Substitution of chemical dehairing by proteases from solid-state fermentation of hair wastes

Juliana Abraham, Teresa Gea, Antoni Sánchez*

Composting Research Group

Department of Chemical Engineering (Escola d'Enginyeria)

Universitat Autònoma de Barcelona

Bellaterra, Cerdanyola del Vallès, 08193 Barcelona, Spain

*Corresponding author:

Tel.: +34 935811019

Fax: +34-935812013

E-mail address: antoni.sanchez@uab.cat

Abstract

The leather industry uses a chemical dehairing process that produces alkaline wastewater and hair as solid waste. A cleaner process is proposed in this work to reduce these environmental impacts. It consists on the valorization of hair waste together with sludge from wastewater treatment by a solid-state fermentation process to obtain proteases that can be used in an enzymatic dehairing process. SSF was undertaken in 4.5 L scale reactors. Neither sterilization of the materials nor inoculation of pure microorganisms was necessary for the development of the process, whereas aeration was provided during the assay to ensure the prevalence of aerobic conditions. Alkaline proteases were produced as a consequence of the degradation of hair. The highest activity of the alkaline protease in crude extracts was determined at 14 days of the process (56270 ± 2632 activity units/g dry sample), after the thermophilic stage. The final organic matter showed a stability degree similar to that of compost in terms of respiration activity. Potential application of the extracted proteases in dehairing cow hides was successfully proved as an alternative to the chemical dehairing process. The results in the efficiency of the dehairing process were similar to those obtained with the chemical process, thus avoiding the need of chemical reagents and strong chemical conditions. The entire process permits the substitution of the chemical process of dehairing by an environmentally friendly enzymatic process, closing the organic matter cycle.

Keywords: hair waste; solid-state fermentation; microbial populations; alkaline proteases; sustainable dehairing.

1. Introduction

The leather manufacturing industry has been facing high demands for process optimization and for reducing environmental impacts in the past decades (Kanth et al., 2009). Due to recent regulations, the adoption of hair-save unhairing techniques has initially solved the problem of the production of high amounts of organic pollutants that typically ended up in wastewater (Valeika et al., 2009). However, this fact implies the production of another solid by-product to be managed and treated. Additionally, conventional dehairing processes use a large amount of fresh water, alkaline substances and sodium hydrosulfide that increases the chemical and biochemical oxygen demand and the dissolved solids in effluents during the process (Thanikaivelan et al., 2004).

The leather industry is a crucial economical sector in Catalonia (Spain), with a turnover of 170 million euros per year and more than 800 direct jobs. The leather tanner's union in Igualada (Barcelona, Spain) comprises 40 companies and treats 100,000 kg of leather daily (14,000 m^2/d). Around 5,000 t/year of hair wastes are produced. The union built a plant to treat the wastewater where the production of sludge rises over 13,000 t/year. The sludge is currently biodried and landfilled.

Regarding the management of hair wastes, previous research has effectively tested co-composting techniques to biodegrade hair waste and sludge (Barrena et al., 2007a). An emerging technology to process organic solid residues is solid-state fermentation (SSF) (Pandey et al., 2008). In this case, the residues can be valorized and degraded obtaining compost-like products, whereas other added-value products can be obtained during the process that are derived from the activity of the microorganisms developed in the process. Some studies have reported how to obtain different products by SSF such as enzymes, organic acids, biopesticides, biosurfactants, biofuels, aroma

compounds, etc. (Pandey et al., 1999; Singhania et al., 2009). SSF can be considered a low-cost biotechnological process as an alternative to the traditional submerged fermentation (SmF) or chemical synthesis (Pandey et al., 1999), especially when wastes are used as bioresources. However, the main challenge for SSF processes full development is the process scale-up due to mass and heat transfer limitations (Pandey et al., 2008). One of the main constraints is temperature rise over optimal values when increasing process scale that results in lower production yields (Mala et al., 2007).

As enzymes, proteases catalyze the hydrolysis of proteins to polypeptides and oligopeptides to amino acids. Microbial proteases are classified into acidic, neutral and alkaline depending on the pH at which they show the maximum activity. Enzymes have been used in a wide variety of applications from industrial sectors to household products and particularly alkaline proteases have been used in products such as pharmaceuticals, detergents (Paul et al., 2013), fertilizers (Han and He, 2010) or textiles and in processes such as food processing (Valdez-Peña et al., 2010), leather industries (Dayanandana et al., 2003) and in the synthesis of oligopeptides (Viswanathan et al., 2012).

The present study describes the production of alkaline proteases by means of an easily scalable SSF process, where wastes from tannery industries are valorized and degraded to compost-like substances. The thermophilic SSF develops when working under near-adiabatic conditions that result in an easier process scale-up when compared to a standard mesophilic SSF with a specific microorganism. This thermophilic SSF is easier to scale-up and the technology exists to implement the process at industrial scale considering composting facilities. The crude extract obtained from the solid-state fermentation is characterized and tested in dehairing cow hides as sustainable alternative to the traditional chemical process, which implies the use of hazardous

products and strong alkaline conditions. This is, to the authors' knowledge, the first study where proteases are produced at bench-scale by SSF and they are used for the dehairing process, where the wastes produced are again used for protease production, thus closing the organic matter cycle.

2. Experimental

2.1. Materials

The materials used in SSF were hair wastes from a local tanning industry and raw sludge (33.02% of dry matter (DM), 84.30% of organic matter (OM) and dynamic respiration index (DRI) of 3.42 g O_2 kg^{-1} DM h^{-1}) from a municipal wastewater treatment plant (both located in Barcelona, Spain). Table 1 shows the main characteristics of hair waste. Wood chips were used as bulking agent in SSF experiments. Cow hides from the same tanning industry were used in the dehairing experiments with the obtained proteases.

2.2. Mixtures

Raw sludge was mixed with hair waste in ratio 2:1 (wet weight ratio) to start up the biodegradation as determined in previous studies (Barrena et al., 2007b). Bulking agent was mixed in a ratio 1:1 (v:v) to provide the proper porosity and to maintain aerobic conditions during the SSF process (Ruggieri et al., 2009). All mixtures were manually prepared with materials at room temperature and were fermented without prior sterilization. Table 1 also shows the main characteristics of the initial mixture.

2.3. SSF

Approximately 1.5 kg of mixture were fermented for 21 days in 4.5 L air-tight reactors (Fig. 1s), working under near-adiabatic conditions and continuous aeration (1 L/min) as described in previous studies (Abraham et al., 2013; Santis-Navarro et al., 2011).

The hydrodynamic behavior of these reactors is that of plug flow reactors (Puyuelo et al., 2010). Water content of the mixture was adjusted to be within the recommended values (40-60%) (Haug, 2013) by adding tap water during the experiments, when necessary. Samples were collected at 0, after 24 hours of thermophilic temperature (about 3 days), 7, 14 and 21 days of process time after manual homogenization of the entire mass in the reactors to obtain the profile of the process and the proteases production. The experiment was undertaken in triplicate. A duplicate of the process was undertaken after three months of the first SSF experiment to evaluate the reproducibility of the process.

2.4. Enzyme extraction

Fermented solid material was mixed thoroughly with 50 mM HCl-Tris (tris(hydroxymethyl)aminomethane) buffer (pH 8.10) in a ratio 1:5 (w:v) for 45 min and the extract was separated by centrifugation at 10000 rpm for 20 min at 4ºC and further filtration through 0.45 μm. The filtered supernatant was used as crude enzyme extract for characterization and dehairing experiments.

2.5. Effect of pH and temperature on protease stability

The effects of pH and temperature (T) on alkaline protease stability were analyzed by a full factorial experimental design consisting of twelve experiments (nine

experiments and three replications at the central point for statistical validation, using the software package Sigmaplot version 10, Systat software Inc.). Temperature was fixed at 30, 50 and 70 ºC and pH at 5.00, 8.00 and 11.00, which are the typical ranges of enzymatic activity. The objective function selected was the residual alkaline protease activity percentage (RA, referred to the initial activity of the extracts) after one hour of incubation. Table 2 resumes the experimental conditions tested for each crude extract. The buffers used for the incubation at the selected values of pH were: Tris-HCl 1 M, pH 8.00; Tris-NaOH 1 M, pH 11.00; acetic acid-sodium acetate 1 M, pH 5.00. These buffers were chosen to maintain the desired value of pH during the whole experiment as shown in previous studies with complex extracts from SSF (Santis-Navarro et al., 2011; Gea et al., 2007).

2.6. Dehairing assay of cow hides

Wet-salted cow hides were washed and cut to the same area (10.68 cm^2) and then were incubated, with 15 mL of enzymatic crude extract (E3, E7, E14, E21) or 15 mL of 50 mM HCl-Tris buffer (pH 8.10) (control) at 37ºC for 24 h on a rotary shaker at 120 rpm. 2% of sodium azide was added to inhibit the microorganisms' growth and to determine only the effect of the enzyme on dehairing. The protease activity and the soluble proteins were determined at 0 and 24 hours of incubation. Hides with the same area were treated sequentially with the following chemicals: sodium carbonate (15 mL, 0.3%, w/v) with a non-ionic surfactant (0.3%, w/v) (soaking, 20 h), calcium hydroxide (50 mL, 1%, w/v, 1 h) and sodium hydrosulfide (50 mL, 1%, w/v, 30 min in orbital agitation) to simulate the chemical dehairing process used in tannery industries. Samples treated with enzymes, chemicals and controls were assayed in triplicate.

Dehairing efficiency was evaluated by scraping gently with tweezers all the hides. After this, the determination of the hair mass for each treatment was evaluated according standard methods for total suspended solids (APHA, 1995). The treatment efficiency for enzyme and control experiments were referred to a percentage of dehairing obtained in the chemical treatment.

The same full factorial experimental design previously described for protease stability was used to determine the optimal pH and temperature for the dehairing enzymatic process. In this case, pH was 8.5, 10 and 11.5 using the same buffers than those of protease stability and temperature was set to 20, 35 and 50ºC.

2.7. Enzymatic activities

Alkaline protease activity was determined using a modified method previously described (Alef and Nannipieri, 1995). 1 ml of the enzyme extract was added to 5 ml of 2% freshly casein solution and incubated at 50ºC and 100 rpm for 2 hours. One unit of alkaline protease activity (UA) was defined as 1 µg of tyrosine released under the assay conditions.

Keratinase activity was determined by using the method described by Rai et al. (2009). 1.0% (w/v) of native sterilized chicken-feather was used as a substrate to incubate the crude extracts at 45ºC for 15 min. Finally, the addition of 0.5 ml of 10% (w/v) ice-cold trichloroacetic acid was added to stop the reaction. Digested protein in the supernatant was determined colorimetrically by using the Folin-Ciocalteu's reagent (Lowry et al., 1951). One unit of keratinase activity (UA) is defined as the µg of tyrosine liberated under the assay conditions.

Collagenase activity was determined according to the methodology proposed in previous works (Nilegaonkar et al., 2007). Briefly, it consists on incubating 1 mL of the extract with 4 mg of azocoll reagent (Sigma Aldrich) in buffer HCl-Tris pH=8.10 at 70ºC for 1 hour and 200 rpm. The reaction was stopped with 15% trichloracetic acid. Then samples were centrifuged at 8500 rpm for 10 min and the absorbance of the supernatant was measured at 520 nm. One collagenase unit is defined as the amount of enzyme required to increase 0.1 units of absorbance per minute at 70ºC.

Soluble Protein (SP) concentration was determined by the method of Bradford using bovine serum albumin (Sigma Aldrich ®) as standard (Bradford, 1976).

2.8. Specific methods for organic mixtures

Respirometric assays were undertaken in a solid-state respirometer using 100 g of sample and continuous aeration and oxygen monitoring, as previously referred (Ponsá et al., 2010). The DRI (dynamic respiration index) represents the average oxygen uptake rate during the twenty-four hours of maximum biological activity observed during the respirometric assay and it reports the stability degree of the sample. The CRI (cumulative respiration index) represents the cumulative oxygen consumption observed during the four days of maximum respiration activity.

FAS (free air space) was determined using an air pycnometer to maintain the proper air circulation through the organic matrix (Ruggieri et al., 2009).

2.9. Polyacrylamide gel electrophoresis (PAGE)

PAGE was carried out with all the crude extracts obtained to determine the molecular weight of the alkaline proteases as generally described (Laemmli, 1970) using

a 12% polyacrylamide (w/v) separating gels (Biorad®). After electrophoresis, gels were stained with Coomasie Blue G-250 (Biorad®). A zymogram was obtained by incubating gels for 1 hour at 50ºC with 2% (w/v) casein in Tris-HCl (pH 8) followed by the same dyeing as explained before. A molecular mass marker (Biorad®) was used to identify the molecular weight of the bands observed.

2.10. Scanning Electron Microscopy (SEM) images

After dehairing, samples were gradually dehydrated and prepared for SEM analysis (Evo ® MA10, Carl Zeiss).

2.11. Standard analytical methods

Water content was determined by gravimetric analyses after drying at a temperature of 105°C until constant weight. The organic matter content was determined from mass loss after heating at 550 ºC according to the standard procedures recommended by the Test Methods for the Examination of Composting and Compost (2011). The total organic carbon (TOC) was determined using an O.I. Analytical Solid TOC Analyser/Win TOC Solids v3.0, and the total nitrogen Kjeldahl (TNK) was determined by standard procedures (APHA, 1995). Protein content was calculated from NTK content by multiplying by 6.25. For the TOC and TNK analyses, the samples were previously dried up and sieved at 0.5 cm. pH and electric conductivity (EC) were determined in an extract 1:5 (w:v) in Milli-Q water and then pH and EC was measured according to standard procedures (Test Methods for the Examination of Composting and Compost, 2011).

2.12. Statistical Analysis for protease activity

Results are presented as mean ± standard deviations of three replicate experiments. A statistical analysis was performed using the Student's t-test and the analysis of variance. A probability level of $p<0.01$ was selected to determine statistically significant differences between enzymatic activities.

3. Results and Discussion

3.1. SSF process performance and reproducibility

Fig. 1a shows the temperature, the oxygen exhaust content and pH during the entire SSF experiment at bench scale (21 days) for one of the three replicates.

As observed, the temperature and pH profiles were similar to that of composting; in which both parameters rise due to the biodegradation of the organic material, as observed in previous SSF and composting experiments with hair wastes (Barrena et al., 2007b). The oxygen levels in the reactor showed the prevalence of the aerobic conditions during the entire process. Differences of process performance in replicates were assessed through the statistical comparison of temperature and oxygen profiles. Area below T curve (ºC·d) is related to heat released in the biodegradation while area below oxygen uptake rate (OUR, in mg O_2/kg DM) equals the total oxygen consumed in the fermentation. T-test and analysis of variance showed no significant differences between experimental trials. Average values plus standard deviation were 724.0±78.6 ºC·d for T curve and 98.5±31.3 mg O_2/kg DM for OUR curve.

The profile of alkaline protease activity determined at the sampling days is also shown in Fig. 1b. The activity was measured in the obtained crude extracts (initial

extract: E0, extract day 3: E3, extract day 7: E7, extract day 14: E14 and extract day 21: E21). The highest activity of the enzyme was observed at 14 days of the SSF process in E14. The Student's t-test revealed no significant differences among the protease activities detected in E14 of each experiment. Similar previous experiments using soy fiber residues as substrate for protease production showed the highest activity just 24 hours after the thermophilic stage (Abraham et al., 2013). These results confirm the difference in protease production due to the different biochemical composition of materials when treated by SSF. Keratinase activity was determined in the extracts and the results showed practically no keratinase activity increase in the process (E0: 1520 UA/g DM, E3: 1780 UA/g DM; E7: 0 and E14: 400 UA/g DM). These results are in agreement with previous studies on the properties of dehairing proteases (Tiwary and Gupta, 2010) although some other studies have shown the relationship among keratinase and dehairing activities (Pillai et al., 2011; Shinivas and Naik, 2011). Additionally, collagenase activity was not detected during the experiments in agreement with other studies (Nilegaonkar et al., 2007). This activity is undesirable in the dehairing process, as it causes damage on the skin matrix (Jian et al., 2011).

Additionally, the dynamic respiration index (DRI) of the mixture was determined at 14 days of SSF process and its value (0.63 g O_2 kg^{-1} DM h^{-1}) indicates the complete stability of the residue, as reported in the composting field (Ponsá et al., 2010).

3.2. Biochemical characterization of crude extracts

The solid and liquid samples of day 14 (when the highest alkaline protease activity was detected) were analyzed to determine the enzyme activity in both phases

(Table 3). As expected, considering the affinity of the enzyme to water, higher alkaline protease activity was found in the liquid samples (crude extract) indicating that buffer extraction is an efficient way to recover and concentrate the desired enzyme from the solid mixture.

Fig. 2s shows the response surface of the residual activity for the three crude extracts assayed to check their stability under different T and pH conditions (Table 2).

According to Table 2 and Fig. 2s, enzymes were more stable at pH 11 confirming that the obtained enzymes were alkaline proteases. The best results for thermal stability were obtained at 50ºC, 30ºC and 30ºC for E3, E7 and E14, respectively. Optimization of the best polynomial fitting to experimental results provided the following optimal pH-Temperature data: 11-37ºC for E3 and 11-30ºC for E7 and E14, respectively. Objective function (residual activity) was 63.3, 56.2 and 67.8% at the optimal value, respectively. A thermostable enzyme could be expected if it is produced by microorganisms that grow at thermophilic temperatures as the alkaline protease produced at E3, whereas mesophilic enzymes are to be produced at mesophilic temperatures such as those of E7 and E14 (Abraham et al., 2013; Zanphorlin et al., 2011). In this case, protease in crude extract E3 showed good stability both at mesophilic and thermophilic temperatures.

Previous studies (Dayanandana et al., 2003; Nilegaonkar et al., 2007; Kandasamy et al., 2012) have inoculated specific microorganisms to produce selected enzymes, whereas in our work we have different microbial populations growing (from the initial microbial consortia from raw sludge) and producing enzymes. For this reason, it should not be expected to find a defined band in a Native-PAGE. Fig. 2a shows the Native-PAGE and Fig. 2b the zymogram for the different crude extracts analyzed. As

observed, several bands appeared with different molecular mass and particularly at E14 the highest quantity of bands was detected. Besides that, the absence of blue color at the zymogram indicates that the bands correspond to that of a catalytic protease activity. These results coincided with the protease activity detected at each extract and the evolution of protease activity (Fig. 1b) and dehairing results.

3.3. Dehairing activity

Table 4 presents the results obtained for dehairing treatments using the proteases produced by SSF.

After 24 hours of incubation, the hides treated with the E7, E14 and E21 showed an easier removal of hair when mechanically scraped with tweezers compared to control experiment with no protease. On the contrary, E3 showed no hair removal as in control experiments. These results suggest that the type of protease produced in the different stages have different dehairing activities. In addition, E14 showed more dehairing activity when compared to the rest of extracts and the same dehairing yield than the chemical process. This confirms that the enzymatic treatment can be a good alternative to chemical dehairing as pointed in others studies carried out on goatskins or buffalo hides (Tiwary and Gupta, 2010; Kandasamy et al., 2012; Saran et al., 2013).

There was a good correlation between protease activity and dehairing capacity, as observed when E14 was diluted, which is in accordance with literature (Kandasamy et al., 2012). These results are consistent with other dehairing assays performed with extracts obtained from replications of the SSF process (data not shown). Finally, Fig. 3 shows the soluble protein (SP) detected in control experiments and the different crude

extracts assayed in dehairing, whereas Fig. 3s shows the remaining protease activity after the dehairing process.

As observed in Fig. 3s, the extract maintained the protease activity after 24 hours of incubation in all the cases. SP determined at 0 and 24 hours of incubation is presented in Fig. 3 and the results revealed the capability of the extracts for degrading the SP released during dehairing because of their protease activity content. Contrarily, the SP released in the control extracts increased significantly because of the lack of proteases. These results suggest the capacity of the enzymatic treatment for dehairing hides while reducing the organic matter and total solids in effluents from tannery industries as referred above (Thanikaivelan et al., 2004). The scanning electron microscope images (SEM) of the unhaired samples after enzymatic and chemical treatments are shown in Fig. 4 with a magnification of 200X and 500X. The enzymatic treated hides appear cleaner and with no superficial damage when compared to the chemical treated hides.

Finally, Table 5 shows pH and temperature effect on the enzymatic dehairing process with E14.

Dehairing appears to be more efficient when increasing pH and temperature in the range of studied parameters; especially when temperatures are around 50ºC and pH around 10. Optimization of the best polynomial fitting to the experimental results show best dehairing yield at 50ºC and pH 11.5 (objective function value 104%).

3.4. Feasibility of the new process in real tanning industry

Implementing this alternative cleaner process in real tanning industry appears feasible from and environmental, technical and economical point of view. Saran et al.

(2013) have recently demonstrated the enzymatic dehairing and degreasing of skins at real scale using 100 kg of hides.

A mass balance done with the data presented in this paper confirms that enough hair and sludge are produced in the region under study (Igualada, Spain) to obtain enough proteases to dehair 14000 m^2/d of leather, without significant protease surplus.

The equipment used for chemical dehairing (stirred tanks or rotating drums and pumps) could be used for the enzymatic process without further modifications. The tanning industry treats the wastewaters in its specific treatment plant, producing sludge that could be used in the SSF process. Proteases could be produced by SSF and further extracted using simple equipment as static bed reactors (used in composting) and stirred tanks, or using SBR type reactors with a sequence of operations. Manufacturers should certainly invest in the proper equipment for proteases production. However, the savings in chemicals and in waste management are expected to compensate this. A preliminary economical balance results in near 2 million € savings per year by eliminating the chemicals cost and internalizing the solid waste management (total costs, 0.391 €/m^2 leather, including: chemicals cost, 0.168; hair waste management, 0.043; sludge management, 0.180).

4. Conclusions

A cleaner production process for the leather industry is demonstrated in this paper. A bench scale production of an alkaline protease has been developed by SSF from hair wastes and wastewater sludge and the proteases produced have successfully tested in the dehairing process of cow hides. The results of this study open the real possibility of substituting a chemical process such as chemical dehairing, which implies

the use of reagents and pollution in wastewater and solid wastes, by a biotechnological process where the substrate is the same waste produced in the dehairing industry. The feasibility of implementing this process at real scale is also supported by the economical savings on chemicals and waste management that have been estimated for the Catalonia region.

Technically, the highest protease activity was detected at 14 days of SSF, being 56270 ± 2632 UA/g dry sample. The characterization of the crude extract indicates that several proteases had been produced by the microbiota developed and they are relatively stable at mesophilic temperatures. A crude extract with a specific activity of 5491±418 UA/cm^2 was successfully assayed in dehairing cow hides as a sustainable alternative while valorizing a by-product of the tannery industry and reducing wastewater pollution.

Further experiments are to be undertaken at higher scale reactors to obtain reliable information on process yield and process emissions. This will allow for a proper economical an environmental assessment.

Further research should be focused on: i) the process characterization at full scale to conduct a reliable technical, economical and environmental analysis, and ii) the complete characterization of the protease obtained and the possibilities of improving its performance using immobilization techniques together with the overall dehairing process optimization (temperature, dosage, reuse, etc.).

Acknowledgements

Authors thank the financial support provided by the Spanish Ministerio de Economía y Competitividad (Project CTM2012-33663). J. Abraham wants to thank the

financial support provided by UAB with a pre-doctoral grant. Authors thank Depuradora del Besós and Igualada Adobers for its collaboration in providing the materials and information for this study.

References

Abraham, J., Gea, T., Sanchez, A., 2013. Potential of solid-state fermentation of soy fiber residues by native microbial populations for alkaline protease production at bench scale. Biochemical Engineering Journal 74, 15–19.

Alef, K., Nannipieri, P., 1995. Methods in Applied Soil Microbiology and Biochemistry, Academic Press Limited, San Diego, USA.

APHA, 1995. Standard Methods for the Examination of Water and Wastewater, 19th edition. American Publishers Health Association, Washington, DC, USA.

Barrena, R., Pagans, E., Vázquez, F., Artola, A., Sánchez, A., 2007a. Full-scale cocomposting of hair wastes from the leather manufacturing industry and sewage sludge. Compost Science and Utilization 15, 16-21.

Barrena, R., Pagans, E., Artola, A., Vazquez, F., Sanchez, A., 2007b. Co-composting of waste from the tanning industry with de-inking and municipal wastewater sludges. Biodegradation 18, 257-268.

Bradford, M.M., 1976. A rapid and sensitive for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72, 248-254.

Dayanandan, A., Kanagaraj, J., Sounderraj, L., Govindaraju, R., Suseela Rajkumar, G., 2003. Application of an alkaline protease in leather processing: an ecofriendly approach. Journal of Cleaner Production 11, 533–536.

Han, W., He, M., 2010. Short-term effects of exogenous protease application on soil fertility with rice straw incorporation. European Journal of Soil Biology 46, 144-150.

Haug, R.T., 1993. The Practical Handbook of Compost Engineering, Lewis Publishers, Boca Raton, Florida.

Jian, S., Wenyi, T., Wuyong, C., 2011. Kinetics of enzymatic unhairing by protease in leather industry. Journal of Cleaner Production 19, 325–331.

Kandasamy, N., Velmurugan, P., Sundarvel, A., Raghava, R. J., Bangaru, C., Palanisamy, T., 2012. Eco-benign enzymatic dehairing of goatskins utilizing a protease from a *Pseudomonas fluorescens* species isolated from fish visceral waste. Journal of Cleaner Production 25, 27-33.

Kanth, S.V., Venba, R., Madhan, B., Chandrababu, N.K., Sadulla, S. 2009. Cleaner tanning practices for tannery pollution abatement: Role of enzymes in eco-friendly vegetable tanning. Journal of Cleaner Production 17, 507–515.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the folin-phenol reagent. Journal of Biological Chemistry 193, 267–275.

Mala, J.G., Edwinoliver, N.G., Kamini, N.R., Puvanakrishnan, R., 2007. Mixed substrate solid state fermentation for production and extraction of lipase from *Aspergillus niger* MTCC 2594. The Journal of General and Applied Microbiology 53, 247-253.

Nilegaonkar, S.S., Zambare ,W.P., Kanekar, P.P., Dhakephalkar, P.K., Sarnaik, S.S., 2007. Production and partial characterization of dehairing protease from *Bacillus cereus* MCM B-326. Bioresource Technology 98, 1238-1245.

Pandey, A., Selvakumar, P., Soccol, C.R., Nigam, P., 1999. Solid-state fermentation for the production of industrial enzymes. Current Science 77, 149-162.

Pandey, A., Soccol, C.R., Larroche, C., 2008. Current developments in solid-state fermentation. Springer, Asiatech Publishers Inc., New Delhi, India.

Paul, T., Das, A., Mandal, A., Halder, S.K., Jana, A., Maity, C., DasMohapatra, P.K., Pati, B.K., Mondal, K.C, 2013. An efficient cloth cleaning properties of a crude keratinase combined with detergent: towards industrial viewpoint. Journal of Cleaner Production, http://dx.doi.org/10.1016/j.jclepro.2013.10.054.

Pillai, P., Mandge, S., Archana, G., 2011. Statistical optimization of production and tannery applications of a keratinolytic serine protease from *Bacillus subtilis* P13. Process Biochemistry 46, 1110-1117.

Ponsá, S., Gea, T., Sánchez, A., 2010. Different indices to express biodegradability in organic solid wastes. Journal of Environmental Quality 39, 706-712.

Puyuelo, B., Gea, T., Sánchez, 2010. A new control strategy for the composting process based on the oxygen uptake rate. Chemical Engineering Journal 165, 161-169.

Rai, S.K., Konwarh, R., Mukherje, A.K., 2009. Purification, characterization and biotechnological application of an alkaline β-keratinase produced by *Bacillus subtilis* RM-01 in solid-state fermentation using chicken-feather as substrate. Biochemical Engineering Journal 45, 218-225.

Ruggieri, L., Gea, T., Artola, A., Sánchez, A., 2009. Air filled porosity measurements by air pycnometry in the composting process: A review and a correlation analysis. Bioresource Technology 100, 2655-2666.

Santis-Navarro, A., Gea, T., Barrena, R., Sánchez, A., 2011. Production of lipases by solid-state fermentation using vegetable oil-refining wastes. Bioresource Technology 102, 10080-10084.

Saran, S., Mahajan, R.V., Kaushik, R., Isar, J., Saxena, R.K., 2013. Enzyme mediated beam house operations of leather industry: a needed step towards greener technology. Journal of Cleaner Production 54, 315–322.

Shrinivas, D., Naik, G.R., 2011. Characterization of alkaline thermostable keratinolytic protease from thermoalkalophilic *Bacillus halodurans* JB 99 exhibiting dehairing activity. International Biodeterioration and Biodegradation 65, 29-35.

Singhania, R.R., Patel, A.K., Soccol, C.R., Pandey, A., 2009. Recent advances in solid-state fermentation. Biochemical Engineering Journal 44, 13-18.

Sundararajan, S., Kannan, C.N., Chittibadu, S., 2011. Alkaline protease from *Bacillus cereus* VITSN04: Potential application as a dehairing agent. Journal of Bioscience and Bioengineering 111, 128-133.

Thanikaivelan, P., Rao, J.R., Nair, B.U., Ramaswami, T., 2004. Progress and recent trends in biotechnological methods for leather processing. Trends in Biotechnology 22, 181-188.

Tiwary, E., Gupta, R., 2010. Medium optimization for a novel 58kDa dimeric keratinase from *Bacillus licheniformis* ER-15: Biochemical characterization and application in feather degradation and dehairing of hides. Bioresource Technology 101, 6103-6110.

Test Methods for the Examination of Composting and Compost, 2001. The US Department of Agriculture and The US Composting Council, Edaphos International, Houston, USA.

Valdez-Peña, A.U., Espinosa-Perez, J.D., Sandoval-Fabián, G.C., Balagurusamy, N., Hernández-Rivera, A., De la Garza-Rodriguez, I.M., Contreras-Esquivel, J.C.,

2010. Screening of industrial enzymes for deproteinization of shrimp head for chitin recovery. Food Science and Biotechnology 19, 553-557.

Valeika, V., Beleška, K., Valeikienė, V., Kolodzeiskis, V. 2009. An approach to cleaner production: from hair burning to hair saving using a lime-free unhairing system. Journal of Cleaner Production 17, 214–221.

Viswanathan, K., Schofield, M.H., Teraoka, I., Gross, R.A., 2012. Surprising metal binding properties of phytochelatin-like peptides prepared by protease-catalysis. Green Chemistry 14, 1020-1029.

Zanphorlin, L.M., Cabral, H., Arantes, E., Assis, D., Juliano, L., Juliano, M.A., Da-Silva, R., Gomes, E., Bonilla-Rodriguez, G.O., 2011. Purification and characterization of a new alkaline serine protease from the thermophilic fungus *Myceliophthora* sp. Process Biochemistry 46, 2137-2143.

Tables

Table 1 Characterization of the hair waste and the initial mixture assayed.

	Hair waste	**Initial Mixture**
Water content (%, wb)	59.9 ± 0.4	44.7 ± 0.6
Organic matter (%, db)	86.0 ± 0.1	88.6 ± 0.9
Dynamic respiration index ($g\ O_2\ kg^{-1}\ DM\ h^{-1}$)	3.0 ± 0.4*	1.3 ± 0.2
Cumulative oxygen consumption (4 days, $g\ O_2\ kg^{-1}\ DM$)	160 ± 12*	66 ± 0.5
Total Nitrogen Kjeldhal (%, db)	12.0 ± 0.1	8.3 ± 0.5
Total Organic Carbon (%, db)	57.2 ± 0.9	64 ± 3
C/N ratio	4.8	7.7
pH	10.76 ± 0.01	6.85 ± 0.01
EC ($\mu S\ cm^{-1}$)	5.03 ± 0.01	2.38 ± 0.01
Free air space (FAS, v/v)	n.d	0.73

Abbreviations: EC: electrical conductivity, wb: wet basis, db: dry basis, DM: dry matter. n.d: not determined. * Ratio 1:2 hair waste: raw sludge (w/w).

Table 2 Full factorial experimental design and results for stability analysis of the crude extracts.

pH	T (ºC)	Residual protease activity (%)		
		E3	E7	E14
5	30	13	9	12
5	50	12	10	13
5	70	1	6	0
8	30	18	23	30
8	50	29	26	32
8	70	10	4	4
11	30	62	55	66
11	50	63	49	59
11	70	17	6	1
8	50	28	25	27
8	50	28	28	28
8	50	31	24	28

Table 3 Summary of determined protease activity in solid and extract samples.

Sample	Enzyme activity (UA/g wet sample)	Total protein (mg)	Specific activity (UA/mg)
Solid	15379±784	170±0.02	90
Extract	31565±1961	0.17±0.01	185676

Table 4 Results of dehairing cow hides experiments for control, enzymatic and chemical treatments.

Sample	Enzymatic activity (UA/cm^2)	Hair removal (%) with respect to chemical treatment (100%)
Control	-	43
E3	4775 ± 1084	33
E7	4173 ± 286	81
E14	5491 ± 418	100
E21	2181 ±1 53	58
Chemical	-	100

Table 5. Full factorial experimental design and results for optimal pH and temperature values for the dehairing process with E14 (5491±418 UA/cm^2).

pH	T (ºC)	Hair removal (%) with respect to chemical treatment (100%)
8.5	20	48.3
10	20	62.1
11.5	20	77.8
8.5	35	52.6
10	35	62.9
11.5	35	70.2
10	35	62.1
10	35	79.3
10	35	73.7
8.5	50	84.7
10	50	99.8
11.5	50	97.6

Legends to figures

Fig. 1 Solid-state fermentation profile (one replicate is shown). (a) Temperature (▬, solid line), percentage oxygen content in exhaust gases (···, dotted line), pH (▲) and (b) protease profile (•).

Fig. 2 Polyacrylamide gel (12%) electrophoresis of crude extracts (15 µL). (a) Native-PAGE, (b) Zymogram.

Fig. 3 Soluble proteins in control experiment and different crude extracts assayed at initial and final (24 hours) of the cow hides enzymatic dehairing process. Initial and final soluble proteins levels in the last step of chemical dehairing process are also shown.

Fig. 4 Scanning electron microscopy of initial hide (a,b), control hide (c,d), treated E14 hide (e,f) and chemically treated hide (h,g).

Fig. 1

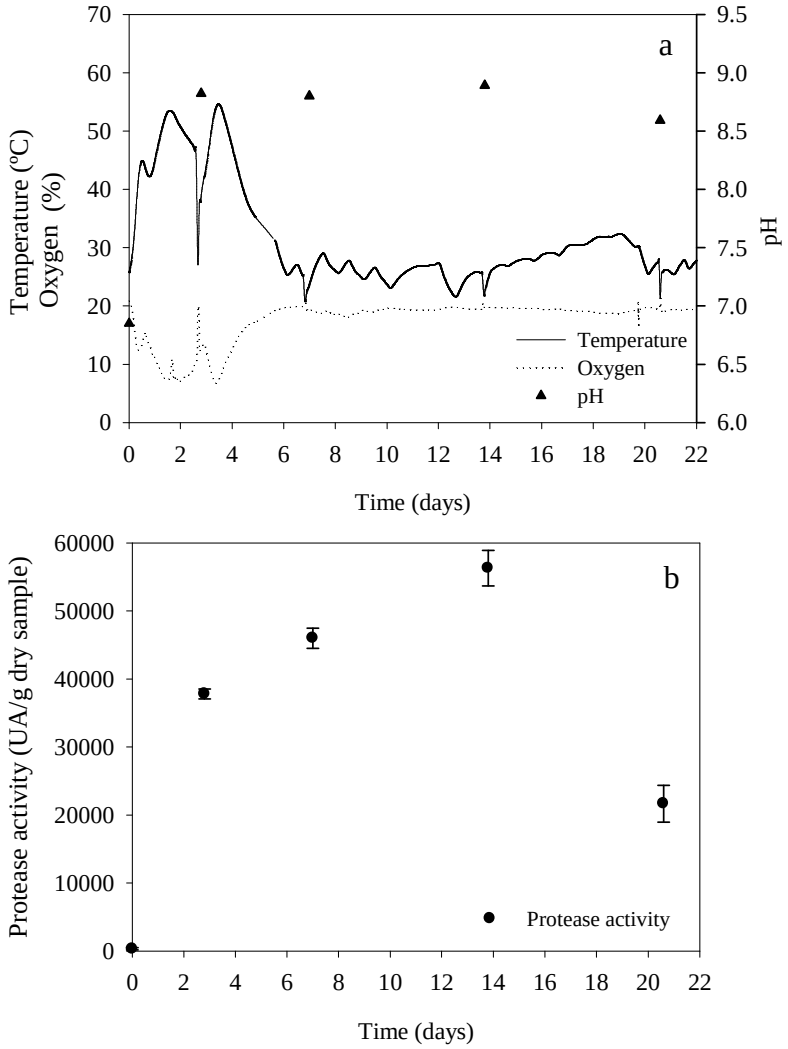
a
Temperature (ºC)
Oxygen (%)
pH
Temperature
Oxygen
pH
Time (days)
b
Protease activity (UA/g dry sample)
Protease activity
Time (days)

Fig. 2

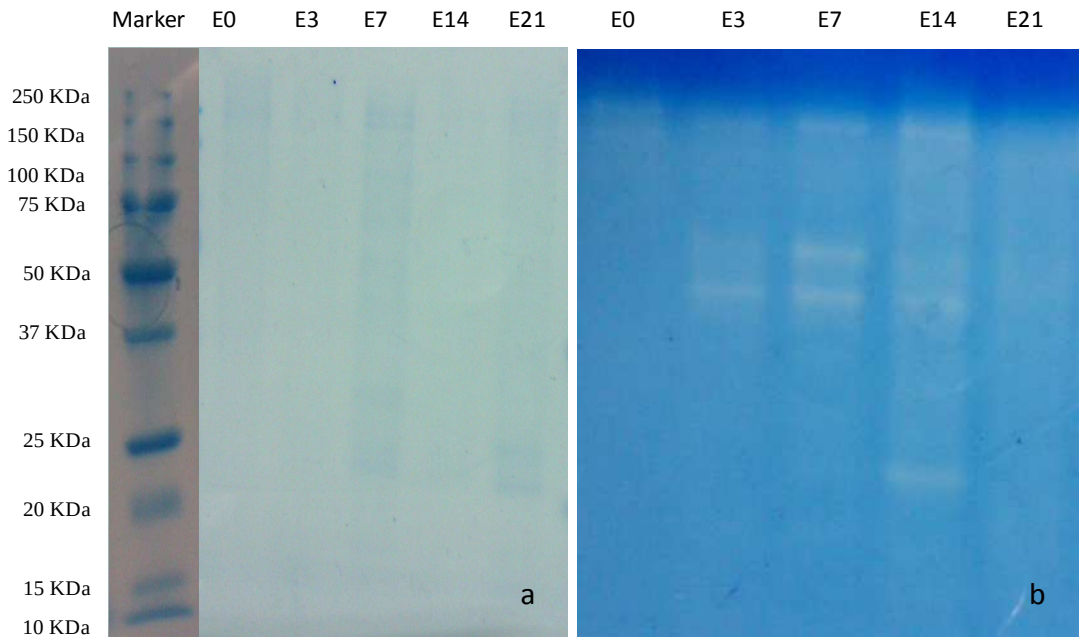
Marker
E0
E3
E7
E14
E21
E0
E3
E7
E14
E21
250 KDa
150 KDa
100 KDa
75 KDa
50 KDa
37 KDa
25 KDa
20 KDa
15 KDa
10 KDa
a
b

Fig. 3

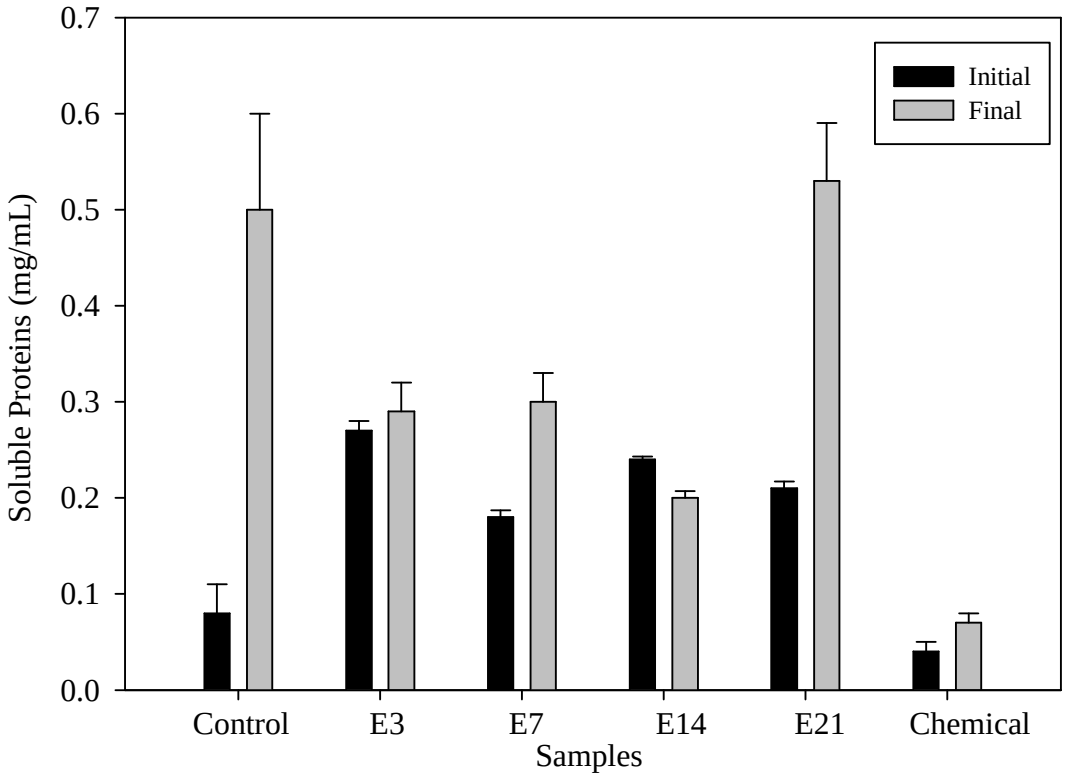
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Soluble Proteins (mg/mL)
Initial
Final
Control
E3
E7
E14
E21
Chemical
Samples

Fig. 4