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Optimization of down-stream for cellulases produced under solid-state fermentation of coffee husk

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Abstract

This work systematically studies the downstream process of the solid state fermentation (SSF) of a mixture of coffee husk and wood chips, inoculated with compost, for cellulase production. Downstream of SSF (at pilot scale) remains as one of the less studied stages of the process, being critical in technical, environmental and economic terms. In this study, the specific downstream points considered were: i) enzyme extraction yield, in terms of extraction ratio solid:solvent, agitation mode and solvent type; ii) enzymatic activity recovery of the lyophilised extract and iii) efficiency of consecutive extractions. Results indicate a maximum activity recovery of 108 ± 30% in the extraction performed at ratio 1:5 solid-solvent, in static mode and with distilled water. Statistical analysis revealed a high dispersion of the results and needs to be considered to extract consistent conclusions in any downstream of SSF. Lyophilisation demonstrated to be an adequate technology for enzymatic activity preservation. Regarding consecutive extractions, yield recovery in the first and second extraction maintain a similar value. In a framework of a zero-waste enzyme production process, different strategies have been tested for the remaining solid after extraction. Respirometric tests reveal that it is possible to aerobically stabilize the remaining solid obtaining a compost like material, whereas anaerobic digestion resulted in low methane yields (51±3 mL methane g^{-1} VS).

Keywords: Cellulase extraction, activity recovery, solid state fermentation, down-stream, zero waste process.

Statement of novelty

Nowadays, most of the studies carried on in the field of enzyme production through solid state fermentation are focused on optimizing the production yield at a laboratory scale. Extraction is mainly performed using a high ratio solid:solvent in the range of 1 to 15 mL per g, suitable for a laboratory scale but non-viable at an industrial one. The present work differs from the others published so far as it focuses on the aspects related to the downstream stage to be implemented at an industrial scale, like the optimization of the extraction conditions in order to save resources, management of the remaining solid after the extraction and conservation of the final product in low-cost mode.

The fact that the production process itself presents many advantages, as it has been proved to be reproducible at a pilot scale, requires a low investment due to the use of a residue as raw material, the equipment is simple and the production yield is high, makes the whole process interesting for implementation at an industrial scale.

The scale of the experiments performed in this work allow to apply the results to the design of an industrial plant for the production of low cost cellulase from coffee husk and to its economic assessment, as well as the conservation of the cellulases by standard ways. The remaining waste is also considered as a source of biogas or compost in a zero waste global strategy.

Introduction

Almost 85% of the energy consumed worldwide was obtained from fossil fuels during 2016 [1]. The excessive use of fossil fuels during the last years has produced an increment of CO_2 level in the atmosphere, which is nowadays reaching an historical maximum of 409 ppm. This rise has a pernicious impact on the global climate creating a greenhouse effect and increasing temperatures around the globe. In the search for new renewable sources of energy, bioproducts like bioethanol are gaining relevance. However, its production process must be optimized in order to achieve a major use compared to fossil fuel. Among the different stages of bioethanol production, hydrolysis of the lignocellulosic material can be performed chemically, by an acid or alkaline reagent, or enzymatically using cellulases, which results in less harsh operational conditions, since most of the enzymes require mild operational conditions, avoiding great energy expenses and corrosion issues [2]. In fact, one of the main uses for cellulases is the conversion of cellulosic and lignocellulosic materials to bioethanol [3, 4]. For this reason, finding a low-cost and robust process for cellulase production is a key point in bioethanol manufacturing.

Cellulases have been in the market for more than 30 years, and they are used in all type of industries. As an example, cellulases have a role in textile industry as biopolishers of fibres or in the pulp and paper field in enzymatic deinking and to improve brightness of the product. Microbial production is the most used method in industry for cellulase production. A wide variety of microorganism like fungi [5], yeast or gram-positive [6] and gram-negative bacteria can be used in the production of cellulase. As a result, the morphology and specificity of the enzyme will depend on the substrate, operational conditions and microorganism used in the production process [7].

Two main methods are used for cellulase production, submerged fermentation (SmF) and solid state fermentation (SSF). Both methods show advantages and disadvantages. No gradients of temperature and media composition are found in submerged fermentation. However, solid state fermentation requires smaller and cheaper equipment, which makes it economically attractive; besides, the option of using a lignocellulosic solid waste as a nutrient source and support for the growing of the microorganism increases the profit obtained in an industrial process and reduces the waste disposed into the environment. Several types of agro industrial wastes have been reported as sources of cellulose: Sun et al. [8] used banana peel obtaining a cellulase activity filter paper unit (FPU) of 5.56 FPU per gram of dry matter; Dhillon et al. [9] also reported a cellulase production of 133.68 U per gram of dry substrate from apple pomace using *Aspergillus niger* NRRL-567 as inoculum. Other residues used as substrate are wheat straw, corn fibre or seaweed [10, 11]. The mentioned cases were all reported at laboratory scale. Few processes have been developed at a scale large enough to be used for industrial purposes, especially when considering all the downstream process. Cerda et al. [12] reported a cellulase production of 9 FPU of enzyme activity per gram of dry matter from coffee husk, a residue obtained after the thermal peeling of the coffee bean. In that case, solid state fermentation was carried on under non-sterile conditions and no temperature control at a 4.5 L scale, allowing the consortia of microorganisms present in the residue to develop. Maximum production was observed after 48 hours of process. Also, Cerda et al. [12] developed a strategy to obtain a continuous stable production of cellulase from the mentioned residue. This process was tested at pilot scale, obtaining higher temperatures, which caused a decrease in the production, but showing that this strategy was valid for scaling up. These results confirmed coffee husk as a promising low-cost source of cellulase, as it cannot be used

as animal feedstock, according to local regulation, and the only possible use would be composting, less profitable than enzyme production.

One of the higher expenses required in solid state fermentation is the extraction of the final product from the solid matrix. In this specific case there is no information regarding recommended extraction agent, ratio solid:liquid for the extraction or agitation method. The optimization of these parameters is not a key point at laboratory scale but at industrial scale will determine the profitability of the process. Also, after the extraction, the solid waste remains, which still can be used in a more profitable way than landfilling. Thus, considering these facts, the current work aims to study different aspects of the downstream process for the production of cellulase from coffee husk, including optimal extraction conditions, taking into account different extraction agents, agitation methods and solid:liquid ratios. Also, the dispose of the solid residue after extraction has been studied, considering its use as feedstock for anaerobic digestion and biogas production, along with the composting of the waste to obtain an organic amendment. Storage of the extracted enzyme was also studied by determining the activity loss of the lyophilised product stored at 4 °C compared to its initial activity.

Materials and methods

Raw material

Coffee husk was provided by Marcilla S.A (Mollet del Vallés, Barcelona, Spain).

Coffee husk or silverskin was produced during coffee grains roasting. The main physico-chemical characteristics of this waste are summarized in Table 1. The residue was collected within 24h after its production and stored at -20 °C until needed. During SSF, coffee husk was mixed in a proportion 9:1 (w:w) with compost provided by the waste biological treatment plant of the Consorci per a la Gestió dels Residus del Vallès

Oriental (Granollers, Barcelona, Spain). Compost was added to increase the initial microbial population.

The mixture of compost and coffee husk was mixed with wood chips as bulking agent to provide an adequate porosity to the final mixture (CH). Wood chips were obtained from Trabede Jorba composting plant (Jorba, Barcelona, Spain), and mixed in a ratio 1:1 (v:v). Moistening of the mixture was not necessary since its value was always near 60% in weight. The suitability of this industrial residue as raw material for cellulase production through SSF has been described for the first time by Cerda et al. [12], where details of the process and cellulase production range are thoroughly provided.

SSF Materials and experimental set up

SSF was carried out in 4.5 and 10 L (working volume) adiabatic reactors, as described in detail in previous works [13, 14]. Both types of reactors were identical in operation. Briefly, air flow was adjusted by an air flow controller (Bronkhorst Hitec, The Netherlands), provided to the bottom of the reactor and evenly distributed by a net placed below the biomass. The oxygen content of output gases was measured by an electrochemical O2-A2 oxygen sensor (Alphasense, United Kingdom) and data were collected using a personal computer HP Compaq LA1951g Intel core ISvpro (Hewlett-Packard, USA), equipped with Ubuntu operation system. Data analysis was carried on by a non-commercial tailor-made software. Temperature was also measured (Transmisor CCPI/T-120, Seneca, Italy). Air flow was supplied to the reactors assuring that oxygen was always above 10% in output gases through a feedback control system in which two oxygen content set points were fixed: 11.5% as a minimum and 12.5% as maximum. Two values of air flow are set by the user, when oxygen concentration is

lower than 11.5%, the system selects the maximum air flow, when oxygen content is higher than 12.5%, the system selects the minimum air flow. Reactors capacity (mass of mixture coffee husk, compost and bulking agent) was 2.7 kg for 10 L reactors and 1.5 kg for 4.5 L reactors.

Cellulase activity recovery – Downstream

Cellulase activity determination – Reference value

To obtain the total cellulase activity, an extraction in agitated mode at 1:15 solid: solvent ratio and buffer citrate mono hydrated 0.05M solution at pH 4.8 (BC) as solvent was performed during half an hour. Pirota et al. [15] performed different experiments for cellulase extraction using ratios of 1:3, 1:6 and 1:9 finding that 1:9 was the optimal extraction ratio. Also Chandra et al. [16] found an optimum extraction ratio for β-endoglucanase at 1:4 ratio w:v from fermented wheat bran. In the case of coffee husk we have observed that an extraction ratio of 1:15 w:v guarantees a good contact between solid and liquid to ensure a total extraction of the enzyme [12].

Activity recovery was calculated referring the activity obtained in the extraction at given conditions as a percentage of the activity in the standard extraction, performed at 1:15 ratio, using BC and orbital agitation, according to equation 1.

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

Since various replicates of extractions under same experimental conditions (6 or 5 depending on the case) were performed, the rule of 25% was applied, discarding the values that differ more than 25% from the average.

187

188 *Extraction parameters*

189 Extractions were performed to evaluate the recovery of cellulase activity under different
190 experimental conditions.

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

193 a) *Solvent type:* Two different solvents were used to perform extractions, distilled
194 water (DW) and BC.

195 b) *Extraction mode:* The extraction modes were three: 1) no agitation (static mode),
196 ii) orbital agitation at 120 rpm, and iii) circulation of the solvent through a
197 column packed with the waste at 140 mL min^{-1} (8.4 kg h^{-1}), which resulted in the
198 renewal of the whole volume of liquid 7 times during the extraction time.

199 c) *Waste to solvent ratio (weight:volume):* Assayed ratios of solid:solvent were 1:2,
200 1:3 and 1:5.

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

202 Between 150 and 200 g of solid from SSF after 48h of fermentation were used in
203 every extraction. The solid and the solvent were in contact during half an hour under the
204 different regimes stated above and then the liquid phase decanted and centrifuged
205 during 10 minutes at 10000 rpm. Cellulase activity of the supernatant was measured and
206 referred to the value considered the total cellulase activity produced.

207 Experimental set up for static and agitated mode extraction consist of a set of
208 plastic containers of equal dimension and shape where solvent and solid were placed.
209 Static extraction was performed placing the fermented solid and the chosen volume of
210 solvent in the plastic container and waiting for half an hour. To obtain the extracted
211 liquid in agitated mode, the same procedure was followed but plastic containers were

placed in a Sanyo orbital shaker IOC402.XX1.C (Sanyo UK) incubator for half an hour at 120 rpm and room temperature.

The experimental set up used in liquid recirculation extraction was composed of a peristaltic pump Watson-Marlow 403U/L2 Ultra compact twin channel pump with variable rotor speed from 0 to 99.9 rpm (Watson-Marlow Alitea, England) and a 2 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 material was placed inside the vessel and each solvent added. The flowrate was set up in the pump and liquid circulation lasted for half an hour.

Number of extraction stages

Enzyme recovery from consecutive extraction stages was assayed performing three 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. Extractions were performed using the lowest-cost agitation mode and extraction solvent, which are static mode and DW as solvent, respectively, in order to evaluate if this strategy implied an increase in activity recovery with no extra cost.

Lyophilisation

Supernatant from extractions at 1:2 and 1:5 solid:solvent ratios, in static mode and using BC as extraction agent were used in this experiment. According to Farinas et al. [17]

optimum cellulase pH regarding stability is between 4 and 5.5, so BC was selected to preserve the enzyme stability.

Samples of 10 ml of the supernatant obtained from the centrifuged extract were frozen at -80°C inside plastic falcons and stored at same temperature. On the other hand, 500 mL of same supernatant were frozen at -80°C into two 250 mL beakers and lyophilized using a Virtis 5L sentry lyophilizer 248627 (Virtis, Gardiner, USA) connected to an Edwards vacuum pump RV5 A653_01_903 (Edward, United Kingdom). The beakers used in the experiment were weighted before and after the removal of the powder and the dry cleaning of the beaker. The obtained powder was easily removed from the walls of the flasks so there were no perceptible losses. After that, the remaining powder was mixed and pulverized using a ceramic mortar in order to completely homogenize the solid. Mass of powder obtained from the 500 mL extract was calculated by weigh difference, and the equivalent quantity to 10 mL of original supernatant was dissolved in DW. The remaining powder was stored at 4 °C.

Cellulase activity for the redisolved solid and for an unfrozen sample of 10 mL was measured for comparison using filter paper units (FPU) of activity for the supernatant obtained after extraction for both 1:2 and 1:5 ratios.

Zero waste strategies

Biomethane Potential (BMP)

Biomass fermented for the production of cellulase was tested after extraction as feedstock for biogas production through anaerobic digestion. Anaerobically digested 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 [18], detailed by Ponsá et al. [19]. According to this standard methodology, BMP tests were carried out under mesophilic conditions and during approximately 20-30 days. Inoculum and biomass from SSF were mixed in a feed to inoculum (F:I) final ratio of 0.5 and placed in hermetic closed bottles. Each sample was tested in triplicate. The ratio F:I was calculated based on substrate and inoculum initial amounts of volatile solids (VS). According to Ponsá et al. [19], this ratio is the optimum to maximize biogas production. The content of volatile solids in fermented CH and anaerobic inoculum were measured, obtaining a percentage of 29.8% and 1.1% on a wet basis, 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 (Memmert, Germany) working at 37ºC for 21-25 days. The amount of biogas produced was calculated from the biogas pressure, measured by an ISE 30A-01-P vacuum switch (SMCpneumatics.com, USA), 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. Percentages of methane and carbon dioxide in the biogas were measured using a gas chromatograph 5890A with a column 17066_F ParcpackQ (250C), support 100/120, tube 3m 1/8”x 5.5 mm. Initial oven temperature was 70°C, final oven temperature was 120°C, determination time was 8 min, injector temperature was 150°C and detector temperature was 180°C. Methane peak was detected at 0.8 minutes. 100 µl of gas sample were required for the analysis.

Composting assays

Solid material after SSF and cellulase extraction was stabilized in order to test its suitability as soil organic amendment. Experiments were carried out in 10 L working volume reactors. Reactors have been previously described [13, 20]. The composting process was performed with air supplied under OUR control [14], which ensures maximum O_2 consumption during the experiment.

Analytical methods

Cellulase activity determination

Cellulase activity was measured using filter paper analysis as described by Ghose [21].

The extract was centrifuged 10 min at 10000 rpm and the supernatant was analysed. An Activity Unit (U) or Filter paper unit (FPU) is defined as 1 µg of glucose released in one hour under the assay conditions.

Respirometric tests

In this case, respirometric tests were performed on the residues before and after SSF in order to determine their stability (Ordinance on Environmentally Compatible Storage of Waste from Human Settlements and on Biological Waste-Treatment Facilities [18]). A dynamic respirometer constructed following the method described by Adani et al. [22] was used. The experimental device was described by Pognani et al. [23]. According to Gea et al. [24] the test was carried out at a constant temperature of 37°C. All the experiments were performed in triplicate. Cumulative oxygen demand at 4 days AT_4 (g

307 O_2 kg^{-1} dry matter) and Dynamic Respirometric Index, DRI_{24} (g O_2 kg^{-1} dry matter h^{-1}),
308 were calculated [24, 25].

309 OUR (g O_2 kg^{-1} dry matter h^{-1}) was calculated during solid state fermentation as
310 a respirometric indicator of the biological activity of the mixture [14]. When obtaining
311 DRI_{24}, fermentations are carried on with temperature control at 37 °C, while for OUR
312 calculation, the fermentation is performed with no temperature control. OUR was
313 expressed as the average value after an hour (OUR1h).

314

315 **Routine analytical methods**

316 pH was calculated by soaking 10 g of sample in 50 mL of distilled water. After 30 min,
317 pH value was measured with a pH meter Crison micro pH 2001. Volatile solids (VS),
318 moisture content, total organic carbon (TOC), total Kjeldahl nitrogen, (TKN) and
319 Soluble N-NH_4^+ were measured according to TMECC [26].

320 TOC (Total Organic Carbon) was determined using an O.I. Analytical Solid
321 TOC Analyser/Win TOC Solids v3.0, and TKN was measured using a Bloc Digester 6
322 (with six tubes capacity) (J.P. Selecta S.A., Barcelona, Spain) and a Büchi Distillation
323 Unit K-355 (Flawil, CH). Fibre content (lignin, cellulose and hemicellulose) was
324 determined by the method of Van Soest et al. [27]. Reducing sugars were determined
325 using the method described by Miller et al. [28]. Air filled porosity was calculated using
326 an air pycnometer, as described by Ruggieri et al. [29].

327

328 **Statistics**

329 t-student mean analysis comparison was performed to compare the mean values of
330 activity recovery obtained under different experimental conditions. Also, three way

331 ANOVA analysis was performed. Sigmaplot 11.0 (Systat software, Inc) was used for all
332 the calculations. Linear regression of the data using Microsoft Excel 2013 was also
333 performed.
334

335 ## Results and discussion

336 ### Waste characterization

337 Table 1 shows the characterization of the waste. Moisture was adequate for solid state
338 fermentation since it falls within the recommended values of 40-70%. Krishna et al.
339 [30] reported a range as wide as 20% to 70% for fungi growth and higher than 80% for
340 bacteria, so no extra moistening was needed. Air filled porosity (AFP) was also
341 adequate to ensure total aeration during the process. Ruggieri et al. [29] reported that the
342 recommended AFP value is highly dependent on the material, and can vary from 30% to
343 60%. Alburquerque et al. [31] found that due to the wide variety of methods for
344 measuring AFP, the same measurement can vary between 26% and 61%, so 77%
345 obtained for coffee husk and bulking agent is acceptable. As the objective of the work
346 was the production of cellulase, coffee husk fibres content was determined. According
347 to literature, measured cellulose content in some lignocellulosic substrates for
348 bioethanol production was within 32-47%, 34-45% and 42% for rice straw, wheat straw
349 and corn straw [32], respectively, so the percentage of fibrous material present in coffee
350 husk makes it suitable for cellulase production. As for the biodegradability of the
351 residue, initial respirometric values on Table 3 show that the raw material is not stable
352 and it can be aerobically degraded [18].
353

354 ### Solid State Fermentation

Figure 1 shows an example of the temperature and OUR1h profiles of a fermentation process performed in 10 L working volume reactors. At 48 hours roughly, according to Cerda et al. [12], the maximum enzymatic activity was reached. Cerda et al. [12] also proved that maximum OUR1h and maximum cellulase activity were achieved simultaneously. Temperatures reached 70 °C in the thermophilic range. Maximum OUR1h achieved was 9 mg O_2 h^{-1} g^{-1} DM. It was observed that the fermentation process is highly reproducible, as the differences in temperature profiles among all replicates were not higher than 6%, presenting an average value of 98.7 °C day (area below temperature curve). Oxygen consumption, on the other hand, presented a maximum deviation of almost 50% of the mean value, which was 1.5 mg O_2 h^{-1} g^{-1} wet matter (WM). This parameter was calculated for each solid state fermentation process as total amount of oxygen consumed (mg) divided by process time (h) and initial quantity of wet matter inside the reactor (g). Mean value of oxygen consumption was calculated as an average of the values obtained in the performed fermentations. Values of oxygen consumption seem to be related to the obtained cellulase activity extracted in standard conditions (1:15 ratio, BC, agitated mode). The highest cellulase activity value was obtained in fermentation processes presenting the highest oxygen consumption, which were 1.3 ± 0.2 FPU g^{-1} DM corresponding to 2.3 mg O_2 h^{-1} g^{-1} WM. Jimenez-Peñalver et al. [33] reported a correlation between sophorolipids production yield by *Starmerella bombicola* in winterization oil cake and oxygen consumption. Among the fermentations performed, a case was found where correlation between cellulase activity and oxygen consumption was not observed (0.45 ± 0.07 FPU g^{-1} DM corresponding to 1.7 mg O_2 h^{-1} g^{-1} WM). However, when extractions under different conditions using this fermented solid were performed and the cellulase activity was compared to the standard, the highest percentage of activity recovery was obtained, indicating that probably extraction

at 1:15 (w:v) did not have the proper efficiency in this case. This can be due to the heterogeneity of the material and the size of the sample extracted as standard.

Cellulase activity recovery - Downstream

Extraction method

After 48 hours of fermentation the process was interrupted and different extraction strategies were performed on quantities of material between 160 and 200 g, under different conditions. Table 2 shows the combination of the different extraction parameters and the activity recovery obtained, as well as the pH and conductivity of each extract. t-student test was performed comparing the means of the activity recovery values for each experimental condition. Results of the test are also presented in Table 2.

As observed, the most favourable extraction mode, according to Table 2, was the one performed at 1:5 ratio in static mode using DW as extraction solvent with an activity recovery of 108 ± 30 %. The less favourable conditions, according to Table 2, are at a ratio 1:5 and DW as extractant but in agitated mode. As t-test showed, no differences due to extraction method can be appreciated between the means of 5-Static-DW, 5-Agitated-CB, 3-StaticDW, 3-Agitated-DW, 2-Static-DW and 5-Static-CB, being all equal to 5-Static-DW. This group of extraction methods that present higher activity recovery include the combinations in which DW and static agitation mode were used, which would be the most favourable conditions from an economic point of view. Linear regression carried out with the obtained results showed a very low correlation coefficient. Coefficients for each variable indicate that the most relevant extraction parameter is the extraction ratio, being 1:5 the most favourable one. On the other hand, solvent type is the less influencing parameter. This result agrees with that deduced from

404 t-student test. According to these results, it is clear that solubility of the produced
405 cellulase is not diminished by the difference in pH observed between BC extract and
406 DW extract. On the contrary, higher pH seems to have a positive influence in the
407 extraction yield. Rezaei et al. [34] reported buffer containing 50 mM sodium acetate at
408 pH 5 to have a positive effect on the recovery of commercial fungal-derived cellulase
409 amended to switchgrass and a negative effect in the recovery of the same enzyme from
410 solid state fermentation samples colonized by the bacterium *Acidothermus*
411 *cellulolyticus.*

412 In the particular case of the extraction of cellulase from a solid matrix of
413 fermented coffee husk it is clear that BC has no positive effect compared to DW
414 extraction. Singh et al. [35] asserted that the ideal buffer would be selective and
415 preferable of the same pH of the fermented substrate. As observed in Table 2, pH of the
416 solid matrix, which corresponds to 5-Agitated-DW experiment, is very similar to the pH
417 achieved in all extractions with DW, where pH value depended only on the electrolytes
418 from the solid matrix, contrarily to what happens with BC extract, which pH is close to
419 6 in all cases, due to the buffer effect of citrate. Comparing initial pH of DW and BC,
420 DW pH is closer to that value. Regarding this, Bera et al. [36] presented the extraction
421 of amylase at different pHs ranging from 2 to 12 and observed no statistical significant
422 differences among the results .

423 According to the data obtained, both agitated and static methods provide an
424 adequate contact between solid and solvent. This can be due to the small size of the
425 coffee husk particles, and consequently no aggregates were formed once in contact with
426 the liquid. Singh et al. [35] reported no differences between static and agitated (220
427 rpm) extraction modes for pectinase from bran, and only an increase of viscosity and
428 colour in the extract. On the other hand, Shata et al. [37] reported a yield in milk-

429 clotting (proteins) extraction from also bran of 3000 U g^{-1} in agitated extraction in front
430 of 500 U g^{-1} in static extraction. Therefore, agitation requirements seem to be highly
431 dependent on the type of enzyme. In the case of coffee husk and cellulase extraction,
432 agitation mode has a low influence in the activity recovery yield.

433 Three independent extractions were performed in recirculated mode with DW as
434 solvent and ratio 1:3 solid:liquid, with 7 times the recirculation of the whole volume of
435 liquid. Also, another three independent extractions were performed using fermented
436 coffee husk from the same process in agitated mode at same ratio and with DW as
437 solvent. Activity recovery for both extraction modes was calculated, obtaining a 98 ± 1
438 % of activity recovery for the agitated mode and a 93.0 ± 0.7 % for the recirculated
439 mode, showing that no improvement is achieved with recirculation.
440
441 **Number of extraction stages**

442 Three extractions in static mode, ratio 1:2 and DW as a solvent were performed and the
443 cellulase activity compared to the standard in order to calculate activity recovery. Figure
444 2 shows cellulase activity recovery in those extractions. As it can be seen, although
445 fermented material came from the same process, one of the replicates differs
446 significantly from the others. One explanation could be the heterogeneity of the
447 material, with an irregular distribution of the bulking agent. A decrease in the activity
448 recovery through the different extraction stages is observed, but a total activity recovery
449 after three extractions of 215% was observed in two of the replicates and a 144% for the
450 remaining one. Activity recovery in the second extraction represents a percentage
451 between 20% and 27% of the activity recovered in the first extraction. In the case of the
452 second replicate. Pirota et al. [15] performed consecutive endoglucanase extractions
453 over the same bran with DW reporting a 75% of recovery in the first extraction and

454 almost a 25% in the second and third. Compared to the results obtained in this work,
455 first extraction has a similar yield than in this case. Diaz et al. [38] reported consecutive
456 extractions of exo-polygalacturonase from fermented grape pomace obtaining almost
457 the same extraction yield up to the fourth extraction, needing at least 6 extractions in
458 order to recover all the enzyme. Although enzyme can be recovered up to third
459 extraction, the enzyme obtained will be much diluted already at the second extraction,
460 which represents increasing costs in processing the extract for lesser yield. According to
461 these data, it is recommended to perform only one extraction.
462

463 **Lyophilisation and activity preservation**

464 Enzymatic activity conservation in the lyophilized product was assessed during 115
465 days. The activity of the resuspended lyophilized enzyme and that of frozen samples
466 were compared to the activity of fresh extract. The weight of remaining powder
467 obtained was 10.881 g for 500 mL of 2-Static-BC lyophilized extract and 8.413 g for 5-
468 Static-BC.

469 Figure 3 shows the activity recovery of lyophilized and frozen extract compared
470 to the activity of the fresh one. For the samples obtained in the 1:2 (w:v) extraction
471 ratio, the activity recovery presented an initial drop of 20% and afterwards presented
472 values overpassing the 100% activity recovery in the last measures. Activity losses can
473 appear in every step from freezing to rehydration. Hédoux et al. [39] reported structural
474 changes in protein lyophilisation after drying step, although the changes in secondary
475 structure were reversible after rehydration. Also, other parameters of the lyophilisation
476 process can affect the subsequent activity recovery, Passot et al. [40] reported that
477 nucleation control by different techniques can improve activity preservation.

478 In our case, values from lyophilised and unfrozen samples can be considered
479 equal, thus, there is no activity loss during the dehydratation process. The observed
480 initial losses can be due to the freezing process and to the error associated to the
481 cellulase determination method, as they are observed in both frozen and lyophilised
482 samples. When comparing lyophilisation and freezing as conservation methods,
483 lyophilisation presents some advantages which are: the concentration of the enzyme
484 facilitating its use and the reduction of the space required for storing and the storage at
485 not very low temperatures for its conservation. For this reason, lyophilisation should be
486 chosen as a preservation method.
487

488 ## Zero waste strategies

489 **Biomethane potential**

490 Bio methane potential (BMP) was assayed using extracted CH as feedstock to evaluate
491 the suitability of the residue after cellulase extraction for the production of biogas.
492 Experiments were carried out during 21 days and biogas produced and methane content
493 were measured. Although recommended ratio of inoculation according to Ponsá et al.
494 [19] is 1:2 F:I (1 g of VS of feedstock per 2 g of VS of inoculum), a first experiment
495 was carried out using different ratios F:I. As the anaerobic biodegradability of this
496 residue had not been measured before, it was necessary to verify that a higher or lower
497 F:I ratio was not needed in order to have an adequate biogas production. Ratios assayed
498 were four; 1:1; 1:1.5; 1:2; 1:2.5 (g of VS of feedstock: g of VS of Inoculum). After 21
499 days of incubation, biogas production was 96 ± 10, 107 ± 7, 128 ± 10 and 138 ± 6 mL
500 biogas g^{-1} VS respectively for the mentioned ratios. In view of the results obtained, ratio
501 1:2 (also according to Ponsá et al. [19]) was selected for future experiments. In the next

set of biogas potential tests, fermented CH after and before extraction from two different fermentation processes was used as anaerobic feedstock in a ratio 1:2 F:I. Tests were performed in triplicate. Biogas and methane productions are shown in Table 4. After 27 days of anaerobic digestion at 37 °C biogas production for fermented material after extraction was 104 ± 7 mL biogas g^{-1} VS in both cases, and 124 ± 6 and 100 ± 11 mL biogas g^{-1} VS for the fermented material before extraction, respectively. As for methane production, extracted samples after 27 days produced 40 ± 8 and 51 ± 3 mL methane g^{-1} VS and for non-previously extracted samples the methane production was 52 ± 3 and 54 ± 6 mL methane g^{-1} VS respectively. Considering the standard deviation, methane production can be considered equal for all samples. As shown in Figure 4, in all cases, more than the 85% of the production was measured during the first 15 days of digestion, which indicates that the length of the process is adequate, since production remarkably decreased from that moment. Regarding biogas production, comparing this material to fresh lignocellulosic materials used as a feedstock for anaerobic digestion such as rice straw, hazelnut skin and cocoa shell, they have been reported to produce 207 ± 22.1, 223 ± 25.1 and 199 ± 22.4 mL CH_4 g^{-1} VS [41], respectively, whereas other lignocellulosic materials as ley, straw or blue mussels produced between 190 and 330 mL CH_4 g^{-1}VS [42]. The low production of methane by coffee husk can be due to the presence of bulking agent in the mixture, which is not easily biodegradable and does not contribute to the production of biogas. Also, coffee husk is generated during the roasting process of coffee grains, which can lead to an easily biodegradable matter loss. Fresh coffee husk itself produced 241 ± 11 mL biogas g^{-1} SV and 141 ± 7 mL CH_4 g^{-1} SV after 28 days of incubation, half of the amount produced by fresh lignocellulosic wastes with no previous roasting. Lee et al. [43] reported different profile in volatile

526 gases during fermentation of coffees with different degree of roasting. Moreover, loss of
527 soluble biodegradable mater also occurs during the extraction of cellulase.
528
529 **Composting**

530 Once the extraction was carried out, the remaining solid waste was stabilized in order to
531 obtain a compost-like material adequate as agricultural amendment, closing the cycle
532 and following a zero waste strategy.
533 As described previously, solid state fermentations lasted 48 hours before being
534 stopped and extraction performed. The remaining solid after extraction was pressed
535 manually in order to remove the maximum amount of moisture. However, the final
536 measured moisture varied between 71% and 75% in weigh, higher than the
537 recommended values for composting, which should be in the range of 40%-60% [44].
538 Three replicates of the composting process were performed, using 10 L working volume
539 reactors. Also, a 10 L reactor was run containing fermented material without extraction
540 in order to compare both materials. Oxygen consumption for the non-extracted sample
541 was 12.5 mg O_2 g^{-1} DM day^{-1}, higher than that of the extracted sample of the same
542 fermentation experiment, which was 4.3 mg O_2 g^{-1} DM day^{-1}. Similar values were
543 measured in the rest of the composting processes, being 4.57 and 4.43 mg O_2 g^{-1} DM
544 day^{-1}. Hygenization temperatures were reached, maximum temperature measured was
545 around 40 °C. However, non-extracted material reached temperatures above 50°C for 24
546 hours approximately. Differences in biological activity of non-extracted material and
547 extracted material can be due to the loss of soluble biodegradable material during the
548 extraction process, since particle size of coffee husk was measured, presenting values
549 between 1.6 mm and 710 µm. Also, biological activity could be hampered by the high
550 moisture of the material after extraction [45]. Table 3 shows the respirometric values

551 measured during the different steps of the process. It is observed that the stability of the
552 waste was reached after 15 days of composting with values of DRI_{24} of 0.33 ± 0.04 g O_2
553 kg^{-1} DM h^{-1} and AT_4 of 29 ± 5 g O_2 kg^{-1} DM. These values were very similar to the ones
554 obtained from a mature compost sample [46]. Waste used in SSF with no extraction was
555 also stable after 7 days of fermentation with values of DRI_{24} and AT_4 of 0.7 ± 0.3 g O_2
556 kg^{-1} DM h^{-1} and 59 ± 21 g O_2 kg^{-1} DM respectively. pH was measured for one replicate,
557 presenting values between 8 and 8.5, which are like those of final compost.
558
559 **Comparison of anaerobic digestion and composting**
560
561 According to coffee production companies, approximately 1% of the weight of final
562 product ends up as coffee husk. In the case of the coffee husk supplier of this study,
563 around 180 t/y of coffee husk are produced. According to our results, anaerobic
564 digestion of this fermented coffee husk would represent a production of approximately
565 3.6 kW of electric power (considering the methane content of the biogas, yield of biogas
566 conversion to electricity, etc.). Although in the case of composting no electricity is
567 generated and there is a net consumption of energy, Zulkepli et al. [47] compared the
568 investment required by an anaerobic digestion plant and by a composting plant for the
569 treatment of municipal solid waste finding that capital cost was for the anaerobic
570 digestion plant is 20 times higher than for the composting plant. In view of these facts,
571 composting would be recommended in the present study as a low cost technology given
572 that the production of electricity from biogas is lower than those reported for other
573 organic wastes. However, anaerobic co-digestion could be considered if other wastes
574 with higher biogas production are available to be treated with coffee husk fermented
575 residues.

Conclusion

Different aspects of the downstream stage in the production of cellulase through solid state fermentation of coffee husk were assessed in this work. Extraction parameters as solid:solvent ratio, agitation mode and type of solvent seem to have low influence in the total activity recovery. Buffering effect of citrate solution does not present any advantage over distillate water on enzyme solubility. Contact between solid and solvent appears to be adequate independently of the agitation provided. Although maximum activity recovery was 108 ± 30 % performing the extraction under 1:5 w:v ratio, static mode and distilled water as solvent, statistically equal result was obtained using 1:2 w:v ratio.

Activity loss after lyophilisation is not observed, as values oscillate around 100% recovery during the first 50 days of lyophilized material storage. For the aerobic stabilization of the extracted material, water can hamper the process, thus, a previous drying step is recommended. At the end of the stabilization process a compost-like product is obtained, which can be used as organic amendment. Biogas production resulted in low values compared with other agricultural wastes. Therefore, anaerobic digestion of the residue is recommended in co-digestion with higher anaerobically biodegradable residues.

In summary, a detailed process to optimize the production of cellulase from lignocellulosic wastes is presented, considering the overall process from SSF to a final lyophilized product. The strategies to manage the spent material after extraction are also presented for a zero waste process.

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736 Analysis of composting and anaerobic digestion in a community: A review. Chem
737 Eng Trans. 56, 1777-1782 (2017)

Tables

Table 1. Characterization of wastes used as substrates in SSF.

Parameter	**Coffee Husk**	**Compost**	**Mixture****
pH	6.4 ± 0.1	7.6 ± 0.5	6.7 ± 0.4
CE (mS cm^{-1})	n.m.	6.3 ± 0.2	n.m.
Moisture (%, wb)	60.2 ± 0.6	35 ± 1	61 ± 1
Dry mater (%, wb)	40.1 ± 0.4	64 ± 1	39 ± 1
Organic matter* (%, db)	90.21 ± 0.01	n.m.	90
Total C* (%, db)	80.1	n.m.	n.m.
Total N* (%, db)	3.5	n.m.	n.m.
C/N ratio	22.9 ± 0.1	n.m.	n.m.
Bulk density* (g L^{-1})	238.1	n.m.	358
Air filled porosity* (%)	78.9	n.m.	77.2
Reducing sugars (%, db)	0.65 ± 0.01	n.m.	n.m.
Glucose (%, db)	0.02 ± 0.01	n.m.	n.m.
Cellulose (%, db)	25.7 ± 0.2	10 ± 1	26 ± 3
Hemicellulose (%, db)	14.6 ± 0.1	10.2 ± 0.1	13.25 ± 0.07
Lignin (%, db)	17.6 ± 0.5	14 ± 1	21.1 ± 1.0

wb: wet basis. db: dry basis; n.a.: not available. AFP: Air filled porosity (v/v, percentage in volume). Values are the average of independent experiments and its standard deviation. (*) s.d.<6% n.m.: not measured. (**) Mixture of compost and coffee husk at 1:1 weight ratio and bulking agent in a volume ratio 1:1

Table 2. Summary of extraction experiments, 30 min extraction time.

Extraction method	pH	Conductivity (mS cm^{-1})	Activity recovery (%)
2-Static-DW [a, b, e, g]	9.0 ± 0.2	3.6 ± 0.2	84 ± 22
2-Agitated-DW [c, d, e, g]	9.0 ± 0.2	3.8 ± 0.3	56 ± 14
2-Static-BC [b, c, f, g]	6.1 ± 0.5	7.8 ± 0.5	58 ± 6
2-Agitated-BC [c, d, e, f, g]	6.2 ± 0.9	7.9 ± 0.6	56 ± 12
3-Static-DW [a]	9.12 ± 0.05	2.6 ± 0.2	95 ± 13
3-Agitated-DW [a, b, d]	9.11 ± 0.07	2.8 ± 0.1	84 ± 15
3-Static-BC [c, d, e]	5.6 ± 0.2	7.2 ± 0.5	61 ± 3
3-Agitated-BC [b, c, g]	5.9 ± 0.4	7.6 ± 0.5	69 ± 12
5-Static-DW [a]	9.21 ± 0.09	1.5 ± 0.1	108 ± 30
5-Agitated-DW [g]	9.1 ± 0.1	1.9 ± 0.1	50 ± 7
5-Static-BC [a, b]	5.2 ± 0.1	6.9 ± 0.5	83 ± 18
5-Agitated-BC [a]	5.4 ± 0.1	7.2 ± 0.5	103 ± 17

BC: Buffer citrate mono hydrate pH 4.8; DW: distilled water; 2: extraction ratio 1:2 (w:v), 3: extraction ratio 1:3 (w:v), 5: extraction ratio 1:5 (w:v).
Subscript indicates the groups of extractions that are considered equal according to t-student test.
Values are the average of independent experiments.

Table 3. Result of respirometric assay. Final SSF values are initial composting values.

	AT_4 (O_2 g^{-1} DM)	**DRI_{24} (O_2 g^{-1} DM h^{-1})**
Fresh material	86 ± 23	1.6 ± 0.2
48 hours of SSF - Before extraction	86 ± 17	1.3 ± 0.3
7 days of SSF - Before extraction	59 ± 21	0.7 ± 0.3
48 hours of SSF - After extraction	47 ± 7	0.70 ± 0.06
Stabilized (previous extraction)	29 ± 5	0.33 ± 0.04
Stabilized (no previous extraction)	20.0 ± 0.7	0.29 ± 0.01

All values are the average of independent experiments.

Table 4. Biogas production of coffee husk anaerobic digestion. For the materials before and after extraction, two independent fermentations were evaluated for biogas and methane production.

	Fresh material	Before extraction		After extraction	
		Fermentation 1	Fermentation 2	Fermentation 1	Fermentation 2
Biogas production (mL g^{-1} VS)	241 ± 11	124 ± 6	100 ± 11	104 ± 7	104 ± 7
Methane production (mL g^{-1} VS)	141 ± 7	52 ± 3	54 ± 6	40 ± 8	51 ± 3

All values are the average of triplicate measurements.

Figure captions

Fig. 1 Temperature (solid line) and OUR 1h (dotted line) profiles of CH solid state fermentation

Fig. 2 Percentage of activity recovery of three consecutive extractions over the same solid. Three independent experiments are showed

Fig. 3 Percentage of activity recovery of: lyophilised samples of CH extracted in Static mode, using BC and ratio 1:2 (w:v) (circles), 1:5 (w:v) (diamonds) and unfrozen samples of CH extracted in Static mode, using BC and ratio 1:2 (w:v) (triangles), 1:5 (w:v) (squares)

Fig. 4 Biogas production from anaerobic digestion of fresh coffee husk (squares), fermented coffee husk before extraction (triangles) and fermented coffee husk after extraction (circles). For materials before and after extraction, two independent fermentations were evaluated.

Fig. 1

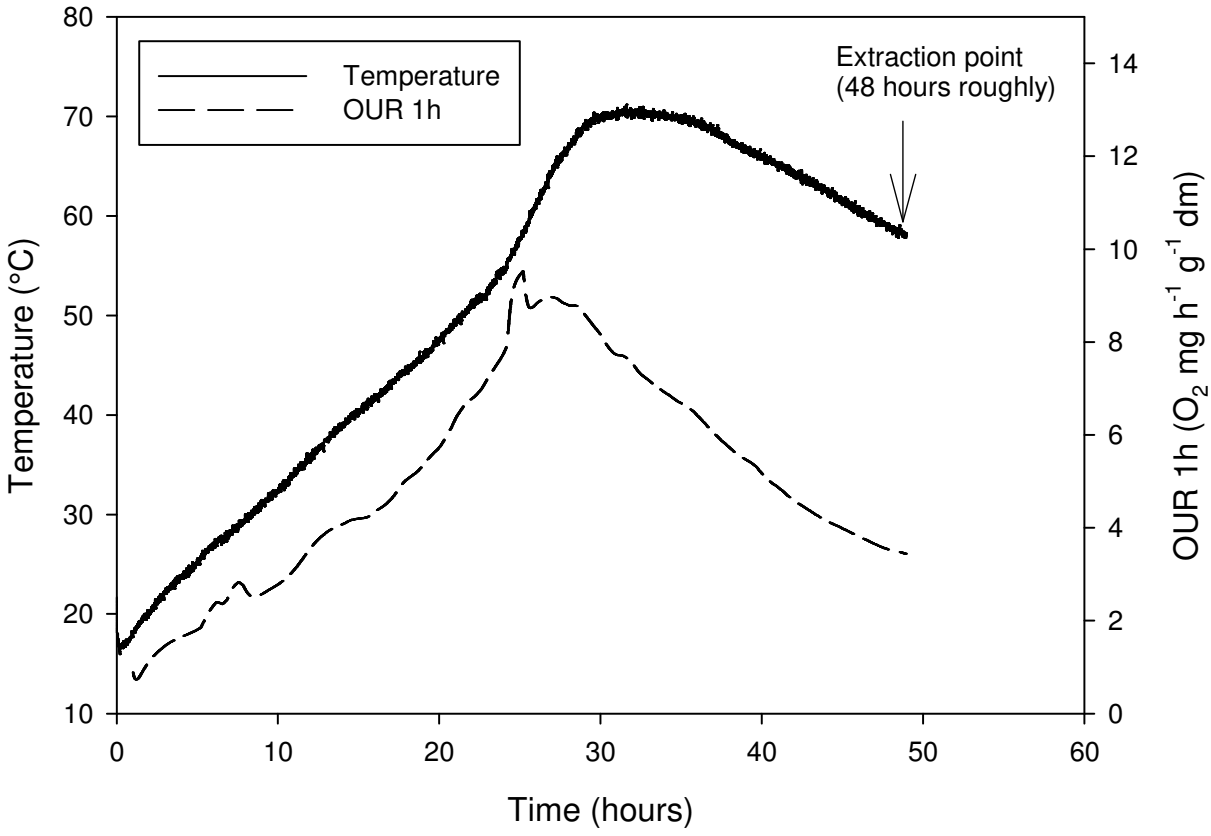

Temperature
OUR 1h
Extraction point
(48 hours roughly)
Temperature (°C)
OUR 1h (O2 mg h-1 g-1 dm)
Time (hours)
80
70
60
50
40
30
20
10
14
12
10
8
6
4
2
0
0
10
20
30
40
50
60

Fig. 2

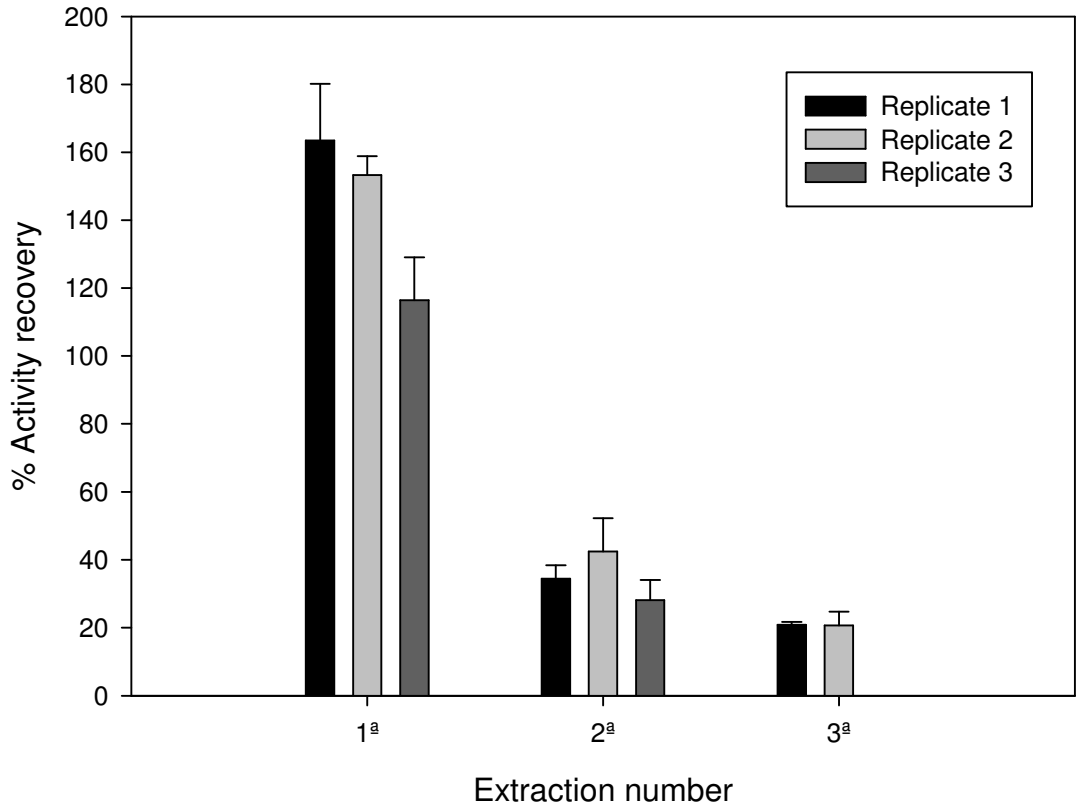

200
180
160
140
120
100
80
60
40
20
0
% Activity recovery
Replicate 1
Replicate 2
Replicate 3
1ª
2ª
3ª
Extraction number

Fig. 3

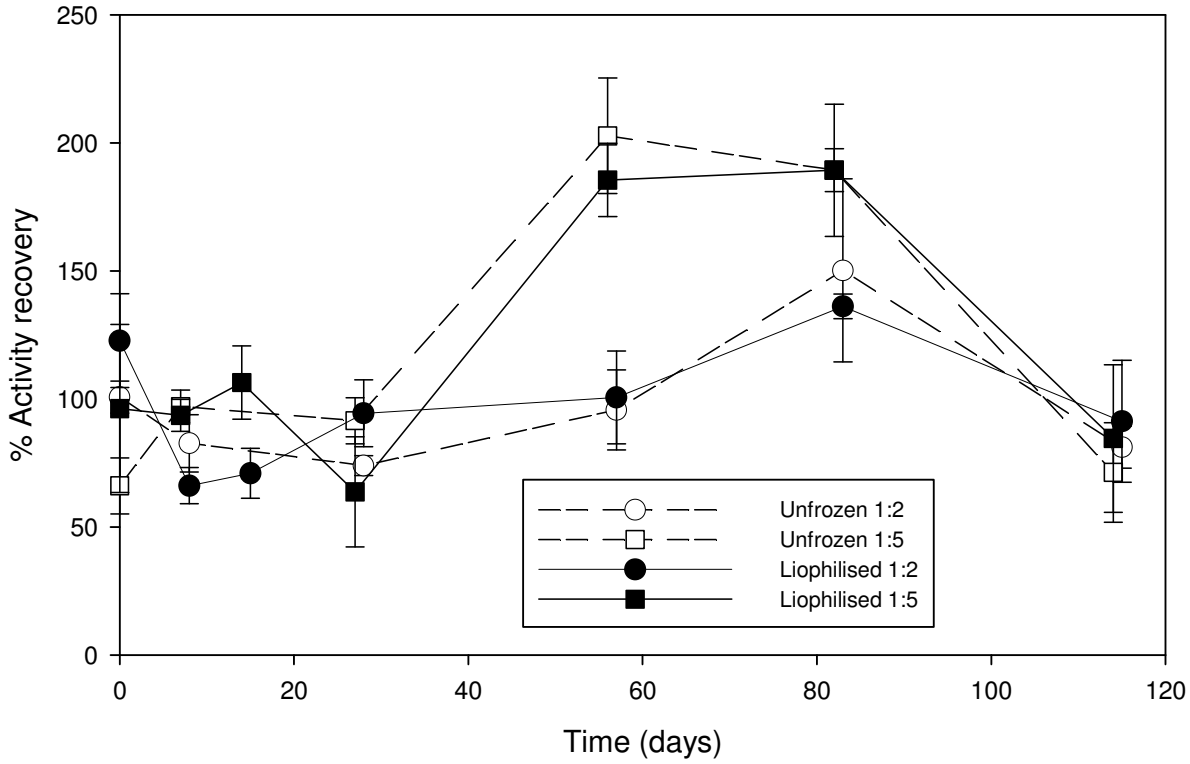
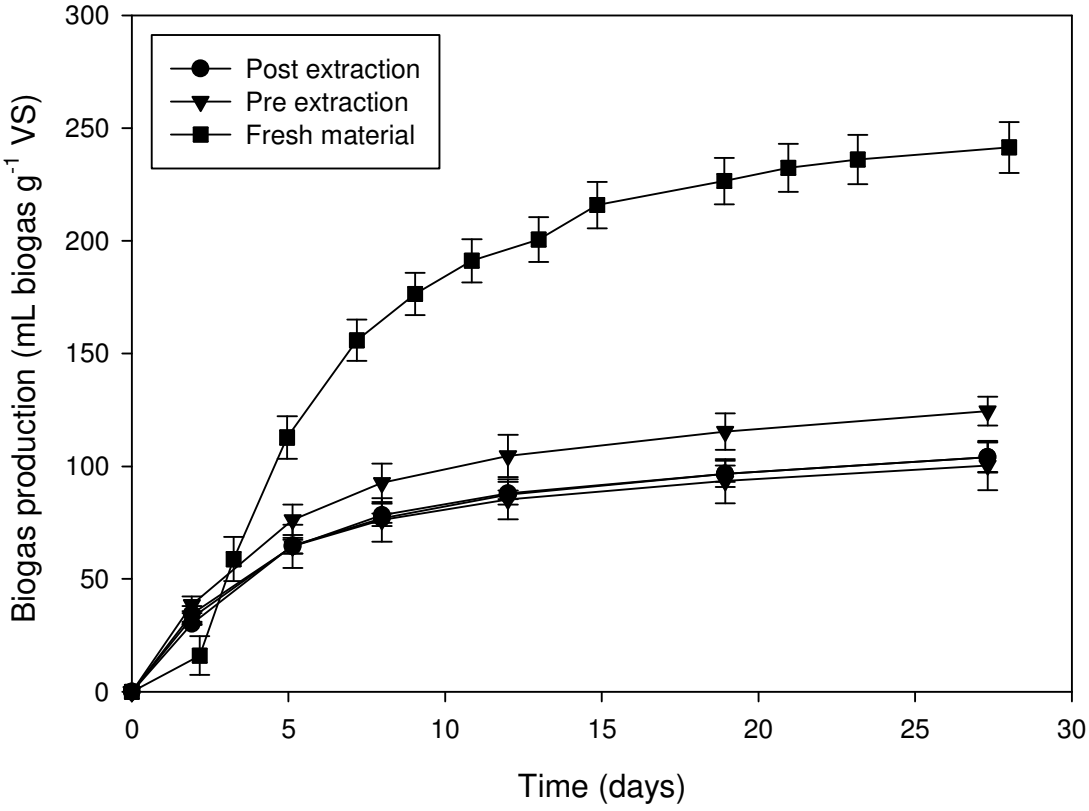

250
200
150
100
50
0
% Activity recovery
0
20
40
60
80
100
120
Time (days)
Unfrozen 1:2
Unfrozen 1:5
Liophilised 1:2
Liophilised 1:5

Fig. 4


Post extraction
Pre extraction
Fresh material
Biogas production (mL biogas g-1 VS)
Time (days)
0
50
100
150
200
250
300
0
5
10
15
20
25
30