and carbohydrates contents of olive residues, in addition to a total loss of reducing sugars (Tab 1). The diminution in lipid content of the OP can be explained by the saponification process. The loss of carbohydrates is mainly caused by peeling and hydrolytic reactions **(Hendriks and Zeeman, 2009).**

**Table 1.** Chemical composition of OP after alkaline pretreatment at 1%, 3%, 5% and 7% NaOH (% Dry Matter).

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Parameter | Untreated OP | 1% NaOH | 3% NaOH | 5% NaOH | 7% NaOH |
| Moisture | 37.64 ± 1.47a | 2.51 ± 0.94b | 0.69 ± 0.55c | 0.83 ± 0.72c | 0.91 ± 0.72c |
| Dry Matter | 62.36 ± 1.47c | 97.49 ± 0.94b | 99.31 ± 0.55a | 99.17 ± 0.72ab | 99.09 ± 0.72ab |
| Ash | 0.70 ± 0.05e | 1.11 ± 0.02d | 1.25 ± 0.10c | 1.51 ± 0.07b | 1.68 ± 0.10a |
| Lipids | 7.68 ± 0.44a | 1.48 ± 0.15b | ND | ND | ND |
| Total Nitrogen | 0.43 ± 0.03a | 0.25 ± 0.01c | 0.32 ± 0.07bc | 0.37 ± 0.04b | 0.32 ± 0.03bc |
| Proteins | 2.68 ± 0.18a | 1.56 ± 0.01c | 2.00 ± 0.46bc | 2.31 ± 0.27b | 2.02 ± 0.19bc |
| Total carbohydrates | 0.86 ± 0.10a | 0.21 ± 0.01b | 0.23 ± 0.01b | 0.22 ± 0.03b | 0.18 ± 0.01b |
| Reducing sugars | 0.68 ± 0.08 | ND | ND | ND | ND |

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

*ND: not detected.*

 In order to better demonstrate the effect of alkaline pretreatment on fiber contents, the NDF, ADF, ADL, cellulose and hemicellulose contents were calculated taking into account the dry matter of untreated olive pomace **(Figure 1)**. It is important to note that NDF comprises lignin, cellulose and hemicellulose, ADF includes lignin and cellulose, while ADL represents only the lignin content. Alkaline pretreatment had no effect on NDF content. Unlike our findings, **Pellera et al. (2016)** found a significant increase of NDF content of OP after alkaline pretreatment with different concentrations of NaOH (1-16%). It can also be seen that ADF and cellulose contents decreased slightly but significantly. The decrease of ADF is mainly the consequence of cellulose diminution after pretreatment. **Rerat (1956)** reported that the alkaline pretreatment of lignocellulosic biomass with 1.25% NaOH resulted in a slight extraction of the cellulose. **Galbe and Zacchi (2007)** explained also the diminution in cellulose content after alkaline pretreatment by the state of swelling which causes an increase in the internal surface area and a decrease in the degree of polymerization and cellulose crystallinity. On the other hand, a prominent increase of more than 60% of cellulose fraction was reported by **Rodríguez-Zúñiga *et al*. (2015)** after alkaline pretreatment (4% NaOH) of sugarcane bagasse substrate. Regarding the ADL fraction, no clear variation was noticed after alkaline pretreatment. Similar result was observed in **Aguilera *et al.* (1986)** study on OP treated with NaOH. The

thermochemical degradation of olive cake follows a complex mechanism **(Ducom *et al*.2019)**. Accordingly, alkaline reagents are less effective for deligniﬁcation of woody biomass (like olive stones) as reported by **Kim *et al*. (2016).** It is interesting to highlight that the variation of NaOH dosage has, in general, no significant effect on the fibers composition, except for the 7% concentration. It appears that the high level of fiber contents in OP samples (58.74%, 45% and 33.33% respectively for NDF, ADF and ADL) could prevent the NaOH effect. **Kumar *et al*. (2009)** reported that the effect of alkaline pretreatment depends on the lignin content of the materials. Moreover, **Rabemanolontsoa and Saka (2016)** explain that the digestibility of alkali-pretreated biomass is reported to be inversely proportional to the lignin content. From the above, the efficiency of alkaline pretreatment depends mainly on the composition of the lignocellulosic substrate and the experimental conditions **(Zheng *et al.*, 2009).** Regarding the hemicellulose fraction, a significant increase was observed after alkaline pretreatments, this could be explained by the decrease of ADF (cellulose and lignin contents) since NDF remained unchanged after pretreatment.

*Vertical bars indicate standard error of three replicates*

*Different letters for the same parameter (a-c) indicate significant differences (p<0.05)*

**Figure 1**. Effect of alkaline pretreatment (1, 3, 5 and 7% NaOH) on fiber composition of OP

**Effect of alkaline pretreatment on the *FPase* activity**

 The different pretreatments used in this study were designed to improve cellulase production by increasing the accessibility of *T. reesei RUT C30* fungi to the cellulosic fraction of the substrate. Alkaline pretreatment is often viewed as a mean to alter the originally complex and recalcitrant chemical structure of lignocellulosic substrates **(Yoon *et al.,* 2014).** It is worth noting that olive pomace used in this study was only moistened with distilled water, without nutrient medium addition for both untreated and pretreated OP. Despite this, rapid and remarkable growth of the mycelium of *T. reesei RUT C30* was observed from the third day of fermentation for untreated and pretreated OP with 1% NaOH compared to those pretreated with 3%, 5% and 7%. As previously mentioned in Tab 1, olive pomace could be considered as an interesting fermentation substrate for the growth of fungi, given its diversified nutrient composition. In fact, the best solid substrate should provide all the necessary nutrients to the growing microorganism for optimal function **(Bansal *et al*. 2012).** As shown in Figure 2 (a), an improvement of the *FPase* activity was obtained after the alkaline pretreatment from 0.83U/gds to 1.28U/gds, respectively for the untreated and the pretreated by 1% NaOH samples. In contrast, for the concentrations of 3%, 5% and 7% NaOH, there was a decrease of cellulolytic activities (0.51U/gds, 0.74U/gds and 0.33U/gds, respectively). **Bali *et al*. (2015),** in a comparison of various alkaline pretreatments, demonstrated that the highest increase in cellulose accessibility was found with dilute NaOH solution (2%). Moreover, **El-Ghonemy *et al*. (2014)** found that treated substrates by 1% NaOH were much more efficient on enzymatic hydrolysis compared to that treated by 4% NaOH. The same trend was reported also by **Rodríguez-Zúñiga *et al*. (2015).** In another study, **Sun *et al. (*2008)** reported that the highest *FPase* activity was found on alkali-treated rice straw compared to the untreated one using *T. reesei* Rut C-30 fungi. The improvement of cellulolytic activity after pretreatment with 1% NaOH, may be due to the swelling of the biomass that becomes more accessible for enzymes after solvation and saponification reactions caused by the alkaline treatment **(Galbe and Zacchi, 2007; Hendriks and Zeeman, 2009).** In fact, cellulose can be swelled or dissolved in NaOH solutions, leading to the decrease of lignocellulosic biomass crystallinity **(Sun *et al.,* 2016).** Besides, the improvement of enzyme activity after treatment may be due to the fact that alkaline treatment did not make changes in the fibers composition but, it depleted the medium of available carbon source readily accessible for the strain used, like the lipids and carbohydrates. This situation promoted the induction of cellulolytic enzyme synthesis by the cellulose of the fermentation medium. Several authors have already affirmed this suggestion. **Ballerini and Alazard-Toux (2006)** explained that the production of cellulases is regulated by the processes of induction and catabolic repression: induction linked to the presence of substrates (in this case cellulose) and repression by the sources of carbon molecules (such as glucose via catabolic repression mechanisms). **Kaur *et al.* (2006)** reported that it is difficult to deduce the nature of the inducers that will be valid for the known cellulolytic microorganisms but it is evident that the insoluble native cellulosic material is certainly the best substrate for the production of cellulases. **Candace and Weimer (1991)** suggested that close physical contact between fungus and cellulose can trigger induction, and assumed that there is an appropriate sites recognized in cellulose. It is also possible that this improvement of *FPase* activity after pretreatment with 1% NaOH is due to the nature of the cellulose used by *T. reesei* RUT C30.Since, there are two types of cellulose in OP composition. The cellulose located in pulp of OP easily accessible for *T. reesei* compared to that located in the OP stones (woody endocarp) which contains, in addition to cellulose, high levels of lignin**.** Knowing thatthe pure pulp of OP, contains around 20% only of the overall crude cellulose as reported by **Sansoucy *et al*. (1984).** Besides the high amount of lignin in biomass, the enzymatic hydrolysis is also controlled by lignin location in biomass and its surface area which play a signiﬁcant role **(Kim *et al*., 2016).** In addition, it was shown that alkali pretreatments are more effective on agricultural residues than on woody materials **(Kumar *et al*., 2009).** In the other hand, **Oke *et al*. (2016)** observed that the untreated mixed lignocellulosic substrates (MS) supported the highest cellulase production, in comparison to MS treated with 1% NaOH. Similar results were also found by **Brijwani and Vadlani, (2011)** when soybean hulls were used as substrate.

Figure 2 (b) presents the soluble protein evolution during the fermentation period using untreated and alkaline pretreated OP. The evolution of soluble proteins, for all used substrates, follows the same progression as the cellulolytic activity, the maximum content of soluble proteins (6.99mg/gds) corresponds to the optimum enzymatic activity for 1% NaOH pretreated OP (24th day of fermentation). It was also seen that extracts from the untreated substrate presented the highest soluble protein content, with a maximum of 8.68mg/gds in the 24th day of fermentation in comparison with the other pretreated samples. Despite the large amount of proteins content, cellulase activity was lower compared to the results of 1% NaOH pretreatment. This may be explained by the existence of a high initial protein content (t = 0) in the extracts of untreated OP and by the new protein charge produced by the extracellular metabolism of *T. reesei*, especially hydrolase enzymes such as xylanases (results not shown).

 The curves of pH evolution, represented in Figure 2 (c), showed the same shape for all the samples analyzed. From different initial values, the pH has decreased during the 12th first days of fermentation to reach values between 5.8 and 6.8, which represents the adequate pH for the production of cellulolytic enzymes by *T. reesei*. **Roussos and Raimbault (1982)** explained the pH diminution by NH4 cations assimilation in the form of NH3 which cause H+ ions accumulation. This diminution can be also related to the production of acidic metabolites that neutralize the NaOH to give lower pH. It was found in this study that a higher concentration of NaOH leads to a higher initial pH, and consequently, a high residual NaOH content in the substrate pores, since an important aspect of alkali pretreatment is that the lignocellulosic biomass on itself consumes some of the alkali: approximately 3g NaOH/100g of total substrate **(Hendriks and Zeeman, 2009).** This affects the good growth of *T. reesei* strain, and resulted in a slow mycelial growth on the solid residue and explained the low celluolytic activities obtained for OP pretreated with concentrations higher than 1% NaOH. **Deswal *et al*. (2011)** observed that increasing the initial pH of the medium from 5.5 to 10.0 leads to a signiﬁcant decrease in cellulases production.

 Taking into account the results of this section where 1% NaOH was the most effective pretreatment on OP substrate for cellulases production (*FPase* activity), this pretreatment was chosen for all further combinations with milling (Section 3.2) and thermal pretreatments (Section 3.3).

*Vertical bars indicate standard error of three replicates.*

**Figure 2.** Evolution of *PFase* activity (a), protein content (b) and pH values (c) of extracts obtained from *T. reesei* culture on alkaline pretreated OP during 30 days of fermentation

**Milling pretreatment**

**Effect of milling pretreatment on the chemical composition of olive pomace**

 Milling was studied here as a pretreatment of OP samples, whereas the majority of authors working on this substrate use it as an important step for sample preparation before each fermentation process, in order to facilitate handling. The effect of milling on OP chemical composition was illustrated in Tab 2. A significant increase was observed in all parameters analyzed after milling process compared to the untreated one. After milling, increase rates of 47.24%, 57.21%, 58.03% and 69.16% were recorded for proteins, carbohydrates, lipids and ash, respectively. This result is the evident effect of milling process which augmented the contact surface in the pretreated OP samples. When the milling is combined with 1% NaOH, the organic matter (proteins, carbohydrates, lipids) has decreased significantly (Tab 2) due to the effect of alkaline pretreatment, as already shown in section 3.1.

**Table 2.** Chemical composition of OP after milling pretreatment (combined or not with 1% NaOH) (% Dry Matter)

|  |  |  |  |
| --- | --- | --- | --- |
| Parameter | Untreated OP | Milled OP | Milled + 1% NaOH OP |
| Moisture | 37.64 ± 1.47a | 4.62 ± 0.04b | 1.44 ± 0.01c |
| Dry Matter | 62.36 ± 1.47c | 95.38 ± 4.62b | 98.56 ± 0.01a |
| Ash | 0.70 ± 0.05c | 2.27 ± 0.03a | 0.87 ± 0.07b |
| Lipids | 7.68 ± 0.44b | 18.30 ± 0.26a | 0.63 ± 0.05c |
| Total Nitrogen | 0.43 ± 0.03b | 0.81 ± 0.04a | 0.18 ± 0.02c |
| Proteins | 2.68 ± 0.18b | 5.08 ± 0.23a | 1.14 ± 0.12c |
| Total carbohydrates | 0.86 ± 0.10b | 2.01 ± 0.05a | 0.27 ± 0.02c |
| Reducing sugars | 0.68 ± 0.08b | 1.33 ± 0.14a | 0.22 ± 0.01c |

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

*ND: not detected.*

 The effect of milling alone or combined with 1% NaOH on fiber contents of OP is depicted in Figure 3. Milling pretreatment caused significant decrease of NDF (29.88%) and ADF (16.22%) fractions compared to untreated OP, whereas, combined pretreatment augmented significantly NDF (31.51%) and ADF (21.01%) fractions compared with the milled OP. It is interesting to note that after milling pretreatment, ADL fraction decreased of about 42% compared to the untreated OP, and a slight decrease of 9.3% was observed after combined pretreatment. It appears that the milling pretreatment was more efficient on ADL fraction than the combined one. This contradiction can be explained by the possible recondensation and redistribution of soluble lignin compounds, solubilized during the alkaline pretreatment **(Hendriks and Zeeman, 2009; Pellera *et al.*, 2016). Vimala Rodhe *et al.* (2011)** observed about 75% loss of lignin on milled sorghum straw pretreated with 0.2M of NaOH. The same fact was also found by **Haddadin *et al*. (1999)** when milled OP pretreated at 3%NaOH was used. Contrary to the trends noted for the previous fractions, the milling pretreatment has augmented significantly the cellulose fraction of OP of almost 35%, the same effect was observed for the combined pretreatment. Milling caused hemicellulose fraction to a significant diminution of 74.60% compared to untreated OP, but, when it was combined with alkaline pretreatment, a significant increase (69%) was reported.

 After milling, the NDF and ADF diminutions are mainly the consequence of ADL decrease. **Mtui (2009)** reported that ADL decrease may be due to reduction of the degree of lignin depolymerization via the cleavage of uncondensed-aryl ether linkages. After combined pretreatment in reference to untreated OP, a significant increase in ADF and cellulose fractions was recorded, whereas NDF and hemicellulose remained unchanged, only ADL fraction was decreased. Our results are in agreement with the findings of **Oke *et al*. (2016)** who found that cellulose content of lignocellulosic substrates increased after milling pretreatment combined with alkali (1% NaOH). In contrast**, Haddadin *et al*. (1999)** observed a significant decrease in cellulose content of milled OP pretreated at 3% NaOH, these findings may be the result of the NaOH high concentration, compared to the dosage used in our research.

*Vertical bars indicate standard error of three replicates .Different letters for the same parameter (a-c) indicate significant differences (p<0.05).*

**Figure 3.** Effect of milling pretreatment (combined or not with 1% NaOH) on fiber composition of OP.

**Effect of milling pretreatment on the *FPase* activity**

 In this part of the study, the first notable result is that a slow fungal colonization was observed with milled OP substrate during the fermentation assay compared to OP pretreated with 1% NaOH. **Sanchez (2009)** thought that the mycelial growth is related to the effective degradation of the lignocellulosic biomass that constitutes its carbon source. Moreover, the slow growth of *T. reesei* strain may be probably due to the heterogenic granulometry of milled sample, which contains particles with different sizes (below 1.25 mm diameter). In fact, the fine particles formed after milling of olive pomace in presence of high lipid contents (18.30%) causes a clogging that prevents an effective transfer of oxygen in the culture medium, unlike larger particles that improve breathing and aeration efficiency by increasing the inter-particle space.

 The evolution of *PFA*se activity during 30 days of fermentation on milled pretreated OP combined or not with 1% NaOH was illustrated in Figure 4 (a). It was clearly shown that the optimal cellulolytic activities obtained in both cases of pretreatments (0.2U/gds for milled and 0.71U/gds for combined pretreated OP) were lower than the activity obtained with 1% NaOH pretreated OP (1.28U/gds). Although milling resulted in a decrease in lignin content, cellulase activity was not improved. According to **Bali *et al*. (2015),** lignin removal has been shown to increase the yield of enzymatic hydrolysis, however, the direct effect of lignin removal on cellulose accessibility is still not fully clear because lignin is also associated with cellulases inhibition, and the relative contributions of these two roles of lignin have not yet been fully defined. In addition, **Berlin *et al*., (2006)** stated that lignin depolymerization has been considered as an effective inhibitor of cellulases. The negative effect of milling process on cellulolytic activity may be due to the formation of inhibitor compounds after degradation of lignocellulose during pretreatment such as soluble phenolic compounds as reported by **Vancov and McIntosh (2011)** and **Sun *et al*. (2016).** In addition, **Pellera *et al*. (2016)** reported that phenolic compounds are produced by the degradation of lignocellulosic materials, and mostly by hemicellulose and lignin solubilization. The low *PFAse* activity obtained on milled OP may also be due to the particle size of this substrate. In fact, several authors concluded that the pore size of the substrate is in relation to the size of the enzymes which constitute an important limiting factor in the enzymatic hydrolysis of lignocellulosic biomass **(Chandra *et al.,* 2007, Alvira *et al*., 2010).** Therefore, **Grethlein (1985)** found linear correlations between the initial hydrolysis rate of pretreated biomass and the pore size accessible to a molecule with a diameter of 51 A° similar to the size of *T. reesei* cellulase components. Consequently, cellulase can get trapped in the pores of substrate if the internal area is much larger than the external area which is the case for many lignocellulosic substrates **(Zhang and Lynd, 2004).**

 An improvement in *FPase* activity was recorded when the milled OP was pretreated with 1% NaOH; therefore, alkaline pretreatment has positively affected the cellulolytic activity. Nevertheless, the inhibitory by-products generated by milling process probably prevented the improvement of *FPase* activity to achieve the best activity obtained with only alkaline pretreatment (1.28U/gds). Several authors reported that when milling is combined with alkaline pretreatment an improvement in cellulolytic activity was noticed. **Bansal *et al*. (2012)** found an enhancement of the cellulase production after alkali pretreatment (1% NaOH) using *Aspergillus niger* fungi cultured on milled agricultural and kitchen wastes. **Belal (2013)** have also observed a positive effect of combined pretreatment (milling+5%NaOH) on conversion of polysaccharide into sugar by *T. reesei* cellulases on milled rice straw substrate. In another study, **Wu *et al.* (2011)** reported that milled bagasse pretreated with 2.5M NaOH has given an enzymatic hydrolysis yield of 98.7%.

 The evolution of soluble protein content of milled OP combined or not with alkaline pretreatment during fermentation (30 days) was represented in Figure 4 (b). A slight decrease in soluble protein content of milled OP was shown during the 6 first days of fermentation (2.37 up to 2.03mg/gds) where a maximum *FPase* activity was recorded. Remarkable decrease in soluble protein content was noted on the 12th day; this may be due to the assimilation of the medium proteins during mycelium growth **(Roussos and Raimbault, 1982).** However, soluble protein content of milled OP pretreated with 1%NaOH increased rapidly during the first 6 days of fermentation (from 0.45 to 2.69 mg/gds), and then stabilized until the end of the fermentation (30 days). This increase can be explained by the secretion of fungal extracellular proteins in the medium. It can also be noted, that protein contents of milled OP combined or not with alkaline pretreatment during the fermentation period were much lower than those recorded in OP pretreated with 1% NaOH, which were proportional to the cellulolytic activity obtained above.

*Vertical bars indicate standard error of three replicates*

**Figure 4.** Evolution of *PFase* activity (a), protein content (b) and pH values (c) of extracts obtained from *T. reesei* culture on milled OP during 30 days of fermentation

As shown in Figure 4 (c), pH evolution of milled OP during fermentation period followed the same trend as soluble proteins, with a remarkable drop in pH value (3.37) at the 12th day; this decrease was previously explained in section 1. The pH values of milled OP pretreated with 1%NaOH decreased during the first 12th days, then stabilized until the end of fermentation. In this case, pH evolution was similar to that noted for OP pretreated with 1% NaOH.

**Thermal pretreatment**

**Effect of thermal pretreatment on the chemical composition of olive pomace**

 In this study, thermal pretreatment was carried out at 100°C during 2h. As shown in Tab 3, generally, significant effect (p<0.05) of thermal pretreatment was observed on chemical composition of OP compared to the untreated one. Ash, lipid and carbohydrate contents decreased significantly after thermal pretreatment with loss rates of 77.14%, 54.69% and 79.07%, respectively. Additionally, a total loss of reducing sugars was also shown. However, no significant effect on proteins after thermal pretreatment was noticed. When OP was subjected to a combined pretreatment (T° + 1% NaOH), a significant variation in ash (increase), protein (decrease) and lipid (total loss) contents were recorded.

**Table 3.** Chemical composition of OP after thermal pretreatment (combined or not with 1% NaOH) (% Dry Matter).

|  |  |  |  |
| --- | --- | --- | --- |
| Parameter | Untreated OP | T° OP | T° + 1% NaOH OP |
| Moisture | 37.64 ± 1.47a | 2.51 ± 0.15b | 1.48 ± 0.11b |
| Dry Matter | 62.36 ± 1.47b | 97.49 ± 0.15a | 98.52 ± 0.11a |
| Ash | 0.70 ± 0.05b | 0.16 ± 0.06c | 1.36 ± 0.21a |
| Lipids | 7.68 ± 0.44a | 3.48 ± 1.38b | ND |
| Total Nitrogen | 0.43 ± 0.03a | 0.41 ± 0.03a | 0.13 ± 0.02b |
| Proteins | 2.68 ± 0.18a | 2.58 ± 0.20a | 0.79 ± 0.15b |
| Total Carbohydrates  | 0.86 ± 0.10a | 0.18 ± 0.01b | 0.15 ± 0.02b |
| Reducing Sugars | 0.68 ± 0.08 | ND | ND |

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

*ND: not detected*

 The effect of thermal pretreatment combined or not with 1% NaOH on fiber contents was exposed in Figure 5. No clear significant effect was observed in the NDF fraction after both pretreatments, while ADF fraction decreased slightly but significantly after thermal process. Regarding ADL and cellulose fractions, no significant effect of thermal pretreatment on olive pomace was remarked whether combined or not with 1% NaOH compared to untreated one. **Aiello *et al.* (1996),** in a study on sugarcane bagasse substrate, found unchanged lignin and cellulose fractions after combined treatment (100°C+ 5% NaOH). The same finding was reported by **Rodríguez-Zúñiga *et al*. (2015)** when hydrothermal pretreatment (190°C, 10 min) was applied on sugarcane bagasse substrate, in term of lignin fraction. Moreover, **Xiao *et al.* (2017)** reported that cellulose and lignin are unaffected by the hydrothermal pretreatment. It seems that, this pretreatment has no effect on lignin fraction, this can be due to the spatial re-localization or reorganization of this later, which can occur with hydrothermal pretreatment **(Kristensen *et al.* 2008).** In addition, **Agbor *et al.* (2011)** explained that not all pretreatments result in substantial deligniﬁcation: the structure of lignin may be altered without extraction due to changes in the chemical properties of the lignin. Thermal pretreatment combined with 1% NaOH caused a slight increase in hemicellulose fraction compared to the untreated OP, whereas, theoretically, thermal pretreatment causes a removal of hemicellulose of the solid fraction. In fact, this increase of hemicellulose was the result of alkaline pretreatment only, and as a consequence, thermal pretreatment had no effect on fiber fractions because of the hardness of olive pomace and the mild temperature applied during pretreatment. The study of **Wu *et al.* (2011)** revealed that low thermal pretreatment (50°C) combined with 2.5M NaOH signiﬁcantly accelerated the removal of hemicellulose and lignin on sorghum bagasse substrate. Further, **Hendriks and Zeeman (2009)** reported that after thermal process, a part of the hemicellulose is hydrolyzed and forms acids. This leads to conclude that the improvement of the digestibility of lignocellulosic biomass depends on the nature of the substrate and the operating conditions (pretreatments combination).

*Vertical bars indicate standard error of three replicates. Different letters for the same parameter (a-b) indicate significant differences (p<0.05).*

**Figure 5.** Effect of thermal pretreatment (combined or not with 1% NaOH) on fiber composition of OP

**Effect of thermal pretreatment on *FPase* activity**

Figure 6 (a) represents the evolution of cellulolytic activity during 30days of fermentation on OP subjected to thermal pretreatment, combined or not with 1%NaOH. A very slow mycelial growth was observed during the fermentation period on OP samples thermally pretreated, combined or not with 1% NaOH. The results of *FPase* activities showed that the highest values were obtained on the 6th day of fermentation on pretreated OP by the temperature alone or combined with 1%NaOH; the activities were respectively 0.15 and 0.09U/gds. These later values were widely lower than the obtained activity with alkali pretreated OP (1.28U/gds). As a result, thermal pretreatments either alone or in combination have a negative effect on the cellulolytic enzyme production. The low *FPase* activities obtained may possibly be the result of changes in the chemical structure of olive pomace after thermal pretreatment because the enzymatic hydrolysis of lignocellulosic substrate could be influenced not only by the efficiency of the enzymes, but also by the physical, chemical and morphological characteristics of these biomass, as reported by **Sun *et al.* (2016).** It could also be explained by the removal of certain nutrients from the substrate after pretreatments **(Abdullah *et al*., 2016).** In our study, a slight production of cellulase was noted, while **Aiello *et al*. (1996)** found no detectable activity during the fermentation using *Trichoderma reesei* QM 9414 on sugarcane bagasse treated with alkali (5%NaOH at 100°C). The authors suggested that the loss of activity could be the result of absorption of cellulase on cellulose and lignin or the inhibition of enzymes by glucose and cellobiose of the fermentation medium.

 This is an indication that in some cases, pretreatment of substrate prior to cellulase production might not be necessarily efficient, it could make a substrate less accessible and less suitable for microbial growth and fermentation when compared with the untreated one **(Yoon *et al*., 2014).** Several studies confirmed this trend, when higher cellulolytic enzyme production was obtained with untreated sugarcane bagasse **(Rodríguez-Zúñiga *et al*., 2014),** wheat straw **(Sharma *et al.*, 2015),** municipal solid wastes **(Abdullah *et al.,* 2016)** and mixed lignocellulosic substrates **(Oke *et al*., 2016)** among others.

 As illustrated in Figure 6 (b), the soluble protein contents during the fermentation period followed the same evolution as *FPAse* activities in enzymatic extracts obtained from OP subjected to whether temperature pretreatment or combined one. Soluble protein contents recorded for both pretreatments were much lower than those noted for alkaline pretreatment, a maximal value of 2.08mg/gds was reported for the combined pretreatment on the 6th day of fermentation and tend to decrease with time.

 From Figure 6 (c), as expected, the pH values obtained for combined pretreatment (temperature+1% NaOH) were close to that of alkaline pretreatment, but higher than the thermal pretreatment. The pH evolution shows stability during the fermentation period maintaining the initial pH values, on average 6.4 and 5.1 for the combined and thermal pretreatment, respectively.

*Vertical bars indicate standard error of three replicates*

**Figure 6.** Evolution of FPase activity (a), protein content (b) and pH values (c) of extracts obtained from *T. reesei* culture on thermal pretreated OP during 30 days of fermentation

**CONCLUSIONS**

 The present study contributes to a better understanding of the most used pretreatment effects on a cheap and abundant agricultural waste (olive pomace) for obtaining high value-added products (enzymes of industrial interest). The effect of three different pretreatments (alkaline, milling and thermal and their combinations) on the chemical composition of olive pomace and on *FPAse* activity is investigated. The rich chemical composition of untreated OP makes it a favorable substrate for growth of lignocellulolytic fungi such as *Trichoderma reesei* RUT C30under SSF. From the results, alkaline pretreatment with concentration of 1%NaOH is the most efficient for cellulase production, while, milling and thermal pretreatments of OP samples have proved to be useless for the improvement of cellulolytic activity, besides being expensive and require high energy consumption.

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**Competing interest**

The authors declare that they have no competing interests.

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