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Solid State and Semi-Solid Fermentations of Olive and Sunflower Cakes with *Yarrowia lipolytica:* Impact of Biological and Physical Pretreatments

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Abstract: Lignocellulosic biomass is a promising feedstock for added value compound production in biotechnological processes such as solid-state fermentation (SSF). Although these solid materials can be directly used as substrates in fermentations in a solid state, a pretreatment is often required, especially if the microorganism selected is unable to produce lignocellulosic enzymes. In the present work, several pretreatment strategies were applied to a 50% (*w/w*) mixture of olive and sunflower cakes before SSF for lipase production by the oleaginous yeast *Yarrowia lipolytica* W29. Co-culture strategies with *Y. lipolytica* and *Aspergillus niger* did not improve lipase production by the oleaginous yeast. Biological pretreatment with a fungal enzymatic extract led to a significant increase in sugar availability in the substrate mixture after a short incubation period, improving yeast growth. Microwave and ultrasound were the physical pretreatments selected and microwave irradiation proved to be the best method, resulting in 44% and 17% increases in yeast growth and lipase production, respectively, compared to the untreated mixture. An improvement in lipase activity was also observed after ultrasonic treatment in semi-solid fermentations, leading to a 2-fold increase in this enzyme activity compared to the control. The utilization of pretreatments before SSF with *Y. lipolytica* can increase sugars availability and result in structural changes in the solid substrate, which can improve the bioprocesses' productivity.

Keywords: *Yarrowia lipolytica*; solid state fermentation; sunflower cake; olive cake; lignocellulosic biomass pretreatment

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1. Introduction

Lignocellulosic biomass has been studied and proposed as substrate in biotechnological processes for biocompound production as an alternative culture medium, making the production process more economically attractive, as well as contributing to a circular economy, with a positive environmental impact [1]. These lignocellulosic materials can be directly used as substrates for solid state fermentation (SSF) processes, functioning both as a solid support and a nutrient source [2]. In some cases, pretreatment of these materials can be performed to improve substrate accessibility, resulting in higher microbial growth and productivity [3–5].

Even though most studies regarding SSF report the utilization of filamentous fungi, some yeast species have received great attention in recent years due to their ability to grow in solid substrates with low moisture content and to produce metabolites at high yields [6]. *Y. lipolytica* is an oleaginous yeast commonly selected for SSF processes with reports on the production of γ-decalactones [7], erythritol [8] and enzymes such as proteases [9] and

lipases [10–14]. Moreover, the biomass of this yeast, which is rich in protein, is considered safe for utilization in food and feed [15].

Most of these reports used agro-industrial by-products as the solid substrates with high fiber contents, including by-products from soybean processing [8,13,14] and olive oil extraction [9,12]. However, because *Y. lipolytica* is unable to produce lignocellulosic enzymes, the hemicellulosic fraction of these materials is underutilized; for this reason, pretreatments before their utilization on biotechnological bioprocesses can be used to overcome this limitation. As an example, alkaline pretreatment of olive by-products prior to SSF resulted in improved lipase and protease production by *Candida utilis* [16] and lipase production by *Y. lipolytica* NRRL Y-1095 [17]. However, this strategy requires the utilization of harmful chemicals and high amounts of water to neutralize the substrate prior to SSF; thus, other approaches for by-product pretreatment with a lower environmental impact must be considered. A pre-fermented mixture of okara and buckwheat husk by *Mucor flavus* was used as the SSF substrate for erythritol production by *Y. lipolytica* M53 [8]. Similarly, co-culture of *Trichoderma* sp. and *Saccharomyces cerevisiae* in SSF using sweet potato flour as a solid substrate resulted in improved bioethanol production by the yeast strain [18]. Using a different approach, Martínez-Avila and colleagues [3] applied fungal enzymatic extracts in enzymatic hydrolysis at high solid loading before SSF for the production of bioplastics by two bacteria strains. The authors from these studies reported that the enzymes produced by the filamentous fungi were fundamental to increase sugar availability in the solid substrate, resulting in improved microbial growth and biocompound production. Besides these biological approaches, physical treatments, such as ultrasound and microwave irradiation, have also been employed in lignocellulosic biomass pretreatment. Ultrasound pretreatment leads to the formation of microbubbles in the materials, and the collapse of these microbubbles can result in structural changes and improve the solubilization of organic matter [19]. In contrast, microwave pretreatment is a technology that allows to selectively heat the lignocellulosic biomass from the inside out, resulting in increased porosity and surface area [20]. Although microwave [5] and ultrasound [4] pretreatments have been used before SSF with filamentous fungi, to our knowledge, the effect of these physical pretreatments on *Y. lipolytica* growth and biocompound production under SSF have not yet been studied.

In the present work, physical and biological pretreatments were performed to induce structural changes in a substrate mixture of 50% (*w/w*, dry basis) OC and sunflower cake (SC) [9], and to increase assimilable sugar availability before SSF for lipase production. Co-culture with filamentous fungi and enzymatic hydrolysis with a fungal enzymatic cocktail were the biological treatments selected as strategies to increase sugar content in the substrate mixture before SSF. Moreover, microwave and ultrasound pretreatments were also applied to the substrate mixture to evaluate their effect on lipase and biomass production by *Y. lipolytica*.

2. Materials and Methods

2.1. Raw Materials

Sunflower, rapeseed and soybean cakes were supplied from a Portuguese vegetable oil production industry (Iberol SA). These materials were obtained in dry conditions and were milled and stored at room temperature. OC was supplied by a two-phase olive mill from the northern region of Portugal (Achsula SA), and was stored at −18 °C due to its high moisture content.

2.2. Microorganisms

Yarrowia lipolytica W29 (ATCC 20460) was stored in 30% glycerol stocks at −80 °C and revived in YPDA (glucose 20 g/L, peptone 20 g/L, yeast extract 10 g/L, agar 20 g/L). For inoculum preparation, yeast cells were collected from an agar plate and cultivated overnight in 500 mL Erlenmeyer flasks with 100 mL of YPD medium, in an orbital incubator, at 200 rpm and 27 °C. *Aspergillus niger* CECT 2915 was obtained from CECT (Colección Española de Cultivos Tipo, Valencia, Spain) and preserved at −80 °C in a glycerol solution.

The microorganism was revived in potato dextrose agar (PDA) plates and, prior to substrate inoculation, the spores from a PDA plate were suspended in 1 g/L peptone and 0.1 g/L Tween 80.

2.3. Co-Culture with *A. niger* and *Y. lipolytica*

Sequential and simultaneous SSF were performed with *A. niger* and *Y. lipolytica* (Figure 1). Fermentations were performed in 500 mL Erlenmeyer flasks with 10 g (dry basis) of 50% (*w/w*) of OC and SC mixed with distilled water to adjust the moisture content. This substrate mixture was previously selected for lipase production by *Y. lipolytica* W29 [9]. In the sequential SSF, 2 mL (10^5 spores per gram of solid substrate) of a spore suspension of *A. niger* was added to the autoclaved substrate, and flasks were kept in an incubator at 27 °C. After 2 days, 2 mL (3.8 mg of cells per gram of dry solid substrate) of a yeast suspension was added to the fermented mixture, adjusting the final moisture content to 75% (wet basis), and flasks were kept at the same temperature for another 2 days. In SSF with simultaneous inoculation, 2 mL of spore and 2 mL of yeast inoculum suspensions were added to the solid substrate, leading to a final moisture content of 75% (wet basis). Flasks were kept at 27 °C for 2 days. A standard SSF assay with a monoculture of *Y. lipolytica* W29 was performed for 2 days, as previously described [9].

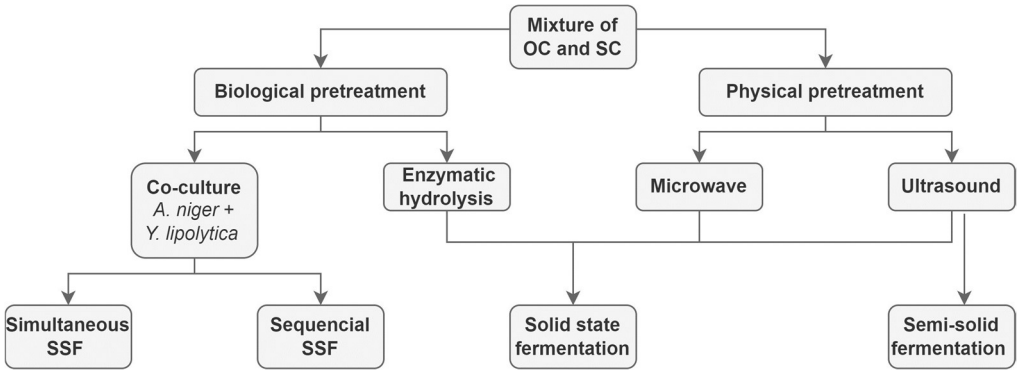

Figure 1. Diagram of the pretreatments applied to the mixture of OC and SC.

2.4. Production of Enzymatic Extract from *A. niger*

The enzymatic extract used in the hydrolysis step was obtained from a SSF process using equal parts of SC, rapeseed and soybean cakes [21]. Flasks were maintained at 25 °C for 7 days. Enzyme extraction was performed by mixing the fermented substrate with 0.05 M citrate buffer (pH = 4.8) with a dry solid/liquid ratio of 1:8 (g:mL). After agitation in an orbital incubator for 30 min at room temperature, the liquid extract was recovered by filtration with a fine-mesh net and centrifuged for 10 min at 8000 rpm and 4 °C. The supernatant was then vacuum filtered with filter paper and stored at −18 °C.

2.5. Enzymatic Hydrolysis Followed by SSF

Enzymatic pretreatment was performed before SSF with *Y. lipolytica* W29 (Figure 1). An amount of 2 g (dry basis) of the mixture of 50% (*w/w*, dry basis) of OC and SC were sterilized for 15 min at 121 °C and, after cooling, the crude enzymatic extract was added to this substrate mixture to achieve a final cellulase concentration of 50, 100 and 150 U/g and a solid loading of 25% (*w/w*, wet basis). A commercial cellulase (Sigma-Aldrich, St. Louis, MO, USA, C1184) in 0.05 M citrate buffer (pH 4.8) was used as a control. Enzymatic hydrolysis was performed at 50 °C and the experiments were followed up for 48 h. After selection of the optimum hydrolysis conditions, 10 g (dry basis) of the optimum substrate mixture for lipase production was autoclaved in 500 mL Erlenmeyer flasks for 15 min at 121 °C and the enzymatic extract was added to the solid materials with a final cellulase concentration of 100 U/g. Flasks were kept in an incubator at 50 °C for 12 h followed by another cycle of sterilization to inactivate the enzymes from the crude extract. The substrate mixture

was inoculated with 2 mL of yeast cellular suspension, leading to 75% (*w/w*, wet basis) moisture. Flasks were placed in an incubator at 27 °C and enzyme production over time was evaluated by sampling the whole fermented substrate in one flask each day. Standard SSF assays were performed for 2 days with water or citrate buffer 0.05 mM (pH 4.8) to adjust the moisture content.

2.6. Physical Pretreatments Followed by SSF

Physical pretreatments were performed with 10 g (dry basis) of the substrate mixture in 500 mL Erlenmeyer flasks, and distilled water was used to moisten the substrate at a solid loading of 25% (*w/w*, wet basis), similar to biological pretreatment (Figure 1). Flasks were placed in an ultrasound bath Sonorex Digitec DT 514 (Bandelin, Germany) at 35 kHz for 15 min and, for microwave irradiation, flasks were placed in domestic microwave equipment (1250 W, model MS2387U, LG) at 680 W for 2 min in cycles of 30 s. After the pretreatments, substrate sterilization and SSF were performed as described above.

2.7. Lipase Extraction

After SSF for lipase production, the fermented solid substrate was mixed with a solution of 10 g/L NaCl and 5 g/L Triton X-100 in a solid/liquid ratio of 1:8 (g:mL) followed by 30 min of agitation in an orbital incubator at 200 rpm and room temperature. A liquid extract was obtained by filtration with a fine mesh net and, after collecting a sample for cell counting, centrifugation was performed at 8000 rpm and 4 °C for 10 min. The supernatant was then stored at −18 °C for further analysis.

2.8. Semi-Solid Fermentation after Ultrasound Pretreatment

The mixture of OC and SC was mixed with distilled water at a solid loading of 10% (*w/w*) and ultrasound pretreatment was performed with an ultrasonic processor Cole-Parmer (Vernon Hills, IL, USA) with 750 W and 20 kHz (Figure 1). The probe of the ultrasonic processor was in contact with the suspension of oil cakes for the required time, which varied from 2 to 15 min. Following sonication, liquid and solid fractions were separated by centrifugation at 8000 rpm for 10 min. The supernatant was stored at −18 °C for further analysis. After selection of the pretreatment duration, 5 g (dry basis) of the pretreated mixture was transferred to 250 mL Erlenmeyer flasks and sterilized at 121 °C for 15 min. After cooling, 1 mL of yeast inoculum suspension was added to the pretreated mixture and flasks were placed in an orbital incubator at 27 °C and 200 rpm. Liquid samples were collected at specific time points and cell counting was performed to estimate cellular concentration in the suspension. The remaining sample was centrifuged at 8000 rpm for 10 min and the supernatant was stored at −18 °C for further analysis. Substrate mixture at 10% (*w/w*) of solid loading without ultrasound pretreatment was used as a control. After 30 h of fermentation, the semi-solid mixture containing the yeast biomass was dried at 60 °C for 48 h and the dry substrate was stored at room temperature.

2.9. Analytical Methods

The aqueous extract obtained after SSF was characterized by reducing sugar content, cellular concentration and enzymatic activities.

Cellular density was estimated by cell counting in an optical microscope, and the cell number was converted to dry cell mass per liter using a conversion factor of 10^{-8}. These values were converted to dry cell mass per gram of dry substrate mixture, taking into account the volumes of aqueous extract and dry solid recovered after SSF.

Lipase and protease activities were determined as previously described [9] using 4-nitrophenyl butyrate and azocasein (Sigma) as substrates, respectively. The activities of cellulase and xylanase after SSF with *A. niger* CECT 2915 were quantified as previously described [22]. Reducing sugars, expressed in mg of reducing sugars per gram of dry substrate (mg/g), were determined using the dinitrosalicylic (DNS) acid reagent method [23], and glucose was used as standard.

Glucose and xylose concentrations were determined as previously described [24] by high-performance liquid chromatography (LC 2060C, Shimadzu, Kyoto, Japan) using an Aminex HPX-87H column (300 mm × 7.8 mm, 8 µm particle size) with temperature set at 60 °C and equipped with RI and UV detectors. 5 mM Sulfuric acid (5 mM), at a flow rate of 0.5 mL/min, was selected as the mobile phase.

Fiber content in the substrate mixture pretreated with the crude enzymatic extract and in the control condition was quantified as previously described [4]. The percentages of hemicellulose and lignin were used to calculate acid detergent fiber (ADF) and neutral detergent fiber (NDF) using the percentages of hemicellulose, cellulose and lignin.

Crude protein and microbial lipids were determined in the fermented solid obtained after semi-solid fermentation. Total nitrogen was determined by the Kjeldahl method and nitrogen was converted into crude protein using a factor of 6.25. Lipid content was determined gravimetrically after extraction with methanol and chloroform (2:1, *v/v*) [25]. The fatty acid profile of the lipids present in the fermented substrate mixture was determined by quantification of fatty acid methyl esters (FAMEs). Fatty acids were converted into FAMEs by methylation using a solution of methanol and sulfuric acid (85:15, *v/v*) [26]. FAMEs analysis was performed by gas chromatography with a CP-3800 gas chromatograph (Varian Inc., Palo Alto, CA, USA) equipped with a flame ionized detector (FID) and TRACSIL TR-WAX capillary column (30 m × 0.25 mm × 0.25 mm, Teknokroma, Barcelona, Spain). The initial temperature of the column was kept at 50 °C for 2 min, followed by an increase of 10 °C/min until reaching 225 °C; this temperature was kept for 10 min. The temperatures of the injector and detector were 220 °C and 250 °C, respectively, and helium was selected was the carrier gas (1 mL/min). Heptadecanoic acid was used as an internal standard and FAMEs standards were used to identify FAMEs in the samples by comparison of the retention times. The ratio between fatty acid concentration (g/L) and the sum of all fatty acids quantified was used to calculate the relative amount of each fatty acid.

2.10. Statistical Analysis

The results are presented as mean ± standard deviation (SD) of two independent replicates. The experimental data were subjected to *t*-test, one-way analysis of variance (ANOVA) and Tukey's test for multiple comparison using GraphPad Prism. All the analyses were performed with a confidence interval of 95%.

3. Results

3.1. SSF with A. niger

Yarrowia lipolytica W29 has been previously selected for enzyme production in SSF using by-products from vegetable oil industries [9]. After substrate mixture and cultivation time optimization, lipase activity of (102 ± 17) U/g was attained after 2 days of SSF with a 50% (*w/w*, dry basis) mixture of OC and SC. While SSF resulted in 29% reduction in the lipid content of this substrate mixture, significant changes in fiber percentage were not registered. This outcome is due to *Yarrowia* species' inability to produce lignocellulosic enzymes; thus, complex polysaccharides, such as cellulose and hemicellulose, are not hydrolyzed during SSF.

Likewise, mixed cultures of microorganisms that do not secrete these enzymes with filamentous fungi producers of lignocellulolytic enzymes could be a suitable strategy to increase substrate utilization and microbial growth, resulting in high biocompound yields. In the present work, two co-culture strategies with *Y. lipolytica* W29 and *A. niger* CECT 2915 were studied and the influence of co-culture on enzyme production was evaluated. Nevertheless, before the co-culture experiments, SSF with *A. niger* CECT 2915 was performed to assess enzyme production on a substrate composed of 50% (*w/w*, dry basis) OC and SC. SSF was followed up to 4 days and the results are present in Table 1.

Table 1. Enzymatic activities and free reducing sugar concentration obtained in SSF with *A. niger* CECT 2915 in 50% (*w/w*) of OC and SC as solid substrate.

Parameters	Sample Collection (Days)	
	2	**4**
Cellulase (U/g)	7 ± 2^{a}	64 ± 5^{b}
Xylanase (U/g)	32 ± 4^{a}	290 ± 74^{b}
Lipase (U/g)	1.95 ± 0.02^{a}	4 ± 1^{b}
Protease (U/g)	13 ± 3^{a}	10 ± 2^{a}
Reducing sugars (mg/g)	39 ± 3^{a}	18 ± 5^{b}

Values represent the mean and SD from two independent experiments. Values with different letters within the same row are significantly different ($p < 0.05$).

Aspergillus niger is widely used in SSF for lignocellulosic enzyme production [4,22,27,28]. The utilization of lignocellulosic materials as solid substrates in SSF can induce the production of lignocellulosic enzymes, which results in the conversion of the lignocellulosic matrix into single sugars that are easily metabolized by microorganisms [1,27]. Furthermore, some studies already reported the use of OC [4,22] and SC [27] as solid substrates for SSF processes with *A. niger*, showing the potential of the substrate mixture used in the present work for fungal growth. Moreover, a 9-fold increase in lignocellulosic enzymes was observed from the second to the fourth day of SSF, highlighting xylanase activity that reached 290 U/g after 4 days of SSF (Table 1). The fact that OC, which corresponds to 50% of the substrate mixture, have higher content in hemicellulose in comparison to cellulose [4] could induce the production of xylanase. Lipase activity increased by 2-fold from the second to the fourth day of SSF; however, this enzyme had the lowest activity detected in these experiments. While the residual oil present in OC could induce lipase production by the filamentous fungi, SSF with *Aspergillus* species for lipase production are often carried for a minimum of 7 days [29,30], which explains the low lipase production observed. Moreover, the low protein content in OC may have a negative effect on protease activity because the production of this enzyme by *A. niger* is induced in protein-rich substrates [31]. Regardless of the high activities of the enzymes cellulase and xylanase, the concentration of reducing sugars, which was around 58 mg/g in the substrate mixture prior to *A. niger* inoculation, decline throughout SSF. This result revealed that the sugar consumption rate by *A. niger* was higher than the sugar release by the action of the lignocellulosic enzymes; thus, an increase in sugar concentration was not detected. Similarly, before *Y. lipolytica* inoculation in pre-fermented okara with the fungi *Rhizopus oligosporus* for 24 h, a decrease in sugar concentration was also observed [32]. In contrast, an increase in reducing sugars after 72 h of SSF with *Mucor flavus* was reported using a mixture of okara and buckwheat husk prior to substrate sterilization and *Y. lipolytica* inoculation [8]. Likewise, the release and consumption of reducing sugars by filamentous fungi in SSF is likely dependent on fungal species and the by-products selected as solid substrates. In spite of the reducing sugar consumption by *A. niger*, the fungal growth in the substrate mixture could release other biomolecules, such as proteins [4], that could improve microbial growth.

3.2. Simultaneous or Sequential SSF with Y. lipolytica and A. niger

Co-culture or sequential SSF with *Y. lipolytica* and filamentous fungi species have been used for substrate biotransformation and biocompounds production [8,32]. In the present work, two strategies were employed to evaluate the effectiveness of co-culture for lipase production by *Y. lipolytica*: a sequential SSF, where *A. niger* CECT 2915 was first inoculated in the substrate mixture and, after two days of SSF, yeast inoculation was performed, and a simultaneous co-culture with the inoculation of the two microorganisms at the same time (Table 2).

Table 2. Enzymatic activities after SSF with a mixed culture of *A. niger* 2915 and *Y. lipolytica* W29 using different co-culture strategies.

Parameters	SSF	
	Sequential	Simultaneous
Maximum lipase (U/g)	8 ± 2^{a}	49 ± 7^{b}
Protease (U/g)	16 ± 1^{a}	12 ± 2^{b}

Values represent the mean and SD from two independent experiments. Values with different letters within the same row are significantly different ($p < 0.05$).

In the sequential SSF, (8 ± 2) U/g of lipase activity was attained in the second day after yeast inoculation (Table 2) and, despite the 2-fold increase in this enzyme activity in comparison to *A. niger* monoculture (Table 1), the production in these conditions was very low. Inoculation of the filamentous fungus two days before *Y. lipolytica* decreased free reducing sugars, as shown above, in spite of the carbohydrases production that contribute to assimilable sugars release. However, sugar concentration in the medium is the balance of the sugars released and sugar consumed by microorganisms. The competition for substrate and other nutrients between the yeast cells and the fungus may limit yeast growth and lipase production because the most active phase of fungal growth seems to be from the second to fourth day of fermentation (Table 1). For this reason, a simultaneous co-culture was performed and, in these conditions, lipase activity reached (49 ± 7) U/g in the second day of SSF, corresponding to a 6-fold increase compared to the two-phase strategy (Table 2). It appears that lipase secretion observed in these experiments is attributed to *Y. lipolytica* because lipase detected after SSF with a monoculture of *A. niger* CECT 2915 for two days was 96% lower (Table 1). Moreover, protease activity in the simultaneous co-culture reached (12 ± 2) U/g after two days of SSF and, in the sequential SSF, an increase of 33% in the activity of this enzyme was registered, which could have also contributed to the lower lipase activity detected in these conditions because lipase can be degraded by proteolysis. In the present study, despite the 6-fold improvement on lipase activity comparing the sequential SSF and the simultaneous co-culture, a standard assay with a monoculture of *Y. lipolytica* W29 was performed and higher lipase activity was achieved, reaching (99 ± 1) U/g, which is similar to the results previously reported with this substrate mixture [9]. These results showed that the mixed cultures of *A. niger* CECT 2915 and *Y. lipolytica* W29 in the co-culture strategies tested in this work did not favour lipase production; thus, application of other pretreatments in the substrate mixture before SSF should be considered.

3.3. Enzymatic Hydrolysis Pretreatment of the Optimum Substrate for Lipase Production

Because mixed cultures with *Y. lipolytica* W29 and *A. niger* CECT 2915 did not improve lipase production by the oleaginous yeast, a different strategy was used to degrade the cellulose and hemicellulose fractions of the substrate mixture and increase sugar availability prior to *Y. lipolytica* inoculation. For this purpose, enzymatic hydrolysis of the substrate mixture was performed using a crude enzymatic extract produced by *A. niger* CECT 2915, as described above; a commercial cellulase was used as a control. The influence of cellulase concentration and incubation time was tested and the time course of reducing sugar release after enzymatic hydrolysis are represented in Figure 2.

Overall, an increase in reducing sugar concentration was obtained after only 6 h of enzymatic hydrolysis in all the conditions tested (Figure 2). However, higher sugar release was observed when the enzymatic crude extract from *A. niger* was used in comparison with the commercial cellulase. This commercial enzyme only hydrolyses endo-1,4-β-D-glycosidic linkages in cellulose and cellooligosaccharides. In contrast, the crude enzymatic extract used in this work has different types of cellulases and other hydrolytic enzymes, resulting in increased reducing sugar release. In fact, besides the endoglucanases (carboxymethyl cellulases) measured herein, β-glucosidase and xylanase were also found in extracts of the fermented mixture [21]. Regarding the results using *A. niger* crude extract, sugars release

was mainly observed in the first 12 h of the hydrolysis process. Indeed, a 46% increase in reducing sugars concentration was observed when the enzymatic extract amount was adjusted to 100 U/g and 150 U/g of cellulase in comparison to the lowest concentration, corresponding to a 3-fold increase of reducing sugars in the substrate mixture after 12 h of treatment. A crude enzymatic extract produced by *A. niger* was also successfully used for enzymatic hydrolysis with high solid loadings of brewer's spent grain and grape pomace, showing the potential of using crude extract in hydrolysis processes before SSF [3]. Because increasing cellulase concentration to 150 U/g or extending enzymatic hydrolysis above 12 h did not increase sugars release, the crude enzymatic extract with a cellulase concentration of 100 U/g and 12 h of incubation time were selected for further experiments. After 12 h of incubation with the crude enzymatic extract, fiber content decreased by 20% in the substrate mixture (Table 3), showing that the enzymatic extract obtained by SSF with *A. niger* effectively degraded the lignocellulosic matrix of the mixture of OC and SC. This outcome is in accordance with reports showing a reduction in fiber content after SSF with *Aspergillus* species [27,33]. Additionally, enzymatic hydrolysis resulted in a 42% increase of glucose in the extracts, and a 3-fold increase in xylose concentration compared to the substrate mixture without pretreatment (Table 3). The differences in the release of these sugars could be related to the production of high levels of xylanase by *A. niger* in the ternary mixture of oil cakes used for the enzymatic crude extract production [21]. Hemicellulose degradation by xylanases could also justify the significant decrease ($p < 0.05$) in NDF (Table 3); the decrease of ADF from the untreated to the hydrolyzed substrate mixture is statistically significant, with a confidence level of 94%.

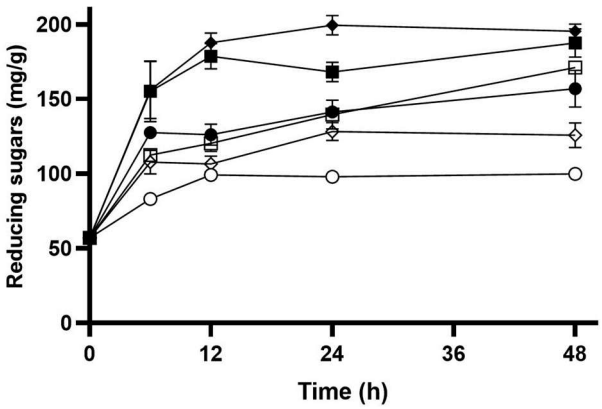

Figure 2. Time course of reducing sugars released after enzymatic hydrolysis with a crude enzymatic extract produced by *A. niger* CECT 2915 (filled symbols) and a commercial cellulase (empty symbols) with a cellulase concentration of units per dry mass of substrate adjusted to 50 U/g (●,○), 100 U/g (♦,◊) and 150 (■,□) U/g. Values represent the mean and SD of two independent experiments.

Table 3. Characterization of the substrate mixture pretreated with a crude enzymatic extract for 12 h with a cellulase concentration of 100 U/g.

Parameters	Time (h)	
	0	**12**
NDF (%)	52 ± 1 [a]	41 ± 3 [b]
ADF (%)	35 ± 2 [a]	28 ± 1 [a]
Glucose (mg/g) *	36 ± 8 [a]	51 ± 1 [b]
Xylose (mg/g) *	9 ± 2 [a]	26 ± 1 [b]

* Sugars quantified in the aqueous extract after extraction of the pre-hydrolyzed substrate mixture. NDF: neutral detergent fiber; ADF: acid detergent fiber. Values represent the mean and SD from two independent experiments. Values with different letters within the same row are significantly different ($p < 0.05$).

In the present study, enzymatic hydrolysis was successfully performed with a high solid concentration of 25% (*w/w*, wet basis), resulting in a 3-fold increase in free reducing sugars in the substrate mixture. Water is essential in hydrolysis reactions, and increasing the solid loading in enzymatic hydrolysis of the lignocellulosic biomass may have a negative impact on the conversion of the lignocellulosic matrix into fermentable sugars [34]. However, the implementation of enzymatic hydrolysis processes with high solid loadings have additional advantages, such as low water usage and reduction in the production costs [34]. The fact that the moisture content used in enzymatic hydrolysis was similar to that used in SSF with *Y. lipolytica* W29 allowed for substrate utilization with minimal processing steps.

3.4. SSF of Enzymatically Pretreated Substrate for Lipase Production by Y. lipolytica

A pre-hydrolyzed mixture of OC and SC was fermented with *Y. lipolytica* W29 for two days, and the lipase activity registered in these conditions was very low (Table 4).

Table 4. Lipase activity, cellular density and pH values obtained after SSF for two days with a mixture of OC and SC with and without enzymatic hydrolysis prior to *Y. lipolytica* W29 inoculation.

Parameters	Pretreated Substrate	Unpretreated Substrate	
		Water	Citrate Buffer pH = 4.8
Lipase activity (U/g)	5 ± 1^{a}	78 ± 2^{b}	60 ± 6^{c}
Cellular density (mg/g)	43 ± 3^{a}	18 ± 2^{b}	15 ± 2^{b}
pH	5.39 ± 0.04^{a}	6.8 ± 0.1^{b}	5.8 ± 0.1^{c}

Values represent the mean and SD from two independent experiments. Values with different letters within the same row are significantly different ($p < 0.05$).

While distilled water was used to adjust the moisture content in the substrate mixture of OC and SC in SSF for lipase production in most of the experiments in the above sections, in these experiments, citrate buffer with a pH of 4.8 was used to guarantee suitable conditions for enzymatic hydrolysis before SSF. Thus, to examine if the low lipase activity observed after enzymatic hydrolysis was related to the changes in the substrate mixture pH, SSF was performed in the mixture of OC and SC without enzymatic hydrolysis using water and citrate buffer (pH 4.8) to adjust the moisture content. It appears that the use of citrate buffer had some influence on lipase secretion because a 29% reduction on lipase activity was observed when the buffer was used instead of water in SSF with the untreated substrate mixture. However, an 11-fold decrease in lipase activity was attained when enzymatic hydrolysis was performed before SSF, compared to the untreated mixture (moistened with citrate buffer), revealing that low pH in these fermentations may not be the only reason for low lipase detection. Another aspect considered in these experiments was the effect of enzymatic hydrolysis on the cell concentration obtained after SSF. Although a similar cellular growth was observed in SSF without enzymatic pretreatment, an almost 3-fold increase in yeast cell concentration was observed when the substrate mixture was pretreated with the fungal enzymatic extract. This outcome could be related to the increased reducing sugars available in the substrate mixture, which improved cellular growth in detriment of biocompound production. To further understand the effect of enzymatic hydrolysis on lipase production, kinetics of SSF was performed for up to 5 days, and the results are represented in Figure 3.

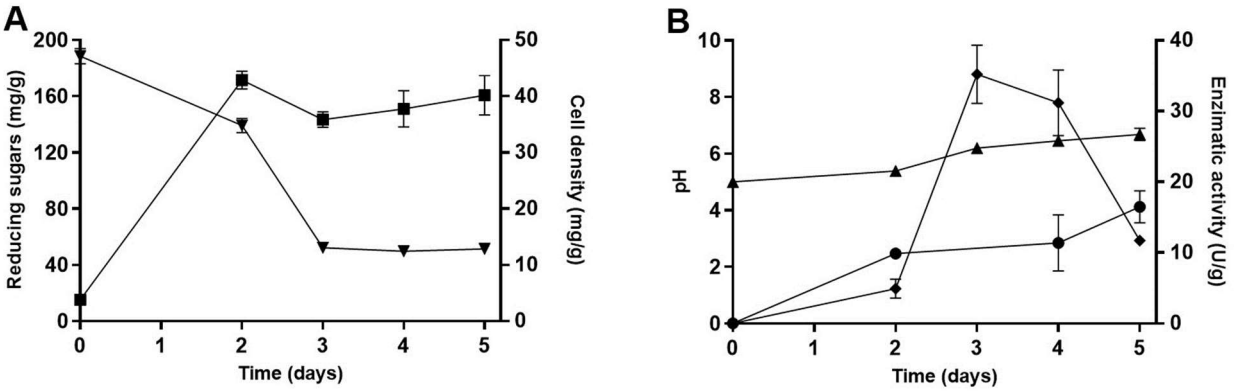

Figure 3. Time course of (**A**) cellular density (■) and reducing sugar concentration (▼), (**B**) activity of lipase (♦) and protease (●) and pH of the solid moistened medium (▲), obtained during SSF with *Y. lipolytica* W29 with a 50% (*w/w*) mixture of OC and SC that was pretreated with an enzymatic extract produced by *A. niger*. Values represent the mean and SD of two independent experiments.

The maximum cellular concentration was attained at the second day of fermentation, and cellular growth stabilised until the end of the experiments (Figure 3A) at higher values than the ones reported in previous work on SSF without pretreatment [9]. Likewise, the 3-fold increase in reducing sugars concentration in the substrate mixture after enzymatic hydrolysis improved *Y. lipolytica* growth. The sugar availability in the substrate mixture allowed *Y. lipolytica* to metabolize around 137 mg/g of reducing sugars. After the third day of SSF, a residual value of reducing sugars was maintained until the end of the experiments, possibly corresponding to monomers that were released during enzymatic hydrolysis, that *Y. lipolytica* is unable to efficiently assimilate. Concerning lipase activity, a 7-fold increase was observed between the second and third days of SSF (Figure 3B). After reaching its maximum on the third day of cultivation, lipase activity decreased and, by the end of SSF, 34% of this enzyme activity was still detected. In these conditions, a decrease in lipase productivity (U/g/day) was observed because the maximum activity was attained later in the SSF process. In particular, 39 U/g/day was obtained in the two-day standard assay with the untreated substrate mixture (Table 4) and a 69% reduction in lipase productivity was observed after substrate hydrolysis. The delayed peak could be explained by the higher concentration of reducing sugars in the SSF medium because lipase secretion into the extracellular medium occurs when the concentration of carbon sources are low [12]. In experiments with *Y. lipolytica* CB7504 cultivated in a medium containing both glucose and olive oil, lipase production peak was delayed and lower values were obtained compared to medium only supplemented with olive oil [35]. While *LIP2*, the gene coding for the most produced extracellular lipase in *Y. lipolytica*, is induced in the presence of oily substrates, it appears that the presence of glucose in the culture medium has a repressive effect on this gene expression [36]. In fact, it has been observed that hexokinase, which is responsible for the phosphorylation of hexoses in the early stage of the glycolytic pathway, is involved in the repression of *LIP2* in *Y. lipolytica* [37]. Even though glucose was present in the substrate mixture before enzymatic hydrolysis, the 42% increase in glucose concentration could be sufficient to alter *Y. lipolytica* metabolism. Moreover, the increase of other reducing sugars' concentrations besides glucose could also play a role in the decreased lipase production observed in this work. Previously, pH values increased until the end of cultivation, with the untreated substrate mixture reaching values above 8 [9]; however, the pH in the present study was kept below 7 until the end of SSF. Furthermore, an increase in medium pH is often related to protease release, leading to lipase degradation by proteolysis both in solid state and submerged fermentations [14,38]. Despite the lower pH observed in this work, protease activity was also detected in the aqueous extract obtained after SSF (Figure 3B). *Yarrowia lipolytica* secretes two enzymes with proteolytic activity, and their secretion is influenced by the medium pH. While the most secreted protease is induced by alkaline

pH, an acid protease is also secreted by the oleaginous yeast [39]. Thus, the lower pH after enzymatic hydrolysis still induced protease production by *Y. lipolytica*, and the activity of this enzyme increased until the end of SSF, possibly resulting in lipase degradation.

Regardless of the lower lipase activity detected in the pretreated mixture of 50% (*w/w*, dry basis) OC and SC, the results obtained in this study showed the great potential of employing crude extracts produced by filamentous fungi in enzymatic hydrolysis processes without any enzyme purification steps. Furthermore, these conditions favoured yeast biomass production, which could result in oil cake biotransformation and improvement of the nutritional value of these materials enriched with microbial protein. Moreover, the fact that the hydrolysis was successfully performed in a high solid loading system allowed the obtention of a solid substrate mixture with increased free and easily assimilable sugarss through an economic and eco-friendly process.

3.5. SSF and Semi-Solid Fermentation after Physical Pretreatments for Lipase Production by Y. lipolytica

Physical pretreatments are often applied prior to enzymatic hydrolysis, resulting in higher yields of fermentable sugars that can be subsequently used in biotechnological processes for value-added compound production [20]. However, these pretreatments can also be beneficial before SSF because the changes in the solid substrate can increase the access of microorganisms to soluble compounds and microbial enzymes to their substrates. Thus, in the present study, the substrate mixture of OC and SC with a solid loading of 25% (*w/w*) was placed in an ultrasonic bath for 15 min or was submitted to microwave irradiation for 2 min before SSF for 2 days with *Y. lipolytica* W29 (Table 5).

Table 5. Effect of physical pretreatments on the release of reducing sugars before SSF, cellular growth and enzyme production by *Y. lipolytica* W29 after 2 days of SSF.

Parameters	Physical Pre-Treatment		Untreated Substrate
	Ultrasound	Microwave	
Reducing sugars released (mg/g)	67 ± 6^{a}	64 ± 1^{a}	58 ± 1^{a}
Cellular concentration (mg/g)	18 ± 2^{a}	26 ± 6^{b}	18 ± 1^{a}
Maximum lipase production (U/g)	$56 \pm 9^{a,b}$	63 ± 4^{b}	54 ± 2^{a}
Protease production (U/g)	9 ± 3^{a}	3.24 ± 0.01^{a}	6 ± 2^{a}

Values represent the mean and SD from two independent experiments. Values with different letters within the same row are significantly different ($p < 0.05$).

Microwave and ultrasound pretreatments resulted in 10% and 16% increases in reducing sugars content in the substrate mixture, respectively, compared to the untreated mixture (Table 5), although the values are not statistically different. These values are significant lower compared to the sugar release observed after enzymatic hydrolysis (Figure 2) because the severity of the pretreatments was not enough to hydrolyse the polysaccharides from the lignocellulosic matrix. Ultrasound pretreatment had no effect on cellular growth; however, when the substrate mixture was irradiated with microwaves, a 44% increase in this parameter was obtained (Table 5). Moreover, the pretreatments used in this work improved lipase production, highlighting the results obtained with microwave pretreatment, which resulted in a 1.2-fold enzyme activity increase in comparison to SSF with the untreated substrate mixture. In a previous study, a 3 min microwave pretreatment of rice hulls and wheat bran before SSF did not significantly alter cellulase production by *Trichoderma* sp. [5]. Additionally, the authors reported that improved enzyme production was only achieved when microwave was combined with an alkaline pretreatment. In the present work, microwave pretreatment was enough to increase lipase production by *Y. lipolytica* W29, resulting in less downstream processing of the solid materials after pretreatment and before SSF. Regarding protease activity, the pretreatments had no effect on this enzyme activity because the differences observed are not statistically significant.

Besides pretreatment with an ultrasonic bath, the substrate mixture at a solid loading of 10% (*w/w*) in water suspension was also pretreated with an ultrasonic probe. This semi-solid mixture was composed of 10.8 g/L of reducing sugars, and the pretreatment led to a slight increase in sugar content (12.2 g/L) after 6 min; extending pretreatment time to 10 and 15 min did not improve reducing sugars release. Although the sonication did not result in significant changes in the reducing sugars concentration in the mixture of OC and SC, it is possible that this pretreatment improved the accessibility of *Y. lipolytica* W29 to other biomolecules present in the substrate. Thus, ultrasound pretreatment for 6 min was applied to the substrate mixture before yeast inoculation. As previously stated, to perform these experiments, the amount of water added to the substrate was higher than in the experiments of SSF, resulting in a semi-solid mixture of OC and SC. Moreover, to minimize the complexity of the bioprocess, which would require the drying of the pretreated substrate followed by moisture adjustment before SSF, the semi-solid mixture was directly inoculated with *Y. lipolytica* W29 after a sterilization step and fermentations were carried for 30 h (Figure 4). A semi-solid fermentation with an untreated semi-solid mixture was performed as a control.

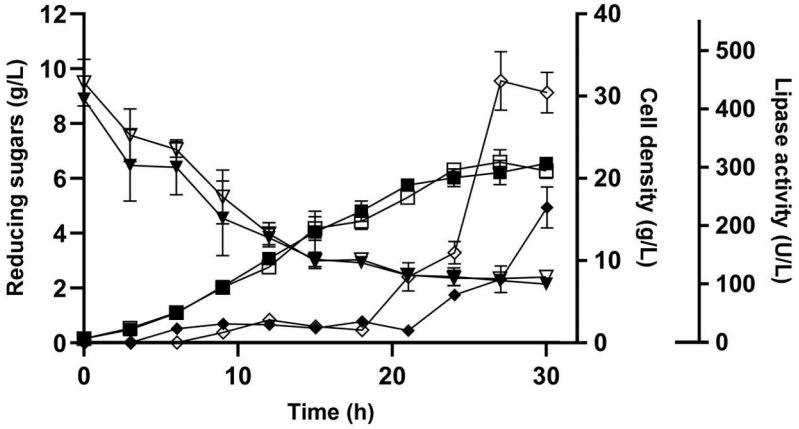

Figure 4. Time course of cell growth (■,□), reducing sugar concentration (▼, ▽) and lipase activity (♦,◊) after semi-solid fermentation with a 50% (*w/w*, dry basis) mixture of OC and SC pretreated with ultrasonic irradiation for 6 min (filled symbols) and the control assays without substrate pretreatment (empty symbols) for 30 h.

The majority of reducingsugars consumption occurred in the first 15 h of cultivation in both untreated and pretreated mixtures (Figure 4). Similar to what occurred in SSF (Figure 3B), a residual value of reducing sugars was detected until the end of the fermentation process. Moreover, ultrasound pretreatment did not result in changes in yeast cell growth with respect to the untreated semi-solid mixture. In particular, after 30 h of cultivation, yeast cells had already entered a stationary phase and a final cell biomass of around 20 g/L was obtained in both conditions tested. In SSF after ultrasonic pretreatment (Table 5), the 18 mg/g of yeast biomass obtained after two days of SSF corresponds to 2.3 g/L; thus, an almost 10-fold increase in cell concentration was achieved in semi-solid fermentation. In these experiments, oxygen transfer was favoured by medium agitation, which could explain the high yeast biomass production in semi-solid fermentation in comparison to SSF. Moreover, the high amount of water in these conditions and the agitation of the slurry medium could increase the solubilization of the substrate mixture, also increasing bioavailability of compounds that could be metabolized by the yeast. Besides reducing sugars and lipids, *Y. lipolytica* may also metabolize phenolic compounds present in the oil cakes used in these experiments [9]. In fact, this yeast has been used to reduce the content of phenolic compounds in olive mill wastewater [40,41]. Additionally, other compounds such as alcohols and organic acids could be present in this substrate mixture, contributing to *Y. lipolytica* growth. Lipase activity quantification was performed and, regardless of

the pretreatment used in the semi-solid mixture, lipase secretion occurred mostly at the end of the experiments (Figure 4). These results are in agreement with a previous study, which showed that lipase secretion to the culture medium by *Y. lipolytica* begins when carbon sources become limited [42]. In the control condition, lipase activity increased until the end of semi-solid fermentation, reaching its maximum value of 233 U/L. Conversely, ultrasound pretreatment improved lipase activity, which peaked after 27 h of fermentation and was 2-fold higher than the maximum lipase activity detected in the control assay. Thus, it is possible that the application of ultrasounds in the mixture of OC and SC could increase the bioavailability of lipids in the substrate mixture and the fact that, in these experiments, the semi-solid mixture was constantly agitated improved the access of *Y. lipolytica* to these lipase inducers. Protease activity was quantified by the end of these experiments and 1576 U/L were obtained with the untreated semi-solid mixture. Furthermore, protease activity in the experiments with the pretreated semi-solid mixture was 21% lower, which could also contribute to the higher lipase activity detected in these conditions.

After semi-solid fermentation, the content of the flasks was dried without yeast biomass separation and the remaining solid was characterized to understand the influence of *Y. lipolytica* growth in substrate composition (Table 6).

Table 6. Effect of semi-solid fermentation on crude protein and total lipids, expressed as % *w/w* (dry basis) and lipid composition on long-chain fatty acids (LCFAs) expressed as % of each LCFA per total detected.

Parameter (%)		Semi-Solid Fermentation		
		Unfermented	**Pretreated by US**	**Untreated**
Crude protein		22 ± 1^{a}	20 ± 1^{a}	20 ± 1^{a}
Lipids		6.1 ± 0.4^{a}	3 ± 1^{b}	3.0 ± 0.3^{b}
LCFAs	C16:0	14 ± 1^{a}	12.2 ± 0.4^{b}	12.1 ± 0.4^{b}
	C16:1	0.6 ± 0.1^{a}	2.0 ± 0.2^{b}	2.1 ± 0.3^{b}
	C17:1	0.2 ± 0.1^{a}	ND	0.2 ± 0.1^{a}
	C18:0	2 ± 1^{a}	1.9 ± 0.4^{a}	1.9 ± 0.4^{a}
	C18:1	62 ± 1^{a}	59.3 ± 0.4^{a}	59.7 ± 0.8^{a}
	C18:2	21 ± 1^{a}	25 ± 1^{b}	24 ± 1^{b}

Values represent the mean and SD from two independent experiments. Values with different letters within the same row are significantly different ($p < 0.05$). LCFA: long-chain fatty acids; ND: not detected.

While yeast growth had no impact on crude protein content, a 50% reduction in lipid content was observed after 30 h of incubation with respect to the mixture without inoculation (0 h) (Table 6). Lipase production by *Y. lipolytica* and the ability of these enzymes to hydrolyse triacylglycerol [6] can explain the decrease in lipid content in the fermented solid. Using the same substrate mixture, SSF with *Y. lipolytica* W29 for two days resulted in a 29% decrease in lipid content [9], showing that in semi-solid fermentation, yeast cells were able to reduce the lipidic content of the substrate mixture more effectively in a shorter period of time. Moreover, SSF with *Y. lipolytica* NBRC-10073 resulted in a lipid content reduction in fish by-products, demonstrating the potential of this microorganism for by-product biotransformation [43]. Moreover, the profile of long-chain fatty acids (LCFAs) were also analyzed after semi-solid fermentation (Table 6). The substrate mixture composed of OC and SC used in this work is characterized by a high oleic acid (C18:1) content, a fatty acid present in high percentages in the by-products obtained after olive oil extraction [44]. Moreover, this value is followed by linoleic acid (C18:2), showing that more than 80% of the fatty acids in this substrate mixture are unsaturated fatty acids. Semi-solid fermentation resulted in a 3.3- and 3.5-fold increase in palmitoleic acid (C16:1) in the substrate mixture treated with ultrasounds and in the untreated mixture, respectively. Moreover, yeast cultivation for 30 h also resulted in around a 16.5% increase in linoleic acid. Although the

contents of heptadecanoic acid (C17:1), stearic acid (C18:0) and oleic acid were unaffected by semi-solid fermentation, this bioprocess resulted in a 14% reduction in palmitic acid (C16:0) percentage.

Fermentations can be used to improve the nutritional properties of agro-industrial by-products before their incorporation into animal feed formulations [27]. Moreover, oilseeds are often used as fat supplementation in some feedstocks to increase the intake of some unsaturated fatty acids, such as oleic and linoleic acids [45]. In the present study, a slight increase in the percentage of unsaturated fatty acids was observed regardless of the utilization of ultrasonic pretreatment. Furthermore, because the content of saturated fatty acids suffered a 10% reduction, the fermented substrate presented a higher ratio of unsaturated to saturated fatty acids, resulting in a nutritional enhancement of the mixture of oil cakes and showing its potential application in animal feed formulations.

4. Conclusions

In the present study, biological and physical pretreatments were used to increase sugar availability and substrate accessibility in a 50% (*w/w*, dry basis) mixture of OC and SC before SSF with *Y. lipolytica* W29 for lipase production. The co-culture strategies employed in this work with the filamentous fungi *A. niger* CECT 2915 did not improve lipase production by the oleaginous yeast. Moreover, enzymatic hydrolysis with a crude enzymatic extract at a high solid loading resulted in high reducing sugars release, which were effectively used by *Y. lipolytica* W29 in SSF, improving yeast growth in comparison to the standard experiment with the untreated substrate mixture. However, in these conditions, a delay on lipase activity peak was observed. Conversely, physical pretreatments before SSF had a positive effect on lipase production, with a highlight to microwave irradiation that not only led to an increase in lipase activity but also resulted in improved cellular growth. Semi-solid fermentation was successfully performed with this substrate mixture and ultrasonic pretreatment led to a 2-fold increase in lipase production. Yeast growth in semi-solid fermentation increased the percentage of unsaturated fatty acids and, in contrast, a reduction of saturated fatty acids was observed; thus, the lipidic profile of the substrate mixture was improved. These results revealed that pretreatments before SSF can improve nutrients accessibility in the substrate, playing an important role on microbial growth and added-value compound production.

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