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

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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 $94 \pm 6\%$ 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, such 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 the recent years, the use of SSF has become outstanding due to its higher yield and the requirement of simpler and smaller equipment (Kriaa and Kammoun 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 such 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 30 °C, 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

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industrial scale is 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 there is 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 that can be used 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 using 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 of 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. (2016a), the listed problems of SSF processes at pilot scale were overcome by 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 enzyme. 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 of 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 and 0.074 μm , while cow hair particle size is about 1 cm long. 79% in weight of bulking agent particle size is between 16 and 3.15 mm. Soy fibre was stored at $-20\text{ }^{\circ}\text{C}$ before the experiments, while fresh sludge and hair were stored at $4\text{ }^{\circ}\text{C}$ due to the structural changes that freezes 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. (2016a), where details of the process and proteases production range are thoroughly provided.

SSF materials and experimental set-up

Solid-state fermentation was carried out in 10 and 50 L (working volume) adiabatic reactors, to minimize environment influence, as described in detail in previous works (Fig. 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. The cap of the reactor has three connections: one of them for a temperature probe, another one for inlet air and the third one for exhaust gases outlet. 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

Fig. 1 **a** 4.5 and 10 L reactors, **b** 50 L reactor. 1: 4.5 and 10 L reactor; 2: 4.5 and 10 L reactor inside; 3: mass of flowmeter; 4: temperature probe; 5: water trap; 6: O_2 electrochemical sensor. 7: 50 L reactor; 8: 50 L reactor inside

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. In each extraction 200 g of the material obtained after fermentation in 10 or 50 L reactors were used. The solid and the solvent were in contact during 1 h under different regimes, at environment temperature, in this case, around 15 °C, and then the liquid phase centrifuged for 10 min at 10,000 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 for 1 h. The extraction ratio was selected in basis on Salariato et al. (2010) work who extracted polygalacturonase with distilled water at a solid: solvent ratio of 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: (1) no agitation (static mode), (2) orbital agitation at 120 rpm, and (3) 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 in Table 2.

Experimental set-up for static and agitated mode consists 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 1 h. 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, 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 (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 hermetically 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 (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 head-space volume. The bottles were manually agitated before and after measurement, 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 3 m 1/8" × 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 min. 100 µl of gas sample were required for the analysis.

Composting assays

Solid material after SSF and protease extraction was stabilized to test its suitability as soil organic amendment. Experiments were carried out in 50 and 4.5 L working volume reactors (Fig. 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. The cap has two connectors, one for a temperature probe and the other as exhaust gases outlet. The outlet air was conducted through a water trap at 4 °C, and oxygen content was 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 assures maximum O₂ 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, to continue the stabilization. During the drying step, the solid was disposed in thin layer out of the reactor for 24 h.

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 and it was 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 was 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 10,000 rpm and the supernatant was analysed. After the 2 incubation hours, 5 mL of trichloroacetic acid (TCA) (15%) was added 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 1 h, to perform a colorimetric determination of the amount of tyrosine present, using the 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) equivalent to 1 µg of tyrosine released after 1 h under incubation conditions. Calculations were carried out according to Eq. 1.

$$\text{Protease activity (U g}^{-1}\text{DM)} = \frac{C15V}{\text{DM}} \quad (1)$$

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 calculation

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

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

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 O₂ L⁻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 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, AT₄ (g O₂ kg⁻¹ dry matter), and Dynamic Respirometric Indices, DRI₂₄ DRI 1 hour (g O₂ kg⁻¹ dry matter h⁻¹), were calculated (Mejias et al. 2017; Almeida 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–NH₄⁺ 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 also calculated using activity recovery data. Excel 2010 data tool was used in both the 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

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

Values are the average of independent experiments and its standard deviation

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)

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 Sukasem 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 *J. 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 and 582 U mL^{-1} , respectively. Although wheat bran also presents a high production value, enrichment with peptone was carried out previously to the SSF process and only *J. 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

Figure 2a and b show an example of temperature and DRI 1 hour ($\text{g O}_2 \text{ kg}^{-1} \text{ DM h}^{-1}$) 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 3rd 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 14th day of fermentation. For the mixture used in this work, the highest value for protease activity was found in the 8th 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. (2016a) characterized the protease obtained from solid-state fermentation of hair and anaerobic sludge determining that the

Fig. 2 Temperature (lines) and DRI 1 hour (short-short) profiles obtained in SSF processes **a** HS fermentation, **b** SF fermentation. DRI 1 hour Dynamic Respirometric Index 1 h average value

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 corncob 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 23,541 U g⁻¹ DM for HS and 8054 to 33,374 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 $91 \pm 8\%$ for SF was achieved in agitated mode at 1:4 using DW as solvent, while a maximum recovery of $121 \pm 22\%$ 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 was present between recoveries using DW or

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

% 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*

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 rises 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 decreases, conductivity increases 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-Holownia 2015). Karatas et al. (2013) compared different solvents for the extraction of proteases and α -amylase from fermented rice husk, showing tap water as 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 has 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⁻¹, which produces 14 times recirculation of the whole volume

Table 3 pH and conductivity of the extracts

SF (fermented soy fibre)					HS (fermented hair + sludge)				
S	EM	RA	pH	Conductivity (mS cm ⁻¹)	S	EM	RA	pH	Conductivity (mS cm ⁻¹)
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⁻¹); TB (HCl—Tris buffer): pH: 8.1, Conductivity: 2.48 (mS cm⁻¹)

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 and 300 rpm for casaba bagasse and palm kernel, respectively. This difference in recovery depending on 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 ratio of solvent–solid were significant.

The small particle size of SF in contact with the solvent losses structure enhancing solid–solvent contact. Vardanega et al. (2009) also reported no influence of the agitation speed on 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 extraction 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. Nabarlantz et al. (2012) compared extraction of protease and lipase from two WWTP sludges 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, is the cause of poorer 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.

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 the recovery at different extraction ratios or difference appears 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 confirms a direct relationship between *w:v* ratio and activity (Vardanega et al. 2009), the opposite result was reported by Volken de Souza et al. (2008) who found a negative effect because 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. Assignment of values to the different extraction parameters to perform the linear regression, as only numbers can be adjusted, was as follows: 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 were designated as follows, S for solvent type, EM for extraction mode, and RA for ratio of extraction *w:v*. The results obtained are displayed below for SF (Eq. 3) and HS (Eq. 4).

$$\begin{aligned} \% \text{ Activity recovery} &= 48.9 + 1.7(S) - 3.4(EM) \\ &+ 9.6(RA) (R^2 = 0.81) \end{aligned} \quad (3)$$

$$\begin{aligned} \% \text{ Activity recovery} &= 51.3 + 5.2(S) - 18.3(EM) \\ &+ 9.0(RA) (R^2 = 0.82) \end{aligned} \quad (4)$$

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 a 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 3a, b shows the percentage of activity recovery for the consecutive extractions performed on 200 g of fermented solid.

Fig. 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

As it can be seen for both residues, approximately 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 fourth one. In this case, recovery in the first and second extraction was very similar, approximately 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 in duplicate. Lyophilisation resulted in high yields of activity recovery, for SF, $95 \pm 4\%$ was recovered as a mean value and $96 \pm 6\%$ 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. (2016a) reported an activity loss of 21% after lyophilisation of protease extract which was also obtained 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 salt 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. (2016b) 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. (2016b) 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 and 20 kg respectively), obtaining the maximum activity achieved in this work, 23,541 U DM⁻¹ for HS and 33,374 U DM⁻¹ for SF, it would be possible to hydrolyse 156.6 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 immobilized protease on ferric nanoparticles solution (8.50 mg mL⁻¹), during 2 h incubation at 50 °C. Hydrolysis yield in this case was lower (9.86%) compared to the one reported by Abu Yazid et al. (2016b) (75 and 50%). Comparing activity to commercial products, neutral protease for beer brewing (Food grade) provided by Creative Enzymes (2017) was taken as example. The mentioned product activity, as stated in manufacturing specifications, is 70,000 U g⁻¹, being an activity unit (U) 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. (2016b) was capable of immobilizing proteases into ferric nanoparticles obtaining an activity of 45.9 U mg⁻¹ of ferric nanoparticles (NP) for HS and 31.9 U mg⁻¹ 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. (2016b) will have an activity of 45,900 U g⁻¹ NP, and will be reusable for at least 3 cycles without activity loss, resulting in a much better yield than 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 and Kim (2013) reported the 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, but Merlino et al. (2012) reported a production approximately of 590 Nm³ t⁻¹ VS of broken soybean after 35 days of digestion at 40 °C, with a methane content of approximately 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 was 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, to decrease its moisture content from 74 to 62%. Before drying, no biological activity occurred despite the aeration provided. According to Chen and He (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. (2016a) also composted a mixture of hair and anaerobic digested sludge after fermentation and extraction of protease

Table 4 Results of respirometric assays

Residue	Solid-state fermentation				Composting	
	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

Final SSF values are initial composting values

Values are the average of independent experiments

AT_4 accumulated O_2 consumption after 4 days, DRI_{24} dynamic respirometric index based on 24 h of maximum O_2 consumption

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. 2016). Table 4 shows DRI_{24} values of 2.0 ± 0.1 and 0.61 ± 0.05 for initial mixture of HS after SSF and composted product,

Fig. 4 Downstream summary. DRI 1 h Dynamic Respirometric Index 1 h 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

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 h (OUR_{24}) 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 24 h, followed by a fast decrease in environment temperature. The maximum temperature values reached were 34 and 42 °C, respectively, and the maximum OUR_{24} values were around 5 g O₂ kg⁻¹ DM h⁻¹, but never reached 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 O₂ kg⁻¹ DM h⁻¹ and AT_4 value of 146 ± 27 g O₂ 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 (Fig. 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 condition for HS. Consecutive extractions in static mode seem an efficient way of also obtaining 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 of waste: solvent $w:v$ becomes the most determining factor in the activity recovery yield, while agitation mode is the key factor when solubility decreases.

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 through composting obtaining a stable product.

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Compliance with ethical standards

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. *Res J Pharm 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 (2016a) Assessment of protease activity in hydrolysed extracts from SSF of hair waste by and indigenous consortium of microorganisms. *Waste Manag* 49:420–426
- Abu Yazid N, Barrena R, Sánchez A (2016b) The immobilisation of protease produced by SSF onto functionalized magnetic nanoparticles: application in the hydrolysis of different protein sources. *J Mol Catal B Enzym* 133(1):s230–s242
- Adani F, Gigliotti G, Valentini F, Lاراia 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* *FDHNI*: production, high yielding recovery optimization through statistical approach and application. *Biocatal Agric Biotechnol* 6:51–57
- Ahmed SA, Mostafa FA (2013) Utilization of orange bagasse and molokhia stalk for production of pectinase enzyme. *Braz J Chem Eng* 30(3):449–456
- Alef K, Nannipieri P (1995) Methods in applied soil microbiology and biochemistry. Academic Press Limited, San Diego, pp 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. *Bioresour 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
- Casciadori 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 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 information (2017). http://www.creative-enzymes.com/product/neutral-protease-for-beer-brewing-food-grade-_3168.html. Accessed 28 Feb 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. *Crit Rev Environ Sci Technol* 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 For Res* 2(63):191–200
- European Commission, Working document, Biological treatment of Biowaste 2nd draft. http://www.compost.it/www/publicazioni_on_line/biod.pdf (2001). Accessed 19 Sep 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 Technol* 48:2119–2126
- Ge X, Vasco-Correa J, Li Y (2016) Solid-State fermentation bioreactors and fundamentals. Current developments in biotechnology and bioengineering: bioprocesses. *Bioreact 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). <http://www.bmu.de/files/pdfs/allgemein/application/pdf/ablagerungsverordnung.pdf>. Accessed 26 Sep 2016
- Gopalan N, Nampoothiri KM (2016) Biotechnological production of enzymes using agro-industrial wastes: economic considerations, commercialization potential, and future prospects. In: *Agro-industrial wastes as feedstock for enzyme production*, pp 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. *Bioresour 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 Biotechnol* 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 Freire 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 Biotechnol* 89:579–586
- 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
- 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. *Eur J Pharm Biopharm* 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
- 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 α -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 Recycl* 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. In: 32nd congress of the International Union of Leather Technologists and Chemist Societies, IULTCS 2013
- Pal A, Khanum F (2010) Production and extraction optimization of xylanase from *Aspergillus niger* DFR-5 through solid-state-fermentation. *Bioresour Technol* 101:7563–7569

- Pan-In S, Sukasem N (2016) Methane production potential from anaerobic co-digestions of different animal dung and sweet corn residuals. *Energy Proced* 138:943–948
- 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. *Bioresour 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 Autònoma 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
- Pourayafar 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* 16:933–938
- 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. *Biocatal Agric Biotechnol* 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. Edaphos International, Houston
- Vardanega R, Remonatto D, Arbter F, Polloni A, Rigo E, Ninow JL, Treichel H, de Oliveira D, Di Lucio M (2009) 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 Technol* 3(3):461–465
- Volken de Souza CF, Rodrigues RC, Heck JX, Ayub MAZ (2008) Optimization of transglutaminase extraction produced by *Bacillus circulans* BL32 on solid-state cultivation. *J Chem Technol Biotechnol* 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