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Production of proteases from organic wastes by solid-state fermentation: downstream and zero waste strategies

Short title: Down-stream of proteases produced by SSF

Maria Marín, Adriana Artola*, Antoni Sánchez

Composting Research Group

Department of Chemical, Biological and Environmental Engineering

Universitat Autònoma de Barcelona

Bellaterra, 08193-Barcelona (Spain)

*Corresponding author: Adriana Artola

Universitat Autònoma de Barcelona, Departament d'Enginyeria Química, Biològica i Ambiental, Escola d'Enginyeria, Edifici Q, Campus de Bellaterra, 08193- Cerdanyola del Vallès, Spain.

Tel.: +34 935814480

Fax: +34 935812013

Email address: adriana.artola@uab.cat

Abstract

Production of enzymes through solid-state fermentation (SSF) of agro-industrial wastes reports high productivity with low investment. The extraction of the final product from the solid waste and solid disposal represent the main cost of the process. In this work, the complete downstream processes of SSF of two industrial residues for the production of proteases, soy fibre (SF) and a mixture of hair and sludge (HS), were studied in terms of activity recovery, using different extraction parameters (extracting solvent, ratio solid:solvent and extraction mode). Activity after lyophilisation was tested. Solid waste valorisation after extraction was studied using respiration techniques and biogas production tests, as part of a zero waste strategy.

Results showed a maximum extraction yield of 91% for SF and 121% for HS, both in agitated mode and distilled water as extraction agent. An average activity recovery of 95 ± 6 % and 94 ± 6 % for SF and HS respectively was obtained after lyophilisation and redissolution. To reduce the cost of extraction, a ratio 1:3 w: v solid-solvent in static mode is advised for SF, and 1:2 w:v extraction ratio in agitated mode for HS, both with distilled water as extracting agent. Both composting and anaerobic digestion are suitable techniques for valorisation of the waste material.

Keywords: downstream, extraction, protease, organic wastes, solid-state fermentation, zero-waste.

Introduction

The use of enzymes in industrial processes is growing every day. They are used, for example, in detergent manufacturing, bioethanol production and food and beverage processing. Their use versus traditional chemical processes allows reducing manufacturing costs and waste generation, also avoiding the need of harsh operational conditions, as high pressure or temperature (Singh et al. 2016).

Two main processes are used to produce enzymes, solid-state fermentation (SSF) and submerged fermentation. In recent years, the use of SSF has become outstanding due to its higher yield and the requirement of simpler and smaller equipment (Kriaa et al. 2016). As substrates, literature provides a wide range of residues, the most popular being agro-industrial residues, like the coffee pulp waste used by Kandasamy et al. (2016) to produce protease. These residues are adequate as a substrate due to their low cost and availability. Besides, several microorganisms have been reported to produce all types of enzymes when inoculated in sterile conditions on an adequate substrate (Riyadi et al. 2017, Lizardi-Jiménez and Hernández Martínez, 2017). In fact, the main part of the studies carried out in solid state and submerged fermentation are focused on the improvement of the production yield by optimizing fermentation parameters as temperature, particle size, moisture or selecting the right strain (Sun et al. 2011; Karpe et al. 2015).

However, several issues regarding operational conditions still need to be solved to ensure a successful scale-up of the SSF processes, like overheating problems (Finkler et al. 2017), or optimization of aeration and agitation modes (Gassara et al. 2013). Also, since most of the optimal working temperatures of SSF microorganisms are around 30ºC, questions like moisture and temperature are critical in the scale-up of SSF

processes. Despite the good yields obtained, SSF is mainly being developed at lab scale (El-Bakry et al. 2015).

Apart from all the technical issues mentioned above, the assessment of the performance of the entire process at industrial scale, results crucial to determine its profitability. Activity losses can occur during extraction due to a low contact area between solid and solvent (Zhang and Sang. 2015), and thus inefficient mass transfer, or due to stability of the enzyme, which is mainly affected by pH and temperature (Silva et al. 2014). Storage of the crude extract can also lead to activity losses through time or during purification (Negi et al. 2011) or lyophilisation stage (Mensink et al. 2017). Also, the disposal of solid biodegradable residues generated after extraction should be studied. These residues still contain a considerable amount of biodegradable matter, a resource to obtain biogas or organic amendments. Despite the relevance of having a better understanding of downstream and global performance of SSF processes, to our knowledge, no complete study at pilot scale has been published.

Some data obtained at lab scale are provided by Rashid et al. (2013), where mannose extraction from 0.9 kg of palm kernel cake was studied, finding that soaking time, nature of solvent, physical state, solid to solvent ratio and number of washes have great influence on enzyme recovery. Chaithanya et al. (2012) also extracted protease from fermented bran, finding that the optimum leaching conditions were glycerol and tap water as solvent, contact time 60 min and agitation at 100 rpm. Both authors obtained the highest recovery yield when agitation was applied and the solvent was a mixture of water and glycerol. A solid-solvent ratio of 1:5 (w: v) was also determined as optimal in both cases. The most influential parameter was the ratio solid-solvent, which produced the highest difference within the maximum and the minimum activity recovery in the extraction of protease.

In previous works by Abraham et al. (2013) and Abu Yazid et al. (2016), the listed problems of SSF processes at pilot scale were overcome, developing and describing robust SSF processes at pilot scale. The operational strategy was based on allowing the native consortia of microorganisms present in the residue to develop, so inoculation and sterilization would not be necessary. Protease activity obtained in those works was remarkably higher than those previously reported and with simpler operational conditions.

Therefore, the aim of this work is to provide a complete picture of the downstream process of two SSF processes for protease production at pilot scale, including activity recovery from enzyme extraction, lyophilisation of the crude extract and activity conservation after dissolving the lyophilised. Downstream processes for two different wastes, soy fibre waste and cow hair, were studied. Influence of extraction parameters (w:v ratio, type of solvent and presence/absence of agitation) was studied to determine the activity recovery obtained with each strategy. Operational conditions were selected considering their suitability for industrial scale. Regarding the management of the substrate after extraction, its potential as feedstock for biogas production was assessed, along with the possible transformation of the solid into an agricultural organic amendment according to its stability for soil application in a zero waste strategy.

Materials and Methods

Raw material

Two wastes were assessed for the extraction of protease after SSF, soy fibre (SF), from a soy beverage manufacturing plant in Castellterçol (Barcelona, Spain), and cow hair,

from a local tannery industry located at Igualada (Barcelona, Spain). Cow hair was mixed with dehydrated sludge from the WWTP (wastewater treatment plant) of Igualada (Barcelona, Spain) as inoculum for the SSF process in a ratio 1:2 hair: sludge (w: w) (HS), and moisture was adjusted to 65%. Neither inoculation nor moisture adjustment was needed for SF as the residue possesses a native population of microorganisms and adequate water content. In both cases, wood chips from a composting plant in Manresa (Barcelona) were used as bulking agent in ratio 1:1 (v: v). SF average particle size is between 800 µm and 0.074 µm while cow hair particle size is about 1 cm long. 79% in weigh of bulking agent particle size is between 16 mm and 3.15 mm, Soy fibre was stored at -20ºC before the experiments, while fresh sludge and hair were stored at 4ºC due to the structural changes that freezing produces in fresh sludge. The suitability of these industrial residues as raw materials for protease production through SSF has been described by Abraham et al. (2013, 2017) and Abu Yazid et al. (2016), where details of the process and proteases production range are thoroughly provided.

SSF Materials and experimental set up

SSF was carried out in 10 L and 50 L (working volume) adiabatic reactors, in order to minimize environment influence, as described in detail in previous works (Figure 1) (Maulini-Duran et al. 2014; Puyuelo et al. 2010). Reactors consist in a Dewar-glass with an outside metallic coverage and an adjusted cap to close the container hermetically. The inward part of the cap is covered with isolating material. In the bottom of the glass, a double plastic net was placed to provide support to the fermentable waste, create a space for lixiviates and distribute evenly the inlet air-flow. In the cap, there are three connexions, two for inlet and outlet gases and the last for a temperature prove. Inside

the reactor, a plastic pipe conducts the inlet air to the bottom of the reactor, below the plastic net. The oxygen content of exhausted gases was measured by an electrochemical oxygen sensor and data were collected using a personal computer. Data analysis was carried on by non-commercial software called Sensor. Temperature and air flow were also measured. A control system for air-flow based in oxygen content in the outlet gases was used, stablishing an upper and lower set point of 12.5% and 11.5% oxygen content and variation of volumetric air-flow between values of 300 and 1000 mL min^{-1}, assuring that oxygen was always above 10% in exhaust gases. Reactors capacity (mass) was 3.8 kg of SF and 2.8 kg of HS for 10 L reactors and 17.5 kg of HS and 20 kg of SF for 50 L reactors, respectively.

Protease activity recovery - Material and experimental set up

Influence of extraction parameters

Extractions were performed to evaluate the recovery of protease activity under different experimental conditions. 200 g of fermented solid from SSF performed in 10 L and 50 L reactors were used in every extraction. The solid and the solvent were in contact during one hour under different regimes, at environment temperature, in this case, around 15 °C, and then the liquid phase centrifuged during 10 minutes at 10000 rpm. Protease activity of the supernatant was measured and referred to a value considering the total protease activity produced.

To obtain the total protease activity, an extraction in agitated mode at 1:5 solid: solvent ratio and HCl-Tris (hydroxymethyl aminomethane) buffer pH 8.1 as solvent was also performed during one hour. The selected ratio was based on Salariato et al. (2010)

169 that extracted polygalacturonase with distilled water at a ratio 1:5. Also, results obtained
170 in this work from consecutive extractions confirm the accuracy of the choice.

171 The experimental parameters assayed were solvent type, extraction mode and w:
172 v solid: solvent ratio. The variations of each parameter were:

173 *Solvent type:* Two different solvents were used to perform extractions, distilled
174 water (DW) and HCl-Tris (hydroxymethyl aminomethane) buffer at pH 8.10
175 (TB).

176 *Extraction mode:* The extraction modes were three: i) no agitation (static mode),
177 ii) orbital agitation at 120 rpm, and iii) circulation of the solvent through a
178 column packed with the waste at 96 mL min^{-1}, which resulted in the renewal of
179 the whole volume of liquid 14 and 7.2 times, for 1:2 w:v and 1:4 w:v extractions
180 respectively, as added volume of solvent in 1:4 extraction is double than for 1:2.

181 *Waste to solvent ratio (weight: volume):* Assayed ratios of solid: solvent were
182 1:1, 1:2, 1:3 and 1:4.

183 The different combinations of these tested variables are shown on Table 2.

184 Experimental set up for static and agitated mode consist of a set of glass
185 containers of different volumes where solvent and solid were placed. Static extraction
186 was performed introducing the fermented solid and the chosen volume of solvent in a
187 beaker and waiting for one hour. To obtain the extract in agitated mode, the same
188 procedure was followed but beakers were placed in a Sony orbital shaker incubator for
189 an hour at 120 rpm and room temperature.

190 The experimental set up used in liquid circulation extraction was composed of a
191 peristaltic pump Watson-Marlow 400L2 with variable rotor speed from 2.5 to 50 rpm
192 and a 0.5 L plastic vessel with two adaptors, one at the top and one at the bottom,
193 connected by plastic tubes to the pump input and output. Inside the vessel, a device was

coupled to let the water fall over the biomass in a drop-shower mode, in order to achieve a homogenous contact with the solid in a percolation mode. Fermented matter was placed inside the vessel and solvent added. The flowrate was set up in the pump and liquid circulation lasted for an hour

Number of extraction stages

Enzyme recovery from consecutive stages was assayed performing four consecutive extractions to the same fermented material. Between extractions, the biomass was drained to reduce its moisture as much as possible. 1:2 solid: solvent w: v ratio was chosen for these experiments. Differences between static/agitated mode and TB/DW were evaluated.

Lyophilisation

15 ml of supernatant obtained after centrifugation of the extraction mixture Solid-DW and Solid-TB were frozen at -80°C and later lyophilized using a Virtis 5L sentry lyophilizer connected to an Edwards vacuum pump RV5 A653_01_903. The protease activity was measured in the dissolved lyophilized solid and the activity recovery calculated.

Zero waste strategies

Anaerobic digestion - Biogas Potential Test (BPT)

Biomass fermented for the production of protease was tested as feedstock for biogas production through anaerobic digestion after extraction. Sludge from an anaerobic

digester of raw sludge from a municipal WWTP in Sabadell (Barcelona) was used as inoculum.

The methodology used was a modified method of the protocol described by the German Institute for Standardization and reported by the Ordinance on environmentally compatible storage of waste from human settlements (2001), detailed by Ponsá et al. (2010) Inoculum and biomass from SSF were mixed in a feed to inoculum (F/I) ratio of 0.5 for both residues and placed in hermetic closed bottles. Each sample was tested in triplicate. The ratio F/I was calculated based on the initial amounts of volatile solids (VS) of substrate and inoculum. According to Ponsá et al. (2010), this ratio is the optimum to maximize biogas production. The content of volatile solids in fermented SF, HS and anaerobic inoculum were measured, obtaining a percentage of 26%, 24%, and 1.5%, respectively. A triplicate with only inoculum was also tested as control and its biogas production subtracted from the sample tests.

All the bottles were placed in an incubator Memmert In750 working at 37ºC for 21 days. The amount of biogas produced was calculated from the biogas pressure, measured by an ISE 30A-01-P vacuum switch, temperature and headspace volume. The bottles were manually agitated before and after measurement, and biogas was periodically released to avoid overpressure.

Representative measures of methane percentage in the biogas were taken at different days of incubation. Percentage of methane and carbon dioxide were measured using a gas chromatograph 5890A with a column 17066_F ParcpackQ (250C), support 100/120, tube 3m 1/8”x 5.5 mm. Initial oven temperature was 70 °C, final oven temperature was 120°C, determination time was 8 min, injector temperature was 150 °C and detector temperature was 180°C. Methane peak was detected at 0.8 minutes. 100 µl of gas sample were required for the analysis.

Composting assays

Solid material after SSF and protease extraction was stabilized in order to test its suitability as soil organic amendment. Experiments were carried out in 50 L and 4.5 L working volume reactors (Figure 1), for HS and SF respectively. Both reactors have been previously described (Sayara et al. 2010; Maulini-Duran et al. 2014). 50 L reactor was a packet bed type reactor, working in almost-adiabatic conditions and made of steel. Similar to the 10 L reactor, the cover had two connectors for the outlet air and the temperature prove, at the bottom of the reactor another connector was placed for the inlet air and also a faucet for lixiviate leaching. The outlet air was conducted through a water trap at 4 °C, and oxygen content measured by an electrochemical sensor. 4.5 L reactor is identical to the 10 L reactor described above but in a smaller size. Composting process was performed with air supplied under OUR control (Puyuelo et al. 2010), a control system based in an algorism which assure maximum O_2 consumption during the experiment. Volumetric flow oscillated between 1300 and 200 mL min^{-1} for HS and between 1000 and 100 mL min^{-1} for SF.

In the case of HS, a drying stage was required after extraction for moisture removal, in order to continue the stabilization. During the drying step, the solid was disposed in thin layer out of the reactor for 24 hours.

Analytical methods

Protease activity determination

Protease activity was determined using an adaptation of the Alef and Nannipieri (1995) method for activity determination in soil (Abraham et al. 2013). According to the

methodology, 1 g of soil is mixed in a falcon tube with 5 mL of HCl-Tris buffer pH 8.1, 5 mL of bovine casein salt (2%) as substrate, covered and incubated for 2 h at 50 °C. During this time, the protease will break the molecules of casein, releasing peptides and free amino acids into the media. In this case, 1 g of soil specified in the methodology was replaced by 1 mL of extract from the SSF and 4 mL of TB were added instead of 5 mL. The conditions for the standard extraction were fixed in a ratio of 1:5 in g of biomass per mL of HCl-tris buffer pH 8.1, agitation mode and environment temperature. The extract was centrifuged 10 min at 10000 rpm and the supernatant was analysed. After the 2 incubation hours, 5 mL of trichloroacetic acid (TCA) (15%) were added in order to precipitate the non-soluble peptides at this TCA concentration. 1.5 mL of an alkaline solution and 1 mL of Folin-Ciocalteu reagent (25%) solution were added to 1 mL of the incubated mixture after centrifugation and then incubated at environment temperature for 1h, in order to perform a colorimetric determination of the amount of tyrosine present, using and spectrometer working at 700 nm. L-tyrosine was used as a standard. So, protease activity was expressed in activity units per gram of dry matter, being an Activity Unit (U) 1 µg of tyrosine released after 1 hour under incubation conditions. Calculations were carried out according to Equation 1.

$$\text{Protease activity (U g}^{-1}\text{DM)} = \frac{C\ 15\ V}{DM} \qquad \text{(Eq 1)}$$

Where C is the concentration of tyrosine expressed as µg of tyrosine per ml in the 15 ml of solution after incubation, 15 is the transformation factor to obtain the concentration in 1 ml of extract; V is the volume of solvent used in the extraction (ml) and DM (g) is the dry matter of fermented solid used in the extraction.

292 *Activity recovery calculation*

293 Activity recovery was calculated referring the activity obtained in the extraction at
294 given conditions as a percentage of the standard extraction according to Equation 2.

295

296

$$\% \text{ Activity recovery} = \frac{100 \times \text{Protease activity (U g}^{-1}\text{ DM)}}{\text{Standard protease activity (U g}^{-1}\text{ DM)}} \quad \text{(Eq 2)}$$

297

298

299 As the extractions were performed by triplicate, rule of 25% was applied,
300 discarding the values that differ more than 25% from the average.

301

302 *Chemical oxygen demand (COD)*

303 2 mL of the extract obtained for activity determination with DW as solvent were used to
304 determine COD with a Lange Kit LCK 514 ranging from 100 to 2000 mg O_2 L^{-1} and
305 COD measures were taken by a Lange Spectrophotometer DR 3900.

306

307 *Dynamic Respirometric Index (DRI)*

308 In this case, respirometric tests were performed on the residues before and after SSF in
309 order to determine their stability (German Federal Ministry for the Environment, Nature
310 Conservation and Nuclear Safety, Ordinance on environmentally compatible storage of
311 waste from human settlements and on biological waste treatment facilities, 2001).

312 A dynamic respirometer following the method described by Adani et al. (2003)
313 was used. The experimental device was described by Pognani et al. (2011). According
314 to Gea et al. (2004) the test was carried out at a constant temperature of 37°C. All the
315 experiments were performed in triplicate. Cumulative oxygen demand, AT_4 (g O_2 kg^{-1}

316 dry matter), and Dynamic Respirometric Indices, DRI_{24} DRI1hour (g O_2 kg^{-1} dry matter
317 h^{-1}), were calculated (Mejias et al. 2017, Almeira et al. 2015).
318
319 *Routine methods*
320 pH was calculated by soaking 10 g of sample in 50 mL of distilled water. After 30 min,
321 pH value was measured with a pH meter Crison micro pH 2001. Volatile solids,
322 moisture content, total organic carbon (TOC), total Kjeldahl nitrogen, (TKN) and
323 Soluble N-NH_4^+ were measured according to TMECC (The U.S. Department of
324 Agriculture and The U.S. Composting Council, 2001).
325 TOC (Total Organic Carbon) was determined using an O.I. Analytical Solid
326 TOC Analyser/Win TOC Solids v3.0, and TKN was measured using a Bloc Digester 6
327 (with six tubes capacity) (J.P. Selecta S.A., Barcelona, Spain) and a Büchi Distillation
328 Unit K-355 (Flawil, CH).
329 Fat content (HEM-Hexane extractable material) was measured using a standard
330 Soxhlet method with n-hexane as organic solvent (The U.S. Environmental Protection
331 Agency, Method 9071B) (The U.S. Department of Agriculture and The U.S.
332 Composting Council, 2001).
333
334 *Statistics*
335 One-way analysis of variance was performed to compare the mean values of activity
336 recovery obtained under different experimental conditions. Multiple factor regression,
337 to obtain a linear equation reflecting the influence of each experimental condition in
338 activity recovery, was calculated using also activity recovery data. Excel 2010 data tools
339 was used in both calculations
340

Results and Discussion

Waste characterization

The results of the characterization of raw materials are summarized in Table 1. As in this work the objective of SSF was the production of protease, nitrogen-rich substrates were used. Air Filled Porosity (AFP) of the mixture was measured to ensure a good aeration of the whole mass. Casciatori et al. (2016) operated with a bed porosity of 0.75 in a mixture of sugarcane bagasse and wheat bran using a packet bed bioreactor. According to Machado de Castro et al. (2016), a high porosity will ensure the oxygen transport, but the initial value will change through the process due to degradation of the support. Porosity seemed to be adequate along the experiment, achieving a good aeration of the whole sample and no heat loss. Biodegradability was evaluated through the dynamic respirometric assay. As Table 1 shows, mixtures assayed presented appropriate initial moisture and porosity, as well as a low C/N ratio. The raw material chosen as a substrate could be a good inducer of the enzyme produced (Gopalan and Nampoothiri 2016). So, for protease production using a consortium of microorganism, the substrate should be a nitrogen-rich one, which means a high content in protein. Compared to other reported wastes used as substrate for protease production like coffee pulp, corn cobs (Kandasamy et al. 2016), wheat bran (Meena et al. 2013) or *Jatropha curcas* seed cake (Thanapimmetha et al. 2012), which presented C/N ratios of 18.9 (Eshetu et al. 2013), 36 (Pan-In and Sukasen 2016), 19.49 and 2.46 (Mishra et al. 2016) respectively, HS and SF present a high content of proteins (nitrogen). Comparing the reported values of protease production in the mentioned examples, and after optimization of the experimental conditions in all cases, maximum production correspond to *Jatropha curcas*, with a range of production near to 1500 U mL^{-1}, which

366 was the residue that presented lowest C/N ratio, while the range of production for wheat
367 bran and the mixture of coffee pulp and corn cobs were 920 U mL^{-1} and 582 U mL^{-1}
368 respectively. Although wheat bran presents also a high production value, enrichment
369 with peptone was carried out previously to the SSF process and only *Jatropha curcas*
370 was specifically chosen for its high N content, according to the authors. In the present
371 work, a high protease production was obtained, compared with the examples above,
372 without any enrichment of the initial mixture, as C/N ratio was already adequate.
373 Material was also biodegradable, as the respirometric values show (Table 4).
374
375 **SSF process evolution in 10 L reactors**
376 Figures 2a and 2b show an example of temperature and DRI 1hour (g O_2 kg^{-1} DM h^{-1})
377 profiles obtained in the fermentation process of HS and SF, respectively. The highest
378 protease activity for HS was always observed in the mesophilic phase after 8 days of
379 process, while SF protease activity presented its maximum at thermophilic temperatures
380 after 5 days of fermentation. Maximum activity point was determined in previous
381 experiments by sampling the reactors periodically and determining the protease activity
382 for both mixtures. Abraham et al. (2013) found that maximum activity for SF was
383 observed at the 3rd day for a mixture equal to the one used in this work. For this residue,
384 maximum biological activity concurred with maximum enzymatic activity. On the other
385 hand, Abraham et al. (2017) found the highest activity value for a mixture of cow hair
386 and digested sludge at the 14th day of fermentation. For the mixture used in this work,
387 the highest value for protease activity was found in the 8th day of fermentation
388 approximately. Difference with Abraham results can be due to the different inoculum,
389 as in this work fresh sludge was used. Abu Yazid et al. (2016) characterized the
390 protease obtained from solid state fermentation of hair and anaerobic sludge

391 determining that the alkaline protease was a serine type in the range between 26 and 100
392 KDa, and exhibit good stability in a temperature range of 30-50 °C and alkaline pH. In
393 literature shorter optimum fermentation times are found for protease production through
394 solid-state fermentation. Kandasamy et al. (2016) found maximum yield of protease
395 production after 60 h of fermentation, using coffee pulp and corncob as a substrate
396 inoculating with *Bacillus sp. BT MASC 3*, Pouryafar et al. (2015) determined an
397 optimum incubation time of 48 h using wheat bran as substrate with *Bacillus*
398 *licheniformis*. However, in the literature consulted, the SSF process was usually
399 interrupted after a fixed time, without taking into account the biological activity. So it is
400 difficult to determine if the maximum production occurred at this time, because no
401 information about the complete production curve is presented. Also, the highest amount
402 of solid used in the references found was 25 g. In consequence, temperature control and
403 oxygen availability was not a limitation of the process. In the present work, SSF was
404 performed at a pilot scale and this approach requires a complete monitoring of the
405 process, which makes that highest protease activity is obtained at different times. Thus,
406 the direct comparison is not possible. High values of DRI 1hour were maintained longer
407 in the case of SF, which indicates a higher content of rapidly biodegradable material.
408 These data are in agreement with previous results obtained by Abraham et al. (2013,
409 2017). The range of protease activity obtained on this work was 6668 to 23541 U g^{-1}
410 DM for HS and 8054 to 33374 U g^{-1} DM for SF. For both residues, pH reached values
411 between 8.0 and 8.8 during the thermophilic phase, and remained in this range until the
412 end of the process.
413
414 **Protease activity recovery**

Factors influencing enzyme extraction from the solid matrix at the point of maximum protease activity during SSF were evaluated for the two wastes under study. As stated above, these factors include solvent type, waste: solvent w: v ratio and operation mode (static, with agitation and recirculation). Table 2 shows the mean and standard deviation of the protease activity recovery achieved during extraction under the conditions listed on the same table. Extractions were performed a minimum of three times for each set of parameters and each residue in agitated and static mode. A duplicate of the extractions was performed in recirculated mode due to experimental constraints.

Maximum activity recovery of 91 ± 8 % for SF was achieved in agitated mode at 1:4 using DW as solvent while a maximum recovery of 121 ± 22% was obtained with the same extraction conditions for HS.

The lowest value of activity recovery for SF was obtained at 1:1 w: v with DW as a solvent and agitated regime. HS presented its minimum at 1:3 w: v extracted with TB and Static mode.

Regarding the mean values of activity recovery of SF, agitated mode presents approximately 10% more efficiency than static mode. These values represent a higher recovery in static mode than the results presented by Mrudula and Kokila (2010) for the extraction of amylase from fermented bran (also a fibrous residue), where a 40% less recovery in static mode than in agitated mode was reported. However, the difference is greater for HS residue, which reached 40% difference between agitated and static mode.

Solvent type

One factor variance analysis was performed comparing the obtained yields between experiments differing in one extraction parameter. Results showed, for SF and HS, no significant difference between recoveries using DW or TB as extractant. Protein

solubility depends mostly on polar interactions between the molecules and the solvent, in some proteins, pH solubility profile presents a minimum value in the isoelectric region, the value of which depends on the structure of the protein (Hu et al. 2017). According to Vuong et al. (2016) salt concentration can also affect solubility, either negatively or positively, as can remove hydrate layers around protein molecules. In this work, both DW and TB are polar solvents than can be adequate for protease extraction, but with different initial pH and salinity. However, certain pH can cause deactivation. Freitas et al. (2013) reported denaturalization of protease from SSF of canola cake at pH 4, so low pH should be avoided. Table 3 show values of pH and conductivity measured in several extracts. As it can be observed, pH for DW and TB extracts presents similar results with a maximum pH of 9 and a minimum value of 8.3. Thus, no buffering properties were needed in the extraction agent to maintain an optimal pH value. pH achieved is due to the solubilisation of salts and other compounds contained in the solid matrix, which rise the pH of DW extracts. Differences between both wastes are reflected in conductivity values, where TB presents higher values due to the Tris salt. As the extraction ratio decrease, conductivity increase due to higher concentration in soluble salts from the matrix. During the SSF of SF and HS, proteases were produced when pH values reached the range of 8-9, thus, these enzymes are expected to be stable under pH extraction conditions presented in Table 3.

According to literature, DW is often used for enzyme extraction with good yields (Pal and Khanum 2010, Zaslona and Trusek-Howolnia 2015). Karatas et al. (2013) compared different solvents for the extraction of proteases and α-amylase from fermented rice husk, showing tap water the best recovery yield for both enzymes over HCl-Tris buffer pH 7 and distilled water. In this case, distilled water showed almost 50% less recovery than the buffer. On the other hand, Negi et al. (2011) found as the

best solvent for extraction of protease and glucoamylase from wheat bran a solution containing 10% of glycerol, other solvents assayed were water, ethanol, acetone and HCl-Tris buffer pH 6.5. Freitas et al. (2013) found that the ideal pH for protease recovery from fermented canola cake was 7, although they achieved good recoveries at a range from 5 to 8.

According to the results of this work, a solvent which a pH lower than 8.1 should be used, as recovery yield using distilled water was higher, having an initial pH lower than TB. However, optimum solvent will vary depending on the substrate and the characteristics of the enzyme produced (Rezaei et al. 2011)

Extraction mode

Three different extraction modes were assayed for protease extraction: static extraction (no agitation), orbital agitation, and recirculation of the solvent at two different extraction ratios, 1:2 w: v and 1:4 w: v, using a flow of 96 mL/min, which produces 14 times recirculation of the whole volume for 1:2 extractions and 7.2 for 1:4 extractions, due to the doubled extracting volume. Results for both residues are shown in Table 2.

One factor variance test was performed for 1:4 and 1:2 w: v experiments for both residues. Activity recovery for SF in agitated and static mode was considered statistically equal in all cases. However, for recirculation mode, differences with agitated mode were found for 1:4 w: v TB.

Sugumaran and Ponnusami (2017) studied the effect of agitation speed in the extraction of pullulan, a polysaccharide, from two fibrous substrates, cassava bagasse and palm kernel, reporting increment of 42% and 46% of the recovery obtained at 100 rpm when increasing the agitation speed to 400 rpm and 300 rpm for casaba bagasse and palm kernel respectively. This difference in recovery depending agitation mode

490 does not agree with the results found in this work. Also, extractions of xylanase from
491 sorghum straw performed by Adhyaru et al. (2016) showed that in this case
492 intermedium agitation speed was the optimum and also that interaction between other
493 extraction parameters like temperature and ration solvent-solid were significant.

494 SF presents very small particle size and once in contact with the solvent losses
495 its structure allowing good contact solid-solvent. Verdanega et al. (2010) also reported
496 no influence of the agitation speed in lipase recovery from soybean meal within a range
497 of 50-150 rpm.

498 When comparing results obtained for HS through one factor variance test,
499 significant differences are shown in all cases between agitated mode and static
500 extraction mode. Explanation for these differences is related to the nature of the residue.
501 Sludge used in HS contains chemical compounds added at the WWTP to produce the
502 flocculation of the solids and their sedimentation. Nabarlatz et al. (2012) compared
503 extraction of protease and lipase from two WWTP sludge using stirring and ultra-
504 sonication, obtaining much better results in almost half of the extraction time for the
505 second strategy. This shows that the presence of flocks, formed by the chemicals added
506 to produce the clumping of the solids during water treatment, causes poor contact
507 liquid-solid.

508 Values of COD for SF and HS extracts, obtained under the same experimental
509 conditions were 7525 and 3280 mg L^{-1} respectively, which indicates a higher
510 solubilisation of SF material than HS.

511

512 *Volume-weight (solvent-waste) ratio*

513

514 Results of one factor variance test of experiments differing from extraction ratio w: v
515 showed, for SF, no statistical difference between extractions performed at 1:3 and 1:4
516 w: v. However, in some cases, significant differences were found between 1:4 and 1:2
517 w:v extractions and even more significant with 1:1 w:v. When the test was performed
518 for HS results, there was no difference between recovery at different extraction ratios or
519 difference appear between 1:4 and 1:3 extraction but not between 1:4 and 1:2 ratios.

520 According to those facts, solubility of protease from SF seems to be high and
521 related to the equilibrium between concentration of protease in the solid matrix and the
522 solvent. In the literature, a direct relation between enzyme activity and w: v ratio is
523 documented. For instance, Adhyaru et al. (2016) assessed the recovery of xylanase from
524 sorghum straw at different ratios from 8 to 16 mL g-1 of substrate, locating the
525 maximum recovery at 12.41 mL g-1 (5069.20 U g-1) when the rest of extraction
526 parameters had been optimized. Although almost all of the studies performed on this
527 issue confirm a direct relationship between w:v ratio and activity (Vardanega et al.
528 2010), the opposite result was reported by Volken et al. (2008) who found a negative
529 effect of the increase of extraction volume during the extraction of transglutaminase
530 from industrial fibrous soy residue. Anyway, it must be pointed that although recovery
531 achieved at higher ratios can be almost total, the obtained extract will present lower
532 enzyme concentration, requiring extra processes for protease concentration or more
533 energy during lyophilisation. This fact is not taken into account in most of the works
534 performed at laboratory scale that do not consider further downstream stages.

535 Linear regression using Excel software was applied to data from Table 2.
536 Assignation of values to the different extraction parameters in order to perform the
537 linear regression, as only numbers can be adjusted, was as follow: values of 1 and -1 to
538 static and agitated mode and also to DW and TB respectively, values of 1, 2, 3, and 4 to

539 extraction ratios of 1:1, 1:2, 1:3 and 1:4, respectively. Recirculation mode results were
540 excluded from calculations, since experiments in this agitation regime were not
541 performed with all ratios. Extraction parameters where designated as follow, S for
542 solvent type, EM for extraction mode, and RA for ratio of extraction w: v. The results
543 obtained are displayed below for SF (equation 3) and HS (equation 4).
544
545 % Activity recovery = 48.9 + 1.7(S) -3.4 (EM) + 9.6 (RA) (R^2 = 0.81) (Eq 3)
546 % Activity recovery = 51.3+ 5.2(S) -18.3(EM) + 9.0 (RA) (R^2 = 0.82) (Eq 4)
547
548 As observed, there is a correlation between the different results, as regression
549 coefficients are 0.81 and 0.82, considered high for experimental values.
550 SF regression shows the highest coefficient for RA, which means that extraction
551 ratio has the greater influence in the extraction of protease. However, for HS, the
552 parameter that presents more relevance is the extraction mode. In both cases, it is clear
553 that agitated mode will produce the maximum recovery yield.
554 To sum up, the facts that can be deduced from those equations agree with the
555 results obtained by one factor variance test, indicating that extraction yield will increase
556 with extraction ratio for SF with little dependence on the agitation mode. When
557 proteases are extracted from HS, agitation must be applied to achieve maximum activity
558 recovery, but a lower extraction ratio is allowed.
559
560 *Number of extraction stages*
561 Consecutive extractions were carried out for HS and SF using DW and TB in a ratio of
562 1:2 w: v with agitation and in static mode. Figure 3 a) and b) shows the percentage of
563 activity recovery for the consecutive extractions performed on 200 g of fermented solid.

As it can be seen for both residues, approximately the 80% of the total activity recovered is extracted during first and second extraction, leaving a small amount of protease in the solid matrix. For both residues, the final percentage of activity recovery is higher for agitated regime. Regarding the total activity recovery after two consecutive extractions, it is possible to conclude that with 1:5 extraction ratio in agitated mode and TB as solvent, practically a 100% recovery of the protease contained in the solid matrix for both residues can be achieved.

Ahmed and Mostafa (2013) reported a recovery of 98% of exo-polygalacturonase produced by solid state fermentation of orange bagasse under optimum leaching conditions after three washes. However, 70% of the enzyme was extracted with the first wash. Also, Abd el Aty and Mostafa (2015) Extracted α-amylase from pre-treated and fermented potato shells with six consecutive washes at the optimum extraction parameters, achieving a recovery of 90.3% at the forth one. In this case, recovery in the first and second extraction was very similar, approximately a 35%. Comparing the results obtained for protease, activity recovery yield was higher in both SF and HS. In view of these results it seems that efficiency recovery of successive extraction stages may be related to the type of enzyme and specifically studied in economic terms.

Lyophilisation process

15 ml of fresh extract were lyophilised and the remaining solid powder dissolved in DW to its original volume. The activity of that solution was measured and compared to the fresh extract to determine the activity recovery of the lyophilisation process. The process was performed by duplicate. Lyophilisation resulted in high yields of activity recovery, for SF, 95 ± 4 % was recovered as a mean value and 96 ± 6 % for HS extracts.

589 No relation between extraction parameters and activity conservation was observed, as
590 samples of each set of extraction parameters were assayed. Minimum activity recovered
591 was 87 ± 3 for HS and 88% for SF. Abu Yazid et al. (2016) reported an activity loss of
592 21% after lyophilisation of protease extract obtained also from the fermentation of hair
593 and sludge. During freezing and drying, protein can suffer denaturation, losing its
594 activity. Bonds between water molecules disappear during drying process, but some
595 components present the ability to stabilize the protein structure and preserve activity
596 (Mensink et al. 2017). The same protein in a different matrix can lead to different
597 activity preservation. Activity losses have been reported by Imamura et al. (2014) for
598 different proteins after drying in sodium and potassium phosphate buffer. The highest
599 recovery with no salts addition reached 31%. Thus, lyophilisation in this case results in
600 low activity losses compared to the results reported, and a possible explanation can be
601 that no unfolding of the protein is happening during lyophilisation.
602
603 **Protein hydrolysis by SSF produced protease**
604 Abu Yazid et al. (2017) described the immobilisation of protease, obtained from the
605 same residues used in this work, onto functionalized magnetic iron oxide nanoparticles
606 through covalent binding method, obtaining a retention yield of 96%. Abu Yazid et al.
607 (2017) also used the immobilized enzymes during at least three cycles for protein
608 hydrolysis with no loss of activity. With an initial protein amount of 5.2 mg from
609 casein, the degree of protein hydrolysis achieved was 75% and 50% of the initial
610 amount of protein for enzymes produced by HS and SF respectively.
611 According to those results, for a 50 L reactor of fermented HS and SF (17.5 kg
612 and 20 kg respectively), obtaining the maximum activity achieved in this work, 23541
613 U DM^{-1} for HS and 33374 U DM^{-1} for SF, it would be possible to hydrolyse 156.6 mg

and 238.3 mg of protein from casein bovine with the recovered protease from HS and SF respectively. For this calculation the immobilized enzyme would be used during 6 cycles, in which initial activity does not diminish until the fourth cycle. Jin et al. (2010), reported hydrolysation of 2.1 mg of protein from rapeseed meals in one cycle using 1 mL of immobilised protease on ferric nanoparticles solution (8.50 mg/mL), during two hours incubation at 50°C. Hydrolysis yield in this case was lower (9.86%) compared to the one reported by Abu Yazid et al. (2017) (75% and 50%). Comparing activity to commercial products, neutral protease for beer brewing (Food grade) provided by Creative Enzymes (Creative enzymes) was taken as example. The mentioned product activity, as manufacturing specifications says is 70000 U/g, being a unit the amount of enzyme that hydrolyses casein to get 1 µg of tyrosine in 1 min. at 30°C and pH 7.5. Abu Yazid et al. (2017) was capable of immobilize 45.9 U / mg of ferric nanoparticles (NP) for HS and 31.9 U / mg nanoparticles (NP) for SF, being a unit (U) the amount of enzyme that hydrolyses casein to get 1 µg of tyrosine in 1 min. at 50°C and pH 8.1, The resulted product described by Abu Yazid et al. (2017) will have an activity of 45900 U / g NP, and being reusable during at least 3 cycles without activity loss, resulted in a much better yield that the commercial one.

Zero waste strategies

As described above, the solid residue obtained after extraction was tested as feedstock for biogas and soil organic amendment production in the framework of a zero waste strategy.

Anaerobic digestion

638 Biogas potential tests were undertaken to determine the suitability of anaerobic
639 digestion to treat the solid waste after enzyme extraction. After 21 days, SF replicates
640 produced 365, 304 and 273 mL of biogas g^{-1} VS with a content of approximately 43%
641 of methane. During the same period of time, HS produced 132 and 111 mL biogas g^{-1}
642 VS, with both presenting 40% of methane content. Biogas production in this case was
643 significantly lower than for SF. This result was expected since HS fermentation lasted 6
644 more days than SF, so HS was more biodegraded at the point of maximum protease
645 production and extraction.

646 Kafle et al. (2013) reported biogas potential of different agricultural and animal
647 wastes like apple waste, bread waste or cutlet fish waste. The values reported ranged
648 from 508 to 617 biogas mL g^{-1} VS, significantly higher than the values reported in this
649 study, since SF and HS were already partially degraded. Values reported by Ponsá et al.
650 (2011) of biogas production for organic fraction of municipal solid waste with no
651 mechanical pre-treatment (340 mL g^{-1} DM) and for municipal solid waste with
652 mechanical pre-treatment (133 mL g^{-1} DM) are in the range of SF and HS biogas
653 production values obtained in the present study. No values of biogas production were
654 found in literature regarding SF and HS before and after SSF and extraction, Merlino et
655 al. (2012) reported a production approximately of 590 Nm^3 t^{-1} VS of broken soybean
656 after 35 days of digestion at 40 °C, with a methane content of approximately a 55%.
657 That data is barely comparable to biogas production of SF since SF was already a
658 fermented material and a part of SF volatile solids were lignocellulosic, non-
659 biodegradable material.

660

661 *Composting process*

662 The stabilization of the solid waste after enzyme extraction under aerobic conditions
663 was also tested. Composting was evaluated as a valorisation technology to obtain an
664 organic soil amendment.

665 HS required a drying stage after enzyme extraction and before composting, in
666 order to decrease its moisture content from 74% to 62%. Before drying, no biological
667 activity was occurring despite the aeration provided. According to Chen et al. (2012),
668 excessive water content can hamper the oxygen transfer.

669 For HS, temperatures up to 55ºC, required for sanitation were not reached during
670 composting. This is probably due to the amount of waste in the reactor (17 kg)
671 compared to industrial scale quantities to which EU regulations apply (European
672 Commission, Working document, Biological treatment of Biowaste 2nd draft, 2001).
673 Abu Yazid et al. (2016) also composted a mixture of hair and anaerobic digested sludge
674 after fermentation and extraction of protease at a 10 L scale for approximately 30 days
675 without reaching thermophilic temperatures. Onyuka et al. (2013) composted a mixture
676 of cow hair and soil achieving maximum temperature of 51 °C at a scale of 2 L. In an
677 industrial scale, it would be expected to reach the required temperature for sanitation, as
678 heat gradient will increase as scale does (Ge et al. 2017). Table 4 shows DRI_{24} values of
679 2.0 ± 0.1 and 0.61 ± 0.05 for initial mixture of HS after SSF and composted product
680 respectively, indicating that the material becomes stable after composting. This stage
681 lasted 5 days for HS.

682 In the case of SF, SSF experiments were stopped for enzyme extraction at the 3rd
683 and 5th day of process, showing values of average oxygen uptake rate during the last 24
684 hours (OUR_{24}) of 3, 4.5, 9 g O_2 kg $^{-1}$ DM h^{-1} in thermophilic range. Thus, SF was
685 clearly a non-stable material at this stage of the process.

686 Composting of SF after SSF and enzyme extraction was carried out in 4.5 L
687 reactors in duplicate. Both replicates showed an increase of temperature and oxygen
688 consumption during the first 24h, followed by a fast decrease to environment
689 temperature. The maximum temperature values reached were 34°C and 42°C
690 respectively, and maximum OUR_{24} values around 5 g O_2 kg^{-1} DM h^{-1}, but never
691 reaching the thermophilic range again.

692 After 9 days of composting process, the residue could not be considered stable
693 as it presented a DRI_{24} value of 2.1 ± 0.4 g O_2 kg^{-1} DM h^{-1} and AT_4 value of 146 ± 27 g
694 O_2 kg^{-1} DM. A longer composting process will be needed, since the decrease of
695 biological activity is slow.

696 According to Barrena et al. (2009), aerobic and anaerobic indices are closely
697 related. Values reported for the ratio between DRI_{24} and biogas production in liquid
698 condition and between AT_4 and biogas production at the same condition for different
699 wastes were 86 and 1.56 respectively. The same ratios were calculated using the values
700 obtained in this study, giving values of 84 and 1.56 respectively. These numbers agree
701 with the parameters reported by Barrena et al. (2009).

702

703 ## Conclusions

704 In this work a complete assessment of the downstream of SSF processes for the
705 production of proteases has been performed (Figure 4). Yields of activity recovery in
706 protease extraction and lyophilisation stages, under a wide variety of conditions, were
707 calculated. According to the results, extraction ratio of 1:3 for SF is recommended and
708 agitation mode at extraction ratio 1:2 would be the adequate conditions for HS.
709 Consecutive extractions in static mode seem an efficient way of obtaining also a good
710 yield, with no agitation but double extraction time. Economical balance between

profitability of the recovered product and water/energy consumption should be performed. Lyophilisation provides a high activity recovery, around 95% as an average. It was also observed that as the solubility of the solid matrix increases, the ratio waste: solvent w: v becomes the most determining factor in the activity recovery yield, while agitation mode is the key factor when solubility decrease.

After enzyme extraction, the use of SF and HS as feedstock for anaerobic digestion reported relatively high biogas production. Also, HS and SF could be treated throw composting obtaining a stable product.

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Conflict of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

References

Abd El Aty AA, Mostafa FA (2015) Production and characterization of fungal α-amylase from marine alternaria alternata utilizing lignocellulosic wastes and its application. Research Journal of Pharmaceutical, Biol. Chem. Sci. 6(3): 813-825

Abraham J, Gea T, Sánchez A (2013) Potential of the solid-state fermentation of soy fibre residues by native microbial populations for bench-scale alkaline protease production. Biochem Eng J 74:15-19

Abraham J, Gea T, Komilis D, Sánchez A (2017) Reproducibility of solid-state fermentation at bench-scale: the case of protease production. Global Nest J 19 (2): 183-190

Abu Yazid N, Barrena R, Sánchez A (2016) Assessment of protease activity in hydrolysed extracts from SSF of hair waste by and indigenous consortium of microorganisms. Waste Manage 49:420-426

Abu Yazid N, Barrena R, Sánchez A (2017) The immobilisation of protease produced by SSF onto functionalized magnetic nanoparticles: Application in the hydrolysis of different protein sources. J Mol Catal B-Enzym 2017:1381-1177

Adani F, Gigliotti G, Valentini F, Laraia R (2003) Respiration index determination: A comparative study of different methods. Compost Sci Util 11:144-151

Adhyaru DN, Bhatt NS, Modi HA, Divecha J (2016) Insight on xylanase from *Aspergillus tubingensis FDHN1*: Production, high yielding recovery optimization through statistical approach and application. Biocatal Agric Biotech 6: 51–57

Ahmed SA, Mostafa FA (2013) Utilization of orange bagasse and molokhia stalk for production of pectinase enzyme. Braz J of Chem Eng 30(3): 449-456

Alef K, Nannipieri P (1995) Methods in Applied Soil Microbiology and Biochemistry, ed by Academic Press Limited, San Diego, USA, 312-315

Almeira N, Komilis D, Barrena R, Gea T, Sánchez A (2015) The importance of aeration mode and flowrate in the determination of the biological activity and stability of organic wastes by respiration indices. Bioresource Technol 196: 256-262

Barrena R, d'Imporzano G, Ponsá S, Gea T, Artola A, Vázquez F, Sánchez A, Adani F (2009) In search of a reliable technique for the determination of the biological stability of the organic matter in the mechanical-biological treated waste. J Hazard Mater 162:1065-1072

Casciatori FP, Bück A, Thomeo JC, Tsotsas E (2016) Two-phase and two-dimensional model describing heat and water transfer during solid-state fermentation with a packed-bed bioreactor. Chem Eng J 287:103-116

Chaithanya R, Bhagavathy CMA, Mrudula S (2012) Optimization of process parameters and leaching conditions affecting protease production under solid state fermentation. Asian J. of Microbiol. Biotechnol. Environ Sci 14(1): 87-94

Chen HZ, He Q (2012) Value-added bioconversion of biomass by solid-state fermentation. J Chem Technol Biot 87: 1619–1625

Creative enzymes, product informaction http://www.creative-enzymes.com/product/neutral-protease-for-beer-brewing-food-grade-_3168.html. Accessed 28 February 2017.

El-Bakry M, Abraham J, Cerda A, Barrena R, Ponsá S, Gea T, Sanchez A (2015) From wastes to high value added products: Novel aspects of SSF in the production of enzymes. Critical Reviews in Environ Sci and Tech 45 (18): 1999-2042

Eshetu B, Eckhardt KU, Leinweber P (2013) Soil organic matter alterations by short-term composted coffee pulp waste: evidence from pyrolysis-field ionisation mass spectrometry. Appl Agric Forestry Res 2(63):191-200

European Commission, Working document, Biological treatment of Biowaste 2nd draft, http://www.compost.it/www/pubblicazioni_on_line/biod.pdf (2001) (accessed 19.09.2016)

Finkler ATJ, Biz A, Pitol LO, Medina BS, Luithardt H, Luz LFDL Jr, Krieger, N, Mitchell DA (2017) Intermittent agitation contributes to uniformity across the bed during pectinase production by *Aspergillus niger* grown in solid-state fermentation in a pilot-scale packed-bed bioreactor. Biochem Eng J 121:1-12

Freitas AC, Castro RJS, Fontenele MA, Egito AS, Farinas CS, Pinto GAS (2013) Canola Cake as a Potential Substrate for Proteolytic Enzymes Production by a Selected Strain of *Aspergillus oryzae*: Selection of Process Conditions and Product Characterization. ISRN Microbiol 2013:8

Gassara F, Ajila CM, Brar SK, Tyagi RD, Verma M, Valero J (2013) Influence of aeration and agitation modes on solid-state fermentation of apple pomace waste by *Phanerochaete chrysosporium* to produce ligninolytic enzymes and co-extract polyphenols. Int J Food Sci Tech 48:2119-2126

Ge X, Vasco-Correa J, Li Y (2016) Solid-State fermentation bioreactors and fundamentals. Current developments in biotechnology and bioengineering: bioprocesses. Bioreactors and Controls 2016: 381-402

Gea T, Barrena R, Artola A, Sánchez A (2004) Monitoring the Biological Activity of the Composting Process: Oxygen Uptake Rate (OUR), Respirometric Index (RI), and Respiratory Quotient (RQ). Biotechnol Bioeng 88:520-527

German Federal Ministry for the Environment, Nature Conservation and Nuclear Safety, Ordinance on environmentally compatible storage of waste from human settlements and on biological waste treatment facilities of 20 February (2001). URL:http://www.bmu.de/files/pdfs/allgemein/application/pdf/ablagerungsverordnung.pdf [accessed 26 Setembre 2016]

Gopalan N, Nampoothiri KM (2016) Biotechnological Production of Enzymes Using Agro-Industrial Wastes: Economic Considerations, Commercialization Potential, and Future Prospects. Agro-Industrial Wastes as Feedstock for Enzyme Production 313-330

Hu H, Fan T, Zhao X, Zhang X, Sun Y, Liu H (2017) Influence of pH and salt concentration on functional properties of walnut protein from different extraction methods. J Food Sci Technol 54(9):2833–2841

Imamura K, Murai K, Korehisa T, Shimizu N, Yamahira R, Matsuura T, Tada H, Imanaka H, Ishida N, Nakanishi K (2014) Characteristics of Sugar Surfactants in Stabilizing Proteins During Freeze-Thawing and Freeze-Drying. J Pharm Sci 103:1628-1637

Jin X, Fang Li J, Huang PY,Dong XY,Guo LL, Yang L, Cao YC, Wei F, Zhao YD, Chen H (2010) Immobilized protease on the magnetic nanoparticles used for the hydrolysis of rapeseed meals. J Magn Magn Mater 322: 2031–2037

Kafle GK, Kim SH (2013) Effects of chemical composition and ensiling on the biogas productivity and degradation rates of agricultural and food processing by-products. Bioresource Technol 142:553-561

Kandasamy S, Muthusamy G, Balakrishnan S, Duraisamy S, Thangasamy S, Seralathan KK, Chinnappan S (2016) Optimization of protease production from surface-modified coffee pulp waste and corncobs using *Bacillus sp*. by SSF. 3 Biotech 6 (2):167

Karataş H, Uyar F, Tolan V, Baysa Z (2013) Optimization and enhanced production of α-amylase and protease by a newly isolated *Bacillus licheniformis ZB-05* under solid-state fermentation. Ann Microbiol 63:45–52

Karpe AV, Beale DJ, Harding IH, Palombo EA (2015) Optimization of degradation of winery-derived biomass waste by Ascomycetes. J Chem Technol Biot 90: 1793–1801

Kriaa M, Kammoun R (2016) Producing *Aspergillus turingensis CTM507* Glucose oxidase by Solid state fermentation versus submerged fermentation: process optimization and enzyme stability by an intermediary metabolite in relation with diauxic growth. J Chem Technol Biot 91: 1540–1550

Lizardi-Jiménez MA, Hernández-Martínez R (2017) Solid state fermentation (SSF): diversity of applications to valorise waste and biomass. 3 Biotech 7 (1): 44

Machado de Castro A, dos Reis Castilho L, Guimarães Freirec DM (2016) Characterization of babassu, canola, castor seed and sunflower residual cakes for use as raw materials for fermentation processes. Ind Crop Prod 83: 140-148

Maulini-Duran C, Puyuelo B, Artola A, Font X, Sánchez A, Gea T (2014) VOC emissions from the composting of the organic fraction of municipal solid waste using standard and advanced aeration strategies. J Chem Technol Biot 89:579-586

Mejias L, Komilis D, Gea T, Sánchez A (2017) The effect of airflow rates and aeration mode on the respiration activity. Waste Manag 65:22-28

Mensink MA, Frijlink HW, van der Voort Maarschalk K, Hinrichs WLJ (2017) How sugars protect proteins in the solid state and during drying (review): Mechanisms of stabilization in relation to stress conditions. European J of Pharma Biopharma 114: 288–295

Merlino G, Rizzi A, Villa F, Sorlini C, Brambilla M, Navarotto P, Bertazzoni B, Zagni M, Araldi F, Daffonchio D (2012) Shifts of microbial community structure during anaerobic digestion of agro-industrial energetic crops and food industry byproducts. J Chem Technol Biot 87: 1302–1311

Meena P, Tripathi AD, Srivastava SK, Jha A (2013) Utilization of agro-industrial waste (wheat bran) for alkaline protease production by *Pseudomonas aeruginosa* in SSF using Taguchi (DOE) methodology. Biocatal Agric Biotechnol 2(3): 210-216

Mishra S, Kumar P, Malik A (2016) Suitability of agricultural by-products as production medium for spore production by *Beauveria bassiana HQ917687*. Int J Recycl Org Waste Agric 5(2, 1): 179-184

Mrudula S, Kokila R (2010) Production of Thermostable a-amylase by *Bacillus cereus MK* in solid state fermentation: Partial purification and characterization of the enzyme. Internet J Microbiol 8(1): 1-2

Nabarlatz D, Stuber F, Font J, Fortuny A, Fabregat A, Bengoa C (2012) Extraction and purification of hydrolytic enzymes from activated sludge. Resour Conserv Recy 59:9-13

Negi S, Gupta S, Banerjee R (2011) Extraction and Purification of Glucoamylase and Protease Produced by *Aspergillus awamori* in a Single-Stage Fermentation. Food Technol Biotechnol 49 (3): 310–315

Onyuka AS, Bates M, Attenburrow G, Covington AD, Antunes APM (2013) Pilot-Scale Composting And Biodegradability Of Bovine Hair. 32nd Congress of the International Union of Leather Technologists and Chemist Societies, IULTCS 2013

Pan-In S, Sukasem N (2016) Methane production potential from anaerobic co-digestions of different animal dungs and sweet corn residuals. Enrgy Proced 138: 943-948

Pal A, Khanum F (2010) Production and extraction optimization of xylanase from *Aspergillus niger DFR-5* through solid-state-fermentation. Bioresource Technol 101: 7563–7569

Pognani M, Barrena R, Font X, Adani F, Scaglia B, Sánchez A (2011) Evolution of organic matter in a full-scale composting plant for the treatment of sewage sludge and biowaste by respiration techniques and pyrolysis-GC/MS. Bioresource Technol 102:4536-4543

Ponsá S (2010) Different indices to express biodegradability in organic solid wastes. Application to full scale waste treatment plants, PhD thesis, ed by Universitat Autonoma de Barcelona, Cerdanyola del Vallés

Ponsá S, Gea T, Sánchez A (2011) Short-time estimation of biogas and methane potential from samples of municipal solid wastes. J Chem Technol Biot 86:1121-1124

Pouryafar F, Najafpour GD, Noshadi N, Jahanshahi M (2015) Thermostable alkaline protease production via solid state fermentation in a tray bioreactor using *Bacillus licheniformis ATCC 21424*. Int J Environ Res 9(4): 1127-1134

Puyuelo B, Gea T, Sánchez A (2010) A new control strategy for the composting process based on the oxygen uptake rate. Chem Eng J 165:161-169

Rashid JIA, Samat N, Yusoff WMW (2013) Studies on extraction of mannanase enzyme by *Aspergillus terreus* SUK-1 from fermented palm kernel cake. Pak J Biol Sci: PJBS 16:933-8

Rezaei F, Joh LD, Kashima H, Reddy AP, VanderGheynst JS (2011) Selection of conditions for cellulase and xylanase extraction from switchgrass colonized by *Acidothermus cellulolyticus*. Appl Biochem Biotechnol 164:793–803

Riyadi FA, Alam MZ, Salleh MN, Salleh HM (2017) Optimization of thermostable organic solvent-tolerant lipase production by thermotolerant Rhizopus sp. using solid-state fermentation of palm kernel cake. 3 Biotech 7 (5): 300

Salariato, D, Diorio LA, Mouso N, Forchiassin F (2010) Extraction and characterization of polygalacturonase of *Fomes sclerodermeus* produced by solid-state fermentation. Rev Argent Microbiol 42(1):57-62

Sayara T, Sarrà M, Sánchez A (2010) Effects of compost stability and contaminant concentration on the bioremediation of PAHs-contaminated soil through composting. J Hazard Mater 179:999-1006

Silva JN, Godoy MG, Gutarra MLE, Freire DMG (2014) Impact of extraction parameters on the recovery of lipolytic activity from fermented babassu cake. Plos One 9 (8): 103176

Singh R, Kumar M, Mittal A, Mehta PK (2016) Microbial enzymes: industrial progress in 21st century. 3 Biotech. 6 (2): 174

Sugumaran KR, Ponnusami V (2017) Conventional optimization of aqueous extraction of pullulan in solid-state fermentation of cassava bagasse and Asian palm kernel. Biocat Agric Biotech 10: 204–208

Sun HY, Li J, Zhao P, Peng M (2011) Banana peel: A novel substrate for cellulose production under solid-state fermentation. Afr J Biotechnol 77: 17887-17890

Thanapimmetha A, Luadsongkram A, Titapiwatanakun B, Srinophakun P (2012) Value added waste of Jatropha curcas residue: Optimization of protease production in solid state fermentation by Taguchi DOE methodology. Ind Crop Prod 37(1): 1-5

The U.S. Department of Agriculture and The U.S. Composting Council, 2001. Test Methods for the Examination of Composting and Compost, ed by Edaphos International, Houston, TX, USA (2001)

Vardanega R, Remonatto D, Arbter F, Polloni A, Rigo E, Ninow JL, Treichel H, de Oliveira D, Di Lucio M (2010) A Systematic Study on Extraction of Lipase Obtained by Solid-State Fermentation of Soybean Meal by a Newly Isolated Strain of *Penicillium sp*. Food Bioprocess Tech 3:461-465

Volken de Souza CF, Rodrigues RC, Heck JX, Ayub MAZ (2008) Optimization of transglutaminase extraction produced by *Bacillus circulants* BL32 on solid-state cultivation. J Chem Technol Biot 83:1306–1313

Vuong HTH, Tran NM, Tran TTT, Ton NMN, Le VVM (2016) Effects of pH and salt concentration on functional properties of rambutan (Nephelium lappaceum L.) seed albumin concentrate. Int J Food Sci Technol 51(5):1212–1219

Zaslona H, Trusek-Holownia A (2015) Enhanced production of polygalacturonase in solid-state fermentation: selection of the process conditions, isolation and partial characterization of the enzyme. Acta Biochim Pol 62(4): 651-657

Zhang H, Sang Q (2015) Production and extraction of xylanase and β-mannanase by *Penicillium chrysogenum QML-2* and primary application in saccharification of corn cob. Biochem Eng J 97: 101-110

Figure captions

Figure 1. a) 4.5 L and 10 L reactors, b) 50 L reactor

1: 4.5 L and 10 L reactor; 2: 4.5 L and 10 L reactor inside; 3: Mass-flowmeter; 4: Temperature probe; 5: Water trap; 6: O_2 Electrochemical sensor. 7: 50 L reactor; 8: 50 L reactor inside

Figure 2. Temperature (lines) and DRI 1 hour (short-short) profiles obtained in SSF processes a) HS fermentation, b) SF fermentation.

DRI 1h: Dynamic Respirometric Index 1 hour average value

Figure 3. Percentage of activity recovery obtained in consecutive extractions of 200 g of fermented solid at 1:2 w: v ratio a) HS b) SF.

HS: Hair and Sludge; SF: soy fibre; TB: HCl-Tris Buffer (pH = 8.10); DW: Distilled water

Figure 4. Downstream summary.

DRI 1h: Dynamic Respirometric Index 1 hour average value; AT_4: Cumulative Oxygen Consumption during 4 days; HS: Hair and Sludge; SF: soy fibre; TB: HCl-Tris Buffer (pH = 8.10); DW: Distilled water; VS: Volatile Solids

Table 1: Characterization of wastes and mixtures used as substrates in SSF

Parameters	Hair waste	Sludge	HS	SF
Moisture (%, wb)	67 ± 8	75 ± 2	62.3 ± 0.8	67 ± 8
Organic matter (%, db)	86 ± 1	67	89 ± 1	96 ± 3
pH	9.9 ± 0.8	7.8 ± 0.4	7.6 ± 0.3	6 ± 2
EC (mS cm^{-1})	4.5 ± 0.8	2.3 ± 0.9	3 ± 1	0.9 ± 0.2
Total organic carbon (%, db)	51 ± 9	n.a.	64 ± 3	69 ± 3
Total Kjeldahl Nitrogen (%, db)	10 ± 4	16	7.3 ± 0.5	4.9 ± 0.6
C/N ratio	6 ± 1	3	7.1 ± 0.8	14 ± 1
Fat content – HEM (%, db)	0.9 ± 0.3	n.a.	n.a.	6 ± 2
Air filled porosity (%)	n.a.	n.a.	77 ± 4	74

wb: wet basis. db: dry basis; n.a.: not available; HEM: Hexane extractable material.

HS: Hair waste:sludge (1:2, w.w) and wood chips (1:1, v:v); SF: Soy fibre and wood chips (1:1, v:v).

Values are the average of independent experiments and its standard deviation.

Table 2: Summary of extraction experiments for fermented soy fibre (SF) and fermented cow hair and sludge (HS) (Extraction time: 1h)

% Activity recovery				Extraction parameters		
SF Mean	SF St deviation	HS Mean	HS St deviation	Solvent (S)	Extraction mode (EM)	Ratio W:V (RA)
53	9	**71**	5	DW	Agitated	1:1
54	10	**66**	3	TB	Agitated	1:1
55	4	**49**	8	DW	Static	1:1
55	9	**52**	4	TB	Static	1:1
83	18	**99**	16	DW	Agitated	1:2
70	6	**93**	19	TB	Agitated	1:2
73	12	**49**	3	DW	Static	1:2
65	8	**50**	6	TB	Static	1:2
63	6	**71**	8	DW	Recirculated	1:2
71	2	**58**	8	TB	Recirculated	1:2
88	20	**114**	18	DW	Agitated	1:3
85	3	**72**	9	TB	Agitated	1:3
74	3	**62**	2	DW	Static	1:3
78	22	**44**	6	TB	Static	1:3
91	8	**121**	22	DW	Agitated	1:4
86	3	**99**	23	TB	Agitated	1:4
79	8	**66**	11	DW	Static	1:4
77	7	**72**	3	TB	Static	1:4
76	19	**93**	21	DW	Recirculated	1:4
72	1	**56**	12	TB	Recirculated	1:4

DW: Distilled water, TB: HCl – Tris buffer (pH = 8.10), EM: Extraction mode, S: Solvent type, RA:Ratio of extraction w:v

Table 3: pH and conductivity of the extracts

SF (Fermented Soy Fibre)					HS (Fermented Hair + Sludge)				
S	**EM**	**RA**	**pH**	**Conductivity (mS/cm)**	**S**	**EM**	**RA**	**pH**	**Conductivity (mS/cm)**
DW	Agitated	1:1	8.9	6.9	DW	Agitated	1:1	8.7	11.46
DW	Agitated	1:2	8.9	4.7	DW	Agitated	1:2	8.83	7.77
DW	Agitated	1:3	8.9	3.2	DW	Agitated	1:3	8.78	5.83
DW	Agitated	1:4	9.0	2.9	DW	Agitated	1:4	8.77	4.76
DW	Static	1:1	8.5	2.4	DW	Static	1:1	8.79	10.16
DW	Static	1:2	8.7	3.0	DW	Static	1:2	8.82	5.99
DW	Static	1:3	8.7	3.5	DW	Static	1:3	8.81	4.34
DW	Static	1:4	8.6	2.7	DW	Static	1:4	8.85	4.32
TB	Agitated	1:1	8.6 ± 0.2	7.5 ± 0.5	TB	Agitated	1:1	8.69	12.04
TB	Agitated	1:2	8.5 ± 0.1	6.1 ± 0.7	TB	Agitated	1:2	8.6	8.76
TB	Agitated	1:3	8.5 ± 0.04	5.2 ± 0.2	TB	Agitated	1:3	8.5	7.09
TB	Agitated	1:4	8.4 ±0.04	4.7 ± 0.4	TB	Agitated	1:4	8.51	6.67
					TB	Static	1:1	8.58	12.55
					TB	Static	1:2	8.54	8.35
					TB	Static	1:3	8.37	5.83
					TB	Static	1:4	8.31	5.09

DW (distilled water): pH: 7.81, Conductivity: 1.86 (mS/cm); TB (HCl – Tris buffer: pH: 8.1, Conductivity: 2.48 (mS/cm)

Table 4. Results of respirometric assays. Final SSF values are initial composting values.

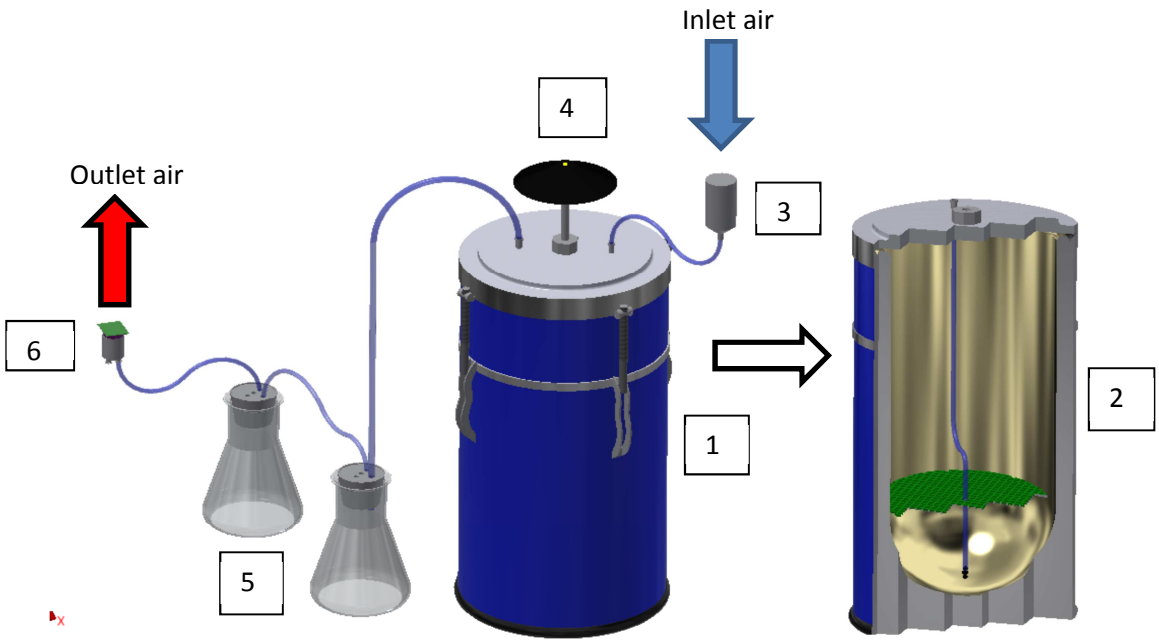
	Solid State Fermentation				Composting	
Residue	Initial AT_4 ($g\ O_2\ kg^{-1}\ DM$)	Final AT_4 ($g\ O_2\ kg^{-1}\ DM$)	Initial DRI_{24} ($g\ O_2\ kg^{-1}\ DM\ h^{-1}$)	Final DRI_{24} ($g\ O_2\ kg^{-1}\ DM\ h^{-1}$)	Final AT_4 ($g\ O_2\ kg^{-1}\ DM$)	Final DRI_{24} ($g\ O_2\ kg^{-1}\ DM\ h^{-1}$)
SF	326 ± 98	185 ± 48	5 ± 2	3 ± 1	146 ± 27	2.1 ± 0.4
HS	113 ± 11	61 ± 6	2.0 ± 0.1	1.0 ± 0.1	48 ± 4	0.61 ± 0.05

AT_4: Accumulated O_2 consumption after 4 days; DRI_{24}: dynamic respirometric index based on 24 hours of maximum O_2 consumption.

Values are the average of independent experiments.

Figure 1

a)



b)

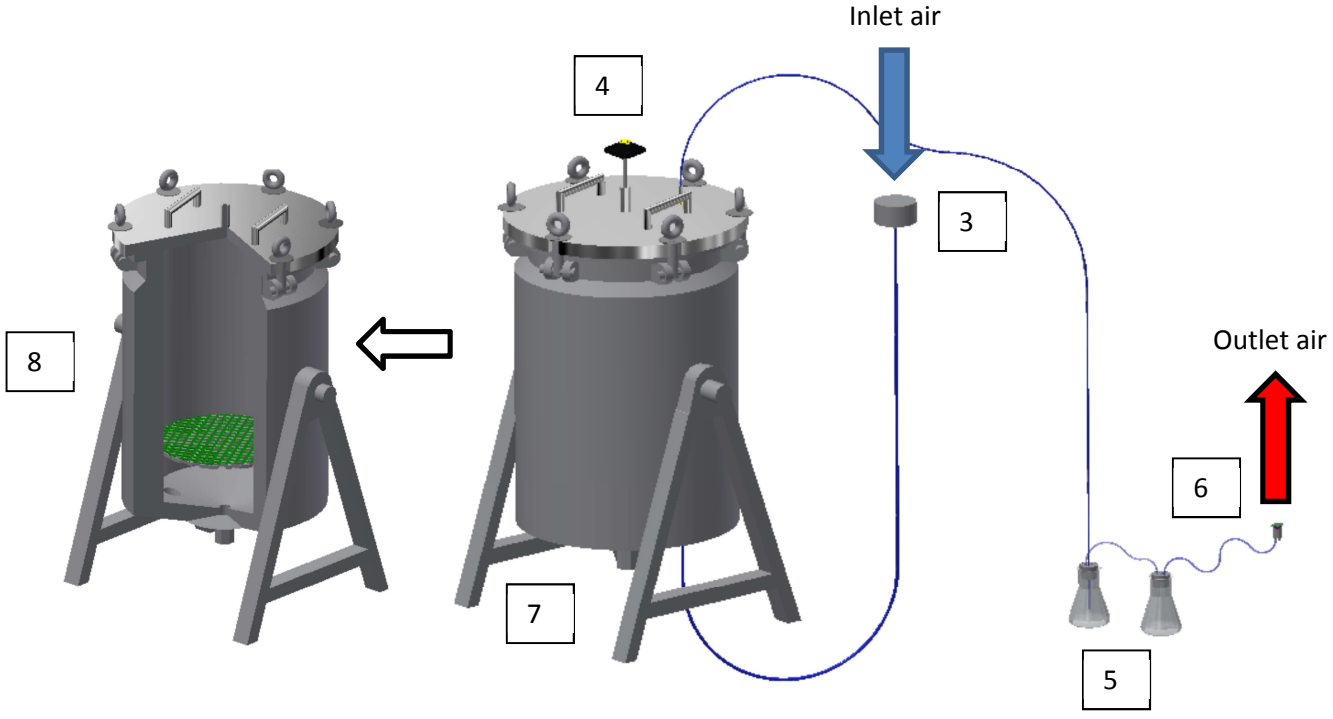
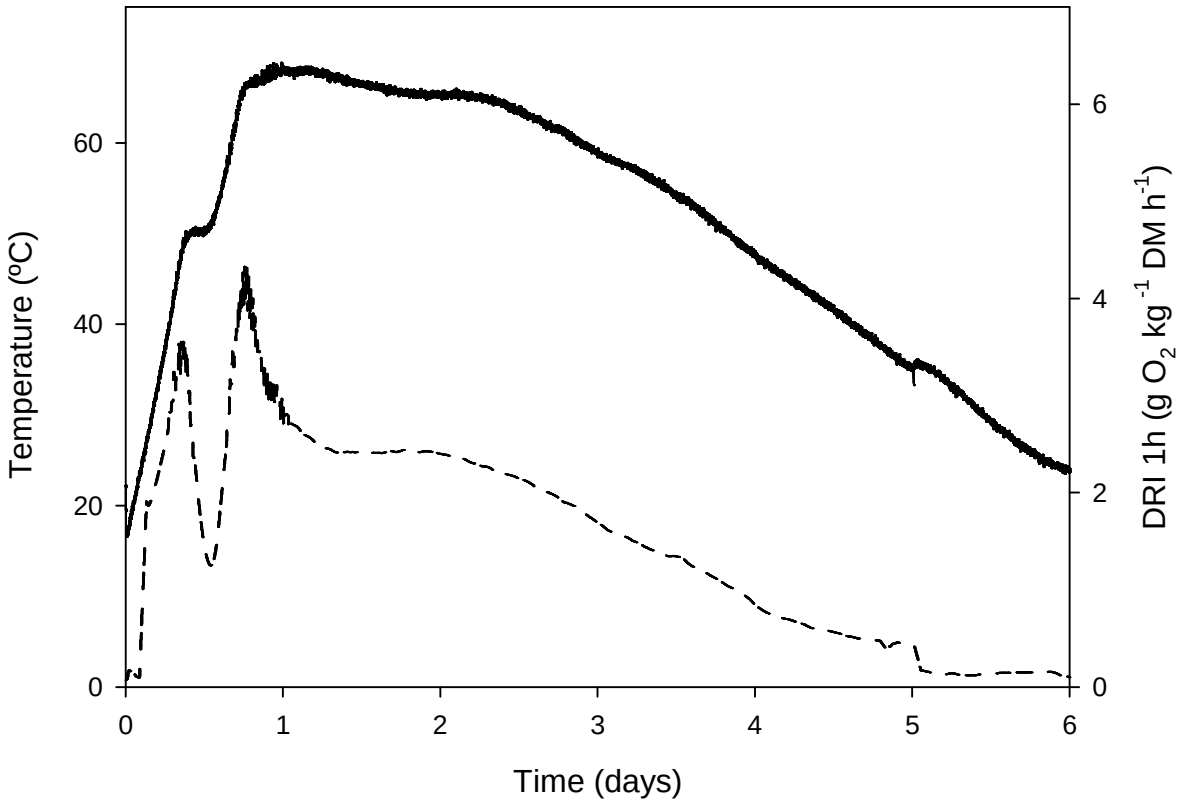


Figure 2

a)


Temperature (ºC)
DRI 1h (g O2 kg-1 DM h-1)
Time (days)
0
20
40
60
0
2
4
6
0
1
2
3
4
5
6

b)

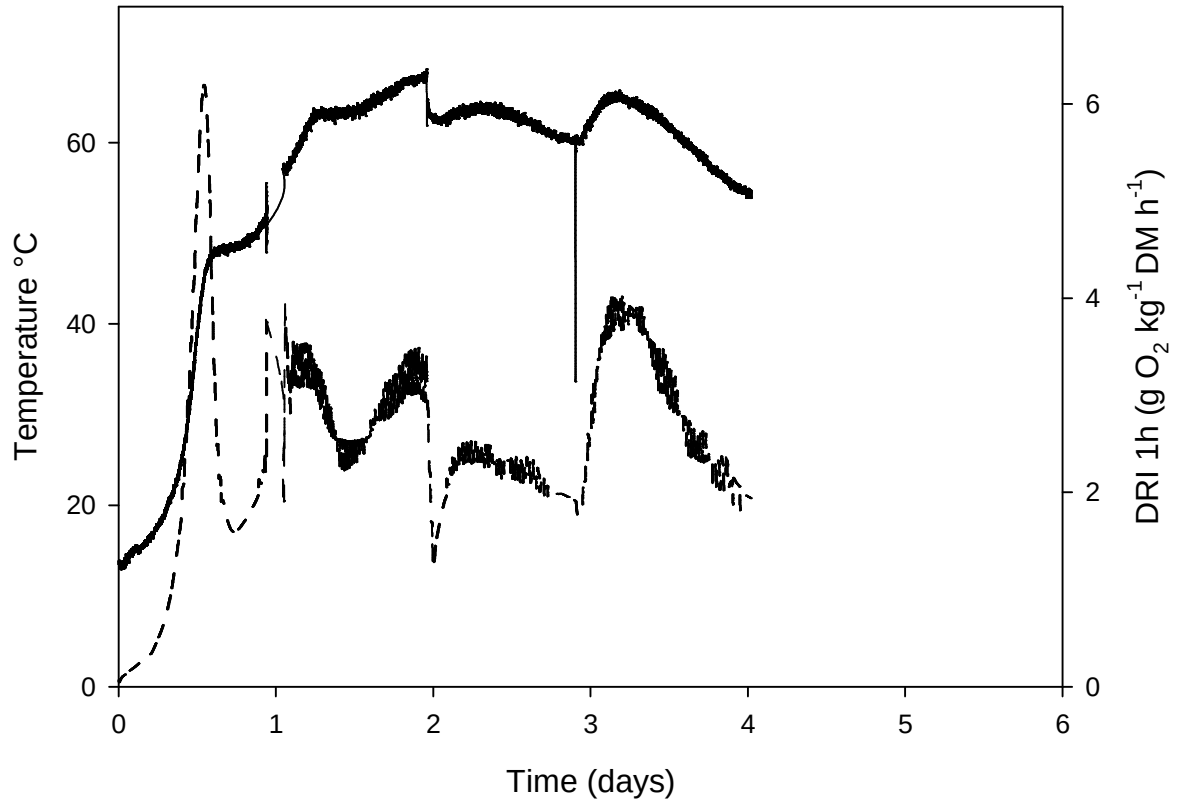

Temperature °C
DRI 1h (g O2 kg-1 DM h-1)
Time (days)
0
20
40
60
0
2
4
6
0
1
2
3
4
5
6

Figure 3

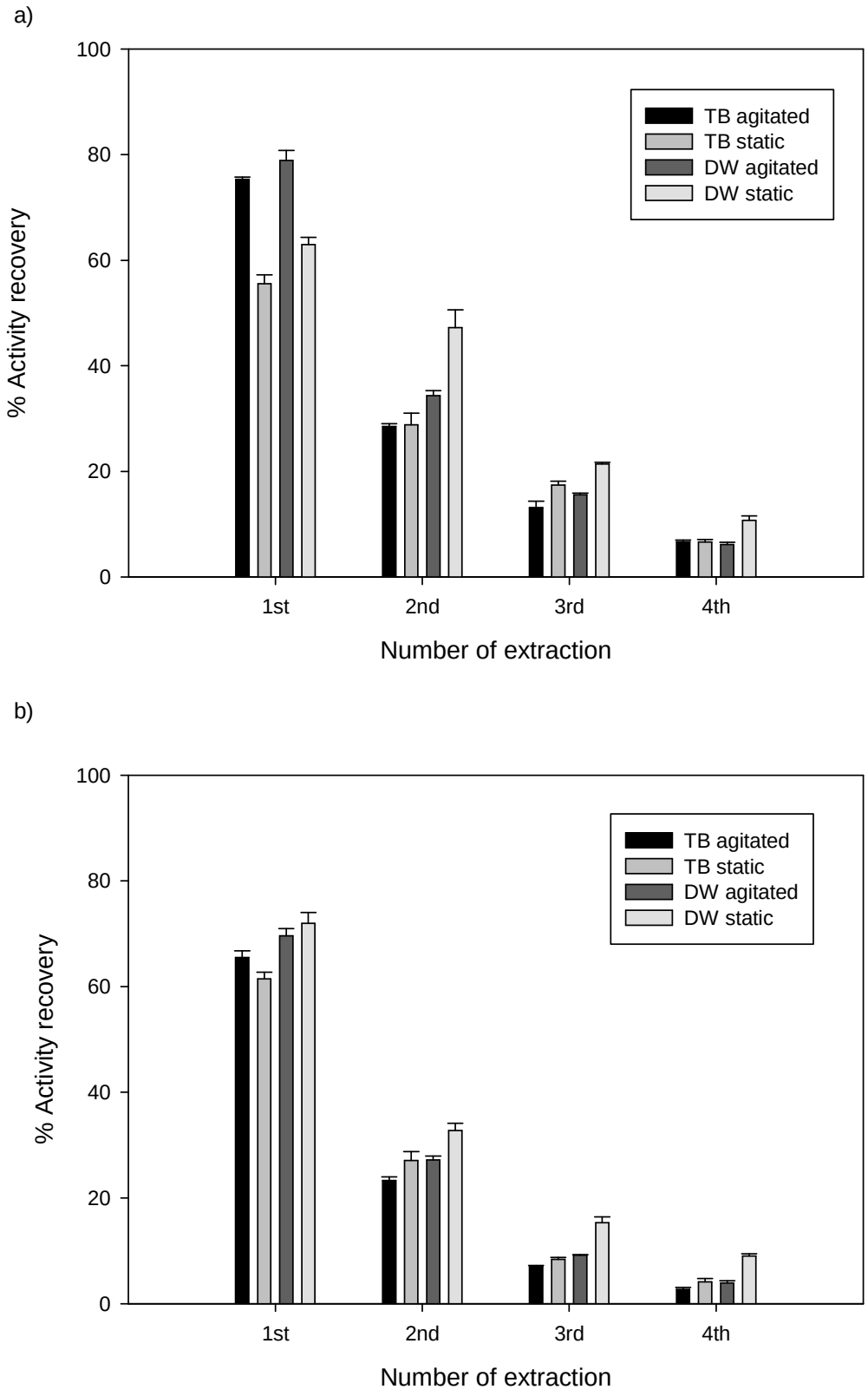
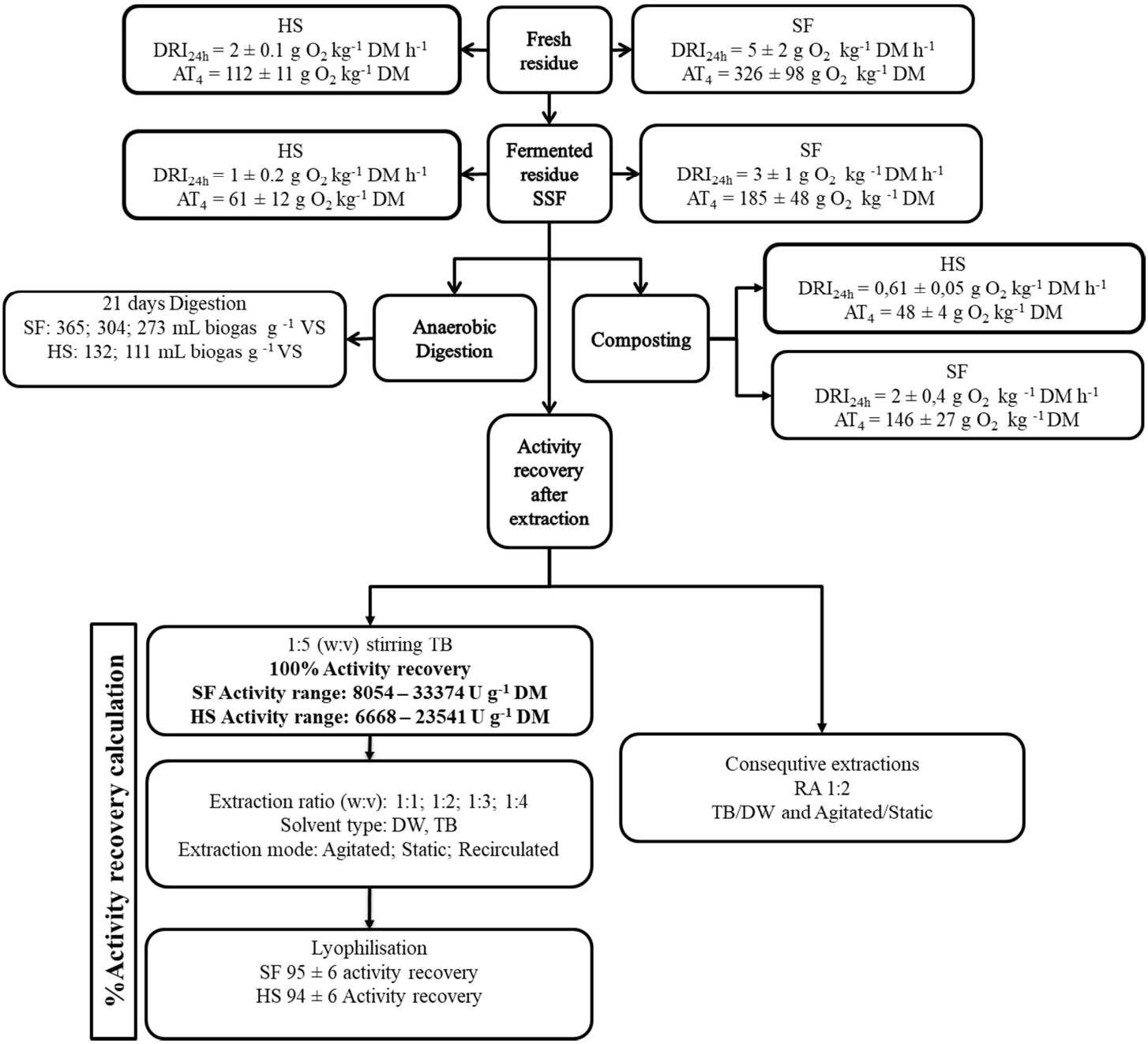

a)
100
80
60
40
20
0
% Activity recovery
TB agitated
TB static
DW agitated
DW static
1st
2nd
3rd
4th
Number of extraction
b)
100
80
60
40
20
0
% Activity recovery
TB agitated
TB static
DW agitated
DW static
1st
2nd
3rd
4th
Number of extraction

Figure 4


HS
DRI24h = 2 ± 0.1 g O2 kg-1 DM h-1
AT4 = 112 ± 11 g O2 kg-1 DM
Fresh residue
SF
DRI24h = 5 ± 2 g O2 kg-1 DM h-1
AT4 = 326 ± 98 g O2 kg-1 DM
HS
DRI24h = 1 ± 0.2 g O2 kg-1 DM h-1
AT4 = 61 ± 12 g O2 kg-1 DM
Fermented residue SSF
SF
DRI24h = 3 ± 1 g O2 kg-1 DM h-1
AT4 = 185 ± 48 g O2 kg-1 DM
21 days Digestion
SF: 365; 304; 273 mL biogas g-1 VS
HS: 132; 111 mL biogas g-1 VS
Anaerobic Digestion
Composting
HS
DRI24h = 0,61 ± 0,05 g O2 kg-1 DM h-1
AT4 = 48 ± 4 g O2 kg-1 DM
SF
DRI24h = 2 ± 0,4 g O2 kg-1 DM h-1
AT4 = 146 ± 27 g O2 kg-1 DM
Activity recovery after extraction
%Activity recovery calculation
1:5 (w:v) stirring TB
100% Activity recovery
SF Activity range: 8054 – 33374 U g-1 DM
HS Activity range: 6668 – 23541 U g-1 DM
Extraction ratio (w:v): 1:1; 1:2; 1:3; 1:4
Solvent type: DW, TB
Extraction mode: Agitated; Static; Recirculated
Lyophilisation
SF 95 ± 6 activity recovery
HS 94 ± 6 Activity recovery
Consequtive extractions
RA 1:2
TB/DW and Agitated/Static