Production of proteases from organic wastes by solid-state fermentation:

downstream and zero waste strategies

Short title: Down-stream of proteases produced by SSF

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

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

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

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

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

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

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

The experimental set up used in liquid circulation extraction was composed of a peristaltic pump Watson-Marlow 400L2 with variable rotor speed from 2.5 to 50 rpm and a 0.5 L plastic vessel with two adaptors, one at the top and one at the bottom, 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 37°C 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 (250°C), 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 probe, 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.

\[
\text{Protease activity (U g}^{-1}\text{DM}) = \frac{C \times 15 \times V}{DM}
\]  

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

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

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

Chemical oxygen demand (COD)

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

Dynamic Respirometric Index (DRI)

In this case, respirometric tests were performed on the residues before and after SSF in order to determine their stability (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, 2001).

A dynamic respirometer following the method described by Adani et al. (2003) was used. The experimental device was described by Pognani et al. (2011). According to Gea et al. (2004) the test was carried out at a constant temperature of 37°C. All the experiments were performed in triplicate. Cumulative oxygen demand, AT\textsubscript{4} (g O\textsubscript{2} kg\textsuperscript{-1}
dry matter), and Dynamic Respirometric Indices, DRI$_{24}$ DRI$_{1hour}$ (g O$_2$ kg$^{-1}$ dry matter h$^{-1}$), were calculated (Mejías et al. 2017, Almeira et al. 2015).

Routine methods

pH was calculated by soaking 10 g of sample in 50 mL of distilled water. After 30 min, pH value was measured with a pH meter Crison micro pH 2001. Volatile solids, moisture content, total organic carbon (TOC), total Kjeldahl nitrogen, (TKN) and Soluble N-NH$_4^+$ were measured according to TMECC (The U.S. Department of Agriculture and The U.S. Composting Council, 2001).

TOC (Total Organic Carbon) was determined using an O.I. Analytical Solid TOC Analyser/Win TOC Solids v3.0, and TKN was measured using a Bloc Digester 6 (with six tubes capacity) (J.P. Selecta S.A., Barcelona, Spain) and a Büchi Distillation Unit K-355 (Flawil, CH).

Fat content (HEM-Hexane extractable material) was measured using a standard Soxhlet method with n-hexane as organic solvent (The U.S. Environmental Protection Agency, Method 9071B) (The U.S. Department of Agriculture and The U.S. Composting Council, 2001).

Statistics

One-way analysis of variance was performed to compare the mean values of activity recovery obtained under different experimental conditions. Multiple factor regression, to obtain a linear equation reflecting the influence of each experimental condition in activity recovery, was calculated using also activity recovery data. Excel 2010 data tools was used in both calculations
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
was the residue that presented lowest C/N ratio, while the range of production for wheat bran and the mixture of coffee pulp and corn cobs were 920 U mL\(^{-1}\) and 582 U mL\(^{-1}\) respectively. Although wheat bran presents also a high production value, enrichment with peptone was carried out previously to the SSF process and only *Jatropha curcas* was specifically chosen for its high N content, according to the authors. In the present work, a high protease production was obtained, compared with the examples above, without any enrichment of the initial mixture, as C/N ratio was already adequate.

Material was also biodegradable, as the respirometric values show (Table 4).

**SSF process evolution in 10 L reactors**

Figures 2a and 2b show an example of temperature and DRI 1 hour (g O\(_2\) kg\(^{-1}\) DM h\(^{-1}\)) profiles obtained in the fermentation process of HS and SF, respectively. The highest protease activity for HS was always observed in the mesophilic phase after 8 days of process, while SF protease activity presented its maximum at thermophilic temperatures after 5 days of fermentation. Maximum activity point was determined in previous experiments by sampling the reactors periodically and determining the protease activity for both mixtures. Abraham et al. (2013) found that maximum activity for SF was observed at the 3\(^{rd}\) day for a mixture equal to the one used in this work. For this residue, maximum biological activity concurred with maximum enzymatic activity. On the other hand, Abraham et al. (2017) found the highest activity value for a mixture of cow hair and digested sludge at the 14\(^{th}\) day of fermentation. For the mixture used in this work, the highest value for protease activity was found in the 8\(^{th}\) day of fermentation approximately. Difference with Abraham results can be due to the different inoculum, as in this work fresh sludge was used. Abu Yazid et al. (2016) characterized the protease obtained from solid state fermentation of hair and anaerobic sludge
determining that the alkaline protease was a serine type in the range between 26 and 100 KDa, and exhibit good stability in a temperature range of 30-50 °C and alkaline pH. In literature shorter optimum fermentation times are found for protease production through solid-state fermentation. Kandasamy et al. (2016) found maximum yield of protease production after 60 h of fermentation, using coffee pulp and corn cob as a substrate inoculating with *Bacillus sp. BT MASC 3*, Pouryafar et al. (2015) determined an optimum incubation time of 48 h using wheat bran as substrate with *Bacillus licheniformis*. However, in the literature consulted, the SSF process was usually interrupted after a fixed time, without taking into account the biological activity. So it is difficult to determine if the maximum production occurred at this time, because no information about the complete production curve is presented. Also, the highest amount of solid used in the references found was 25 g. In consequence, temperature control and oxygen availability was not a limitation of the process. In the present work, SSF was performed at a pilot scale and this approach requires a complete monitoring of the process, which makes that highest protease activity is obtained at different times. Thus, the direct comparison is not possible. High values of DRI 1hour were maintained longer in the case of SF, which indicates a higher content of rapidly biodegradable material. These data are in agreement with previous results obtained by Abraham et al. (2013, 2017). The range of protease activity obtained on this work was 6668 to 23541 U g⁻¹ DM for HS and 8054 to 33374 U g⁻¹ DM for SF. For both residues, pH reached values between 8.0 and 8.8 during the thermophilic phase, and remained in this range until the end of the process.

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

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

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

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

\textit{Volume-weight (solvent-waste) ratio}
Results of one factor variance test of experiments differing from extraction ratio w: v showed, for SF, no statistical difference between extractions performed at 1:3 and 1:4 w: v. However, in some cases, significant differences were found between 1:4 and 1:2 w:v extractions and even more significant with 1:1 w:v. When the test was performed for HS results, there was no difference between recovery at different extraction ratios or difference appear between 1:4 and 1:3 extraction but not between 1:4 and 1:2 ratios.

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

Linear regression using Excel software was applied to data from Table 2. Assignation of values to the different extraction parameters in order to perform the linear regression, as only numbers can be adjusted, was as follow: values of 1 and -1 to static and agitated mode and also to DW and TB respectively, values of 1, 2, 3, and 4 to
extraction ratios of 1:1, 1:2, 1:3 and 1:4, respectively. Recirculation mode results were
excluded from calculations, since experiments in this agitation regime were not
performed with all ratios. Extraction parameters where designated as follow, S for
solvent type, EM for extraction mode, and RA for ratio of extraction w: v. The results
obtained are displayed below for SF (equation 3) and HS (equation 4).

\[
\% \text{ Activity recovery} = 48.9 + 1.7(S) -3.4 \text{ (EM)} + 9.6 \text{ (RA)} \quad (R^2 = 0.81) \quad (\text{Eq 3})
\]

\[
\% \text{ Activity recovery} = 51.3+ 5.2(S) -18.3(EM) + 9.0 \text{ (RA)} \quad (R^2 = 0.82) \quad (\text{Eq 4})
\]

As observed, there is a correlation between the different results, as regression
coefficients are 0.81 and 0.82, considered high for experimental values.

SF regression shows the highest coefficient for RA, which means that extraction
ratio has the greater influence in the extraction of protease. However, for HS, the
parameter that presents more relevance is the extraction mode. In both cases, it is clear
that agitated mode will produce the maximum recovery yield.

To sum up, the facts that can be deduced from those equations agree with the
results obtained by one factor variance test, indicating that extraction yield will increase
with extraction ratio for SF with little dependence on the agitation mode. When
proteases are extracted from HS, agitation must be applied to achieve maximum activity
recovery, but a lower extraction ratio is allowed.

Number of extraction stages

Consecutive extractions were carried out for HS and SF using DW and TB in a ratio of
1:2 w: v with agitation and in static mode. Figure 3 a) and b) shows the percentage of
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.
No relation between extraction parameters and activity conservation was observed, as samples of each set of extraction parameters were assayed. Minimum activity recovered was 87 ± 3 for HS and 88% for SF. Abu Yazid et al. (2016) reported an activity loss of 21% after lyophilisation of protease extract obtained also from the fermentation of hair and sludge. During freezing and drying, protein can suffer denaturation, losing its activity. Bonds between water molecules disappear during drying process, but some components present the ability to stabilize the protein structure and preserve activity (Mensink et al. 2017). The same protein in a different matrix can lead to different activity preservation. Activity losses have been reported by Imamura et al. (2014) for different proteins after drying in sodium and potassium phosphate buffer. The highest recovery with no salts addition reached 31%. Thus, lyophilisation in this case results in low activity losses compared to the results reported, and a possible explanation can be that no unfolding of the protein is happening during lyophilisation.

**Protein hydrolysis by SSF produced protease**

Abu Yazid et al. (2017) described the immobilisation of protease, obtained from the same residues used in this work, onto functionalized magnetic iron oxide nanoparticles through covalent binding method, obtaining a retention yield of 96%. Abu Yazid et al. (2017) also used the immobilized enzymes during at least three cycles for protein hydrolysis with no loss of activity. With an initial protein amount of 5.2 mg from casein, the degree of protein hydrolysis achieved was 75% and 50% of the initial amount of protein for enzymes produced by HS and SF respectively.

According to those results, for a 50 L reactor of fermented HS and SF (17.5 kg and 20 kg respectively), obtaining the maximum activity achieved in this work, 23541 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 hydrolysis 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
Biogas potential tests were undertaken to determine the suitability of anaerobic digestion to treat the solid waste after enzyme extraction. After 21 days, SF replicates produced 365, 304 and 273 mL of biogas g\(^{-1}\) VS with a content of approximately 43% of methane. During the same period of time, HS produced 132 and 111 mL biogas g\(^{-1}\) VS, with both presenting 40% of methane content. Biogas production in this case was significantly lower than for SF. This result was expected since HS fermentation lasted 6 more days than SF, so HS was more biodegraded at the point of maximum protease production and extraction.

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

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

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

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

In the case of SF, SSF experiments were stopped for enzyme extraction at the 3rd and 5th day of process, showing values of average oxygen uptake rate during the last 24 hours (OUR) of 3, 4.5, 9 g O₂ kg⁻¹ DM h⁻¹ in thermophilic range. Thus, SF was clearly a non-stable material at this stage of the process.
Composting of SF after SSF and enzyme extraction was carried out in 4.5 L reactors in duplicate. Both replicates showed an increase of temperature and oxygen consumption during the first 24h, followed by a fast decrease to environment temperature. The maximum temperature values reached were 34°C and 42°C respectively, and maximum OUR$_{24}$ values around 5 g O$_2$ kg$^{-1}$ DM h$^{-1}$, but never reaching the thermophilic range again.

After 9 days of composting process, the residue could not be considered stable 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 O$_2$ kg$^{-1}$ DM. A longer composting process will be needed, since the decrease of biological activity is slow.

According to Barrena et al. (2009), aerobic and anaerobic indices are closely related. Values reported for the ratio between DRI$_{24}$ and biogas production in liquid condition and between AT$_4$ and biogas production at the same condition for different wastes were 86 and 1.56 respectively. The same ratios were calculated using the values obtained in this study, giving values of 84 and 1.56 respectively. These numbers agree with the parameters reported by Barrena et al. (2009).

**Conclusions**

In this work a complete assessment of the downstream of SSF processes for the production of proteases has been performed (Figure 4). Yields of activity recovery in protease extraction and lyophilisation stages, under a wide variety of conditions, were calculated. According to the results, extraction ratio of 1:3 for SF is recommended and agitation mode at extraction ratio 1:2 would be the adequate conditions for HS. Consecutive extractions in static mode seem an efficient way of obtaining also a good 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.

**Acknowledgements**

This work could not be completed without the help of Noraziah Abu Yazid and Raquel Barrena. We would also like to thank the Spanish Ministerio de Economía y Competitividad, which gave financial support (Project CTM2015-69513-R). Maria Marín also thanks the Universitat Autònoma de Barcelona for a predoctoral scholarship.

**Conflict of interest**

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


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


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: O₂ 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; AT₄: 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

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

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)

<table>
<thead>
<tr>
<th>SF</th>
<th>Mean</th>
<th>St deviation</th>
<th>HS</th>
<th>Mean</th>
<th>St deviation</th>
<th>Solvent (S)</th>
<th>Extraction mode (EM)</th>
<th>Ratio W:V (RA)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>53</td>
<td>9</td>
<td>71</td>
<td>5</td>
<td>4</td>
<td>DW</td>
<td>Agitated</td>
<td>1:1</td>
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<tr>
<td></td>
<td>54</td>
<td>10</td>
<td>66</td>
<td>3</td>
<td>49</td>
<td>TB</td>
<td>Agitated</td>
<td>1:1</td>
</tr>
<tr>
<td></td>
<td>55</td>
<td>4</td>
<td>52</td>
<td>4</td>
<td>DW</td>
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<td>70</td>
<td>6</td>
<td>93</td>
<td>19</td>
<td>DW</td>
<td>Agitated</td>
<td>1:2</td>
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<td></td>
<td>73</td>
<td>12</td>
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<td>3</td>
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<td>Static</td>
<td>1:2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>65</td>
<td>8</td>
<td>50</td>
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<td>Static</td>
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<td></td>
<td>63</td>
<td>6</td>
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<td>8</td>
<td>DW</td>
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<td>1:2</td>
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<td>12</td>
<td>TB</td>
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<td>1:4</td>
<td></td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th></th>
<th>SF (Fermented Soy Fibre)</th>
<th></th>
<th>Conductivity (mS/cm)</th>
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<th>HS (Fermented Hair + Sludge)</th>
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<th>Conductivity (mS/cm)</th>
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<tbody>
<tr>
<td></td>
<td>S</td>
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<td>RA</td>
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<td></td>
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<td>2.7</td>
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<td></td>
<td>TB Static</td>
<td>1:3</td>
<td>8.37</td>
<td>5.83</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TB Static</td>
<td>1:4</td>
<td>8.31</td>
<td>5.09</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Residue</th>
<th>Solid State Fermentation</th>
<th></th>
<th></th>
<th>Composting</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial AT&lt;sub&gt;4&lt;/sub&gt;</td>
<td>Final AT&lt;sub&gt;4&lt;/sub&gt;</td>
<td>Initial DRI&lt;sub&gt;24&lt;/sub&gt;</td>
<td>Final DRI&lt;sub&gt;24&lt;/sub&gt;</td>
<td>Final AT&lt;sub&gt;4&lt;/sub&gt;</td>
<td>Final DRI&lt;sub&gt;24&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td>(g O&lt;sub&gt;2&lt;/sub&gt; kg&lt;sup&gt;-1&lt;/sup&gt; DM)</td>
<td>(g O&lt;sub&gt;2&lt;/sub&gt; kg&lt;sup&gt;-1&lt;/sup&gt; DM)</td>
<td>(g O&lt;sub&gt;2&lt;/sub&gt; kg&lt;sup&gt;-1&lt;/sup&gt; DM h&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>(g O&lt;sub&gt;2&lt;/sub&gt; kg&lt;sup&gt;-1&lt;/sup&gt; DM h&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>(g O&lt;sub&gt;2&lt;/sub&gt; kg&lt;sup&gt;-1&lt;/sup&gt; DM)</td>
<td>(g O&lt;sub&gt;2&lt;/sub&gt; kg&lt;sup&gt;-1&lt;/sup&gt; DM h&lt;sup&gt;-1&lt;/sup&gt;)</td>
</tr>
<tr>
<td>SF</td>
<td>326 ± 98</td>
<td>185 ± 48</td>
<td>5 ± 2</td>
<td>3 ± 1</td>
<td>146 ± 27</td>
<td>2.1 ± 0.4</td>
</tr>
<tr>
<td>HS</td>
<td>113 ± 11</td>
<td>61 ± 6</td>
<td>2.0 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td>48 ± 4</td>
<td>0.61 ± 0.05</td>
</tr>
</tbody>
</table>

AT<sub>4</sub>: Accumulated O<sub>2</sub> consumption after 4 days; DRI<sub>24</sub>: dynamic respirometric index based on 24 hours of maximum O<sub>2</sub> consumption.

Values are the average of independent experiments.
Figure 1

(a)

(b)
Figure 2

a) 

b)
Figure 3

(a) and (b) show the percentage activity recovery for different numbers of extractions with TB agitated, TB static, DW agitated, and DW static conditions.
Figure 4

**HS**
DRI$_{24h}$ = 2 ± 0.1 g O$_2$ kg$^{-1}$ DM h$^{-1}$  
AT$_4$ = 112 ± 11 g O$_2$ kg$^{-1}$ DM

**SF**
DRI$_{24h}$ = 5 ± 2 g O$_2$ kg$^{-1}$ DM h$^{-1}$  
AT$_4$ = 326 ± 98 g O$_2$ kg$^{-1}$ DM

**Fresh residue**

**Fermented residue**

**SSF**

21 days Digestion  
SF: 365; 304; 273 mL biogas g$^{-1}$ VS  
HS: 132, 111 mL biogas g$^{-1}$ VS

**Anaerobic Digestion**

**Activity recovery after extraction**

**Composting**

**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

**Consecutive extractions**  
RA 1:2  
TB/DW and Agitated/Static