Assessment of protease activity in hydrolysed extracts from SSF of hair waste by and indigenous consortium of microorganisms

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Abstract

Hair wastes from the tannery industry were assessed for its suitability as substrates for protease production by solid-state fermentation (SSF) using a pilot-batch mode operation and anaerobically digested sludge as co-substrate. Maximum protease activity (52230 ± 1601 U g^{-1} DM) was observed at the 14^{th} day of SSF. Single step purification resulted in 2 fold purification with 74% of recovery by ultrafiltration with 10 kDa cut-off. The recovered enzyme was stable at a temperature of 30ºC and pH 11; optimal conditions that were determined by a central composite full factorial experimental design. The enzyme activity was inhibited by phenylmethylsulfonyl fluoride, which indicates that it belongs to serine protease group. The remaining solid material after protease extraction could be easily stabilized to obtain a final good quality compost-like material as the final dynamic respiration index was lower than 1 g O_2 kg^{-1} OM h^{-1}. The lyophilized recovered enzymes were a good alternative in the process of cowhides dehairing with respect to the current chemical treatment, avoiding the production of solid wastes and highly polluted wastewaters. In conclusion, the entire process can be considered a low-cost sustainable technology for the dehairing process, closing the organic matter cycle in the form of value added product and a compost-like material from a waste.

Keywords: Protease; dehairing; protein purification; solid-state fermentation; hair waste.

1. Introduction

Traditionally, the tanning industry has been always associated with high pollution of the environment, which is mainly related to the use of toxic chemicals and the production of huge amounts of highly polluted wastewaters and solid wastes that impart a great challenge to the environment (Ahmad and Ansari, 2013). Over the years a large quantity of solid waste is generated worldwide from tanning processes such as skin trimming, shaving, dehairing, fleshing and production of buffing waste, which are associated to the increasing of high oxygen demand in water, discharging of highly toxic metal salts and the emission of unpleasant odour and atmospheric pollutants (Kanagaraj et al., 2006). Consequently, these processes lead to health and safety issues due to the land, air, surface, and groundwater contamination.

Approximately 5% to 10% of dry hair per ton of animal is being disposed to land or sanitary landfills (Onyuka et al., 2012). The main composition of hair waste is protein that constitutes 65% to 95% of hair weight where the rest consists of water, lipids, pigments and trace elements (Dawber, 1996). Furthermore, keratin, which is a constituent of protein in hair waste has a structure that take long time to degrade under natural environmental conditions, therefore landfill is not the best option to manage this material and it can cause severe environmental problems (Gupta and Ramnani, 2006).

Due to the environmental concern, EU tanners' environmental costs for hair waste disposal are estimated about 5% of their turnover (IPCC, 2003). Hence, any alternative treatment for hair waste would be attractive to tanning industry. In some cases, due to its high content of nitrogen, composting of hair waste has been proposed as a friendly environmental technology to produce a valuable organic amendment (Barrena et al., 2007a). However, for an efficient waste management the organic solid waste abundantly produced from tanneries could be recycled and exploited into other

value added products. Currently, only few works regarding the recycle of hair waste into these products are reported. One of the preferable ways of hair waste valorisation is its bioconversion into proteolytic enzymes that could be used in the same tanneries for dehairing (Wang et al., 2007). This strategy demonstrated to be a viable technique through solid-state fermentation (SSF) (Abraham et al., 2013).

Due to this origin, hair waste are highly alkaline and do not present the appropriate characteristics to be degraded alone and the use of a co-substrate and an inoculum is necessary to activate the biological transformation and accomplish the biodegradation process of hair waste. Dewatered sewage sludge has been effectively tested as a co-substrate to biodegrade hair waste (Barrena et al., 2007a; Abraham et al., 2014). Other economical option is to use as co-substrate anaerobically digested sludge (ADS), which has been successfully used as a co-substrate for different organic wastes (Barrena et al., 2007b). Furthermore, ADS is available in sufficient quantity and can be easily obtained from local wastewater treatment plants. To date, the use of hair waste and ADS as co-substrates for the production of alkaline protease has not been studied.

Literature regarding the production of alkaline protease by SSF is relatively abundant (Haddar et al., 2010; Mukherjee et al., 2008; Uyar and Baysal, 2004). However, most of the protease production via SSF is carried out with few grams of material, which hampers the development of the process at industrial scale (Ravindran et al., 2011). Moreover, the information about the recovery yields of protease activity is very scarce. As alkaline proteases have numerous potential applications in detergent industries, food processing, tanning and leather processing, waste treatment, textile and pharmaceutical industry (Abidi et al., 2008; Gupta et al., 2002; Khan, 2013; Raj et al., 2012), it is essential to develop highly scalable process of SSF that could use wastes as substrates or the hydrolysed materials for applications as biofuels (Han et al., 2015a).

In this context, the present study highlighted the protease production and extraction using a pilot-batch mode operation presenting the yields and recovery achieved on an easily scalable low-cost downstream process. In addition, a first approach to the characterization of enzyme produced is also presented. It also examines the possibility to continue the SSF process after the protease extraction in the same reactor after final material stabilization.

2. Materials and Methods

2.1. Materials

The main waste used for this research was hydrolysed hair produced during the dehairing of bovine hide and obtained from a local tanning industry located in Igualada (Barcelona, Spain). ADS from a local wastewater treatment plant were used as co-substrate for the SSF process. The complete characterization of substrates in terms of physical, chemical and overall biological activity is presented in Table 1. For the mixture, firstly, hair waste and ADS were mixed in a weight ratio of 1:2, as described in previous studies (Abraham et al., 2014; Barrena et al., 2007a). Afterwards, wood chips were used as bulking agent to the mixture hair: sludge in a 1:1 volumetric ratio to create the proper porosity in the mixtures (Barrena et al., 2007b).

Protease inhibitorsphenylmethylsulfonyl fluoride (PMSF), pepstatin A and trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (e64) were purchased from Sigma-Aldrich (St. Louis).

2.2. Solid state fermentation

Several fermentations were carried out under - aerobic environment. A preliminary fermentation was done to establish a reference profile for protease production in SSF using the above-explained mixture. The mixtures (approximately 3.7

kg) were prepared in duplicate (C1 and C2) in 10-L air tight reactors and fermented for 23 days, which was considered the control experiment. Samples of 100 g were collected at 0, 3, 6, 14, 20 and 23 days alter manual homogenization of the entire mass in all the reactors. SSF for protease extraction with the same mixture content was done in triplicate (R1, R2 and R3) using the same 10-L reactors. These reactors are used to produce the proteasesand to extract them in the same reactor in the moment of higher protease activity detected in the control experiment. The extracts were purified, characterized and used in the dehairing process, as explained later.

The experiments were performed under near-adiabatic conditions with continuous aeration at a minimum rate of 0.1 L/min. The reactors included a data acquisition system with a PLC (programmable logic controllers), which allowed data reading every minute. Particularly, PLC system reads the values of oxygen, airflow and temperature, which are connected to a personal computer, and it enables on-line complete monitoring. The oxygen was regulated by means of airflow manipulation in the exhaust gas to maintain the system in favourable aerobic conditions (oxygen content above 12%), as previously described (Puyuelo et al., 2010).

2.3. Enzyme extraction

For the control experiment, in which the protease activity was only monitored, about 10 g of homogenized solid samples were taken from the reactors at different days of SSF, as explained. The samples were mixed and extracted with 50 mM HCl-Tris buffer (pH 8.10) at 1:5 (w/v) ratios for 45 minutes at room temperature. Then the mixtures were separated by centrifugation at 10000 rpm for 10 minutes. The supernatant was collected and filtered through a 0.45 μm filter and used as crude enzyme extract for further characterization and use. All the extractions were done in triplicates.

To ensure the reproducibility, extraction of the whole fermented mass was performed in triplicate 10-L reactors at day 14, which was the time when the control experiment showed the highest production of protease according to the protease profile. In this mode of extraction the entire batch was submerged in each reactor with 50 mM HCl-Tris buffer (pH 8.10) at 1:1 (v/v) ratio for 1 hour. Then the extracts were sieved with 2.0 mm stainless steel sieve and centrifuged as aforementioned. The supernatant was taken as the crude enzyme extract, whereas the solid remaining residues continued the SSF process until 42 days for final stabilization.

2.4. Lyophilisation

The crude enzyme extracts obtained after the extraction at 14th day of SSF (R1, R2 and R3) were frozen to -80 ºC prior to lyophilisation using vacuum with a bench top VirTis Sentry 5L freeze dryer. The frozen samples were attached to quickseal valves on stainless steel drum manifolds. The lyophilisation process lasts approximately 24 hours. The lyophilised extracts were collected and preserved at 4 ºC for further use.

2.5. Partial purification and characterization of protease

The crude extracts were concentrated using Amicon® Ultra-15 centrifugal filter devices (Milipore, Ireland) with 10k molecular weight cut-off (MWCO) Ultracel® low binding regenerated cellulose membrane The concentrated ultrafiltered liquid was recovered and stored for further experiments of protease characterization. In these processes, recovery yield is defined as the percentage of residual activity with respect to the initial activity of crude extract, whereas purification fold is the quotient between specific enzymatic activity after purification with respect to the specific activity of the initial crude extract.

Molecular weight of proteases was estimated by SDS-PAGE electrophoresis using 12% polyacrylamide (w/v) precast gels (Biorad®). The gel was stained with Coomassie Blue G-250 (Biorad®). Zymogram was carried out using casein (Sigma-Aldrich) as copolymerized substrate to reveal protease profile from the extracts (Abraham et al., 2014).

The stability of the enzyme was determined by incubating the lyophilised extracts for 1 h at different temperatures within 30ºC-70ºC and different pH values using the following buffers: Acetic acid-sodium acetate 1 M (pH 5), Tris-HCl 1 M (pH 8), Tris-NaOH 1 M (pH 11). The stability was analysed using the Design-Expert software (version 6.0.6) using a full central composite design (CCD) that consisted of 13 experimental points, including five replications at the central point and four star points ($\alpha = 1$) Residual activity was selected as objective function (in percentage) for each pH and temperature tested assuming that the initial activity of the enzyme is 100%.

The effect of various protease inhibitors such as phenylmethylsulfonyl fluoride (PMSF), trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (e64), pepstatin A, and ethylene diamine tetra acetic acid (EDTA) were determined by the addition of the corresponding inhibitors at 1 mM and 10 mM (final concentration) to the aliquot of the protease. The reaction mixtures were pre-incubated at 37 ºC for 1 h without the substrate fraction and assay under standard conditions. The recorded residual activities were compared with that of the control (without inhibitors).

2.6. Application of alkaline protease in cowhides dehairing

The cowhide was cut in small pieces with the same area (10.68 cm^2) for the dehairing process. About 0.4 g of lyophilized enzyme extracts was dissolved in 15 mL of Tris-HCl buffer to incubate the hides. The dehairing efficiency of the enzyme was

assessed in comparison with the chemical dehairing process, where the initial amount of hair was assumed to be the same for each piece. Additionally a commercial powder provided by for tanning industry was tested. The dehairing process was performed by scrapping the hair using tweezers in a plate fill with water after 24 h incubated with protease or chemicals. The hair was harvested and measured as TSS (total suspended solids). Dehairing was expressed as percentage removal where the hair being removed by the chemical treatment was considered 100%. Briefly, the chemical treatment consists of a sequential treatment of the cow hides with the following reagents: sodium carbonate (15 mL, 0.3%, w/v) with a non-ionic surfactant (0.3%, w/v) (soaking, 22 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.

2.7. Analytical methods

2.7.1. Dynamic respiration index (DRI)

On the basis of the methodology to assess the degree of biological stability of the remaining solid material from SSF, the dynamic respiration index (DRI) was measured using a dynamic respirometer (Ponsá et al., 2010). The determination consists of placing 100 g of sample in a 500-mL Erlenmeyer flask and incubating the sample in a water bath at 37 ºC. A constant airflow was supplied through the sample, and the oxygen content in the outgoing gases was measured. From this assay, DRI was determined as the maximum average value of respiration activity measured during 24 hours, expressed in g O_2 kg^{-1} OM h^{-1} (Adani et al., 2006, Ponsá et al., 2010). ().

DRI was used as a test to determine the stability of organic matter after SSF. In this sense, lower respiration activity can be considered as stable compost (Adani et al., 2004).

2.7.2. Protease activity assay

The protease activity was measured using casein (2%) as substrate according to Alef and Nannipieri (Alef and Nannipieri, 1995), with a slight modification as described previously (Abraham et al., 2013). One unit of alkaline protease activity was defined as 1 μg of tyrosine liberated under the assay conditions.

2.7.3. Protein determination

Protein concentration was determined by the modified method of Lowry (Lowry et al., 1951) using bovine serum albumin (BSA) as standard protein. The protein content was estimated by measuring the absorbance at 750 nm using Varian Cary® 50 UV-visible spectrometer.

2.7.4. Routine analytical methods

Dry matter (DM) and organic matter contents (OM), total suspended solid (TSS), bulk density and pH in solid samples were determined according to standard methods (TMECC, 2001).

3. Results and Discussion

3.1. Solid-state fermentation

Primarily, the initial DRI of the mixture was considered high, 4.7±0.7 g O_2 kg^{-1} OM h^{-1} (Table 1) to initiate aerobically degradation during SSF process. The moisture

content (>60%) and C/N ratio (11) of the mixture were maintained to favour degradation process to produce protease enzyme during thermophilic and mesophilic condition. The results presented in Fig. 1 showed the protease production and fermentation profiles (temperature and oxygen exhaust content) of SSF. No lag phase was observed because of the previous hydrolysis of hair waste, as pointed in other studies (Du et al., 2008). The fermentation showed a normal operating condition where the thermophilic temperature (60ºC) occurred at the beginning of the SSF (day 2) and decreases towards the end of the fermentation. In Fig. 1, oxygen contents were low during the thermophilic stage as an evidence of high aerobic respiration activity during that period in the profile (Gea et al., 2004). The oxygen profiles showed a similar trend to that of co-composting hair waste and raw activated sludge profiles (Abraham et al., 2014; Barrena et al., 2007b).

To validate the processes performances of the replicates, they were assessed through the statistical comparison of temperature and oxygen profiles that have been summarized in Table 2. Thus, to evaluate the performance of mixtures of hair waste and anaerobically digested sludge (ADS) the area below the temperature curve, the area below sOUR (specific oxygen uptake rate), the maximum protease activity, the specific activity and dry mass reduction were calculated until 14 days of SSF. Apparently, there was no considerable difference between replicates for the temperature curve, where the average was 499 ± 15ºC day^{-1}. The coefficients of variation (CV) for all the fermentations were lower than 5%, thus validating statistically the process and confirming non-statistically differences between replicates (de Guardia et al., 2010). Additionally, the maximum specific oxygen uptake rate (sOUR max) and accumulated oxygen content throughout the SSF process was correlated well with a correlation coefficient of 0.951. Overall, the correlation between the area below the temperature

curve and the maximum protease activity reached, the sOUR max and the maximum protease activity were significant at $p < 0.001$. As sOUR max value also correlated to the increasing value of the area below sOUR (Table 2) ($p=0.017$), it can be stated that the SSF for the production of proteases from hair and ADS under this pilot conditions is highly reproducible and the results are consistent.

3.2. Enzymatic activity profile and extraction

Protease profile is shown in Fig. 1 at day 14th, the highest protease activity production was observed, which resulted in a e value of 48971 ± 872 U g^{-1} DM with a CV equal to 1.8%. These results are in agreement with previous studies using 4.5 L reactors where the highest activity of protease was observed at 14 days of SSF with hair waste and different co-substrate (Abraham et al., 2014, 2013). Therefore, this time was selected to carry out the extraction of the enzyme in further experiments.

The SSF process was continued until 42 days with the mixture after extraction to complete the entire process that implies the stabilization of the remaining material. The activity of protease at the end of SSF process (42 days) was 3273 ± 1342 U g^{-1} DM, showing a significant decrease of activity at end of the fermentation.The average of dynamic respiration index (DRI) of the mixtures at the end of the process (42 days) was lower than 1 g O_2 kg^{-1} OM h^{-1}, which indicates the degradation of biodegradable matter and show that a further stabilization is useful to obtain a very stable compost-like material. Thus, it can be concluded that, after extraction, DRI showed a high biological stability, as reported in previous studies (Gupta et al., 2002; Ponsá et al., 2010).

3.3. Partial purification and characterization

Table 3 shows the summary of the partial purification steps tested for alkaline protease in the three replicates (R1, R2 and R3). In all cases, the partial purification of protease resulted in a 2 fold purification factor with 74% of recovery by ultrafiltration using Amicon Ultra-15 centrifugal filter device with 10kDa MWCO. Additionally, protease activity was not detected in permeate as the size of alkaline proteases produced were in range between 26-100 kDa. Some studies reported that there was no protease activity detectable in all permeates due to the lost as a deposit in the membrane of the tube (Bezawada et al., 2011). However, during lyophilisation the loss of activity resulted in 21%, being this step the most critical for the recovery of protease.

The response surface of protease in front of several conditions of pH and temperature was determined to assess the stability of the protease. For this, it was analysed using the regression equation obtained from the analysis of variances (ANOVA) by the Design Expert software to determine the suitability of the model (Eq. 1). In the model, *pHT* indicates the interaction term between temperature and pH. The negative coefficient of *T* (Eq.1) suggested that high temperature has a pronounced effect on activity and, as shown in Fig. 2, pH effect becomes insignificant at higher temperatures.

$$\textit{Residual activity (\%)} = 52.79 + 4.17pH - 29T - 2.78pH^2 - 3.25pHT \quad \text{(Eq. 1)}$$

Table 4 shows that the regression for residual activity yield model was significant (242.97) and the lack of fit was not significant (0.58) at $p < 0.0001$ relative to pure error. The lack of fit of the F-value for the residual yield was less than the $F_{critical}$ value ($\alpha = 0.05$ at degree of freedom 4,3) of 9.12, which indicates that the treatment differences were highly significant. The fit of the models were checked by the determination of correlation coefficient, R^2. In this case, the value of the coefficient for residual activity was $R^2 = 0.9943$. The values showed that only 0.57% of the variables

behaviour is not explained by the model (Amini et al., 2008). The predicted R^2 (0.9797) for the model was in reasonable agreement with the adjusted R^2 of 0.9902, therefore it can be concluded that the proposed model adequately approximated the response surface and it could be used to predict the values of the variables within the experimental domain (Gilmour, 2006; Myers et al., 2004).

The partially purified protease was found to be an alkaline protease displaying the residual activity more than 70% when approaching alkaline pH up to 11 (Fig. 2). As it was stable in alkaline pH up to 11, it can be used for industrial purposes where commercially proteases have highest activity in alkaline pH ranges of 8-12 (Gupta et al., 2002; Kumar et al., 1999). For thermal stability, the enzymes exhibited good activities over a temperature range 30 to 50 ºC in a wide range of alkaline pH from pH 7-11. The optimum enzyme activity (49347 ± 5487 U g^{-1} DM) was obtained at 30ºC and pH 11. However, the enzymes were rapidly inactivated and retained approximately 4% to 8% of residual activity after incubation at 70ºC at any tested pH (Fig. 2). These results were in accordance with previous studies (Abraham et al., 2014, 2013) reported that the protease produced from hair waste was highly stable in alkaline and mesophilic temperatures, which coincides with the conditions of the SSF when it is mainly produced (Fig. 1).

The proteolytic activity of concentrated alkaline protease (R1, R2, and $R3_2$) subjected to a zymogram revealed four clear hydrolytic zones around the blue background with molecular weight from 26 kDa to 100 kDa (Fig. 3). The molecular weights of the proteases coincided with previous study (Abraham et al., 2014) that were obtained after 14 days of SSF in 4.5 L reactors, when the best results in dehairing were observed. Probably, these proteases were produced by the same type of microorganisms that may coexist in the mixture of hair waste and ADS for protease production.

In the present study, pepstatin A (acid protease inhibitor), e64 (cysteine protease inhibitor), and EDTA (metalloprotease inhibitor) had a minimal effect on the protease activity (Table 5). On the contrary, PMSF (serine protease inhibitors) inhibited the enzyme activity at very low concentration (1mM). Therefore, the protease produced in this study can be considered as serine protease type. Since serine proteases generally are active at pH 7-11 and have broad substrate specificities it may be used in the dehairing process as reported in other studies (Huang et al., 2003; Ito et al., 2010; Wang et al., 2007).

3.4. Dehairing of cowhides

In order to evaluate the feasibility of the proposed process at industrial scale, the protease produced was tested on dehairing of high pigmentation cowhides. Normally, in tanning industries the solubilisation of black and brown hair is slower compared to the white and calfskin due to melanin and colour pigmentation (Onyuka, 2009).

To validate the possibility of using proteases from SSF in the dehairing process, the lyophilized enzyme (R1, R2 and, R3) with similar initial enzymatic activities of 7556±129.8 U cm^{-2}, 8561±12.0 U cm^{-2} and 9373±48.6 U cm^{-2} respectively, were used for dehairing of black cowhides. Fig. 4 indicates the percentage of hair removal of the raw hide that was processed for dehairing using Tris-HCl buffer as control, enzymatic and chemical treatments for comparison. Additionally, the dehairing activity of the obtained enzyme was compared with a commercial powder used for dehairing that was being used in the tanning industry with a similar activity (74885 ± 3137 U mL^{-1} and 87588 ± 1110 U mL^{-1} for lyophilized extract and commercial powder respectively).

Approximately, between 90-95% of hair removal with respect to chemical treatment was observed in treatment with proteolytic enzymes from R2 and R3 after 24

hours of incubation. The commercial powder was very close to the chemical treatment (Han et al., 2015b). These results suggest that using an appropriate enzymatic conditions results in good dehairing performance as pointed out in other studies (Asker et al., 2013; Dayanandan et al., 2003; Sundararajan et al., 2011). In the case of R1, protease showed a weaker activity with only 50% of hair removal probably due to the lower specific protease activity already detected in SSF (Table 3). Sivasubramanian et al. (2008) suggested that dehairing of hides is substantially difficult as the structural features and thickness of skin and hide vary greatly; therefore the dehairing efficiency of enzyme may vary accordingly.

Furthermore, it can be stated that the protease produce in this work presents the possibility to be an alternative to chemical dehairing as reported in other studies carried out at lower scales (Dayanandan et al., 2003; Saravanan et al., 2014; Sivasubramanian et al., 2008; Sundararajan et al., 2011).

4. Conclusions

The use of hair waste in SSF was found to be a practical approach to produce alkaline proteases that can be used in the dehairing process. Moreover, in-situ protease extraction could make SSF process easier to scale up. Additionally, after extraction, SSF can be continued with the same mixture to reach final stabilization, similar to that of compost. With regard to the enzyme properties, protease activity was only inhibited by PMSF, suggesting that it belongs to the serine protease group. The enzyme produced has highly and stable alkaline properties with moderate heat stability (30ºC-50ºC), which is of relevance during industrial dehairing application. In conclusion, the process presented can be considered a complete alternative to chemical dehairing. In future works, to improve the efficiency of wastes utilization, the use of hair waste should be

investigated with a special focus on biorefinery concepts (possibility of obtaining other valuable products). At the same time, next studies should be focused on a deep economic and environmental study of the entire process.

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Tables

Table 1. Characterization of anaerobically digested sludge, hair waste and initial mixture for SSF process

Characteristics	Hair waste	Anaerobically digested sludge	Mixture*
Physical characteristics			
Organic matter (% db)	85.5 ± 0.7	71.3 ± 0.6	84.6 ± 0.4
Water content (% wb)	62 ± 3	86 ± 4	72 ± 4
Dynamic respiration index (DRI) ($g\ O_2\ kg^{-1}\ OM\ h^{-1}$)	N.D.	2.4 ± 0.3	4.7 ± 0.7
Electrical conductivity ($mS\ cm^{-1}$)	4.1 ± 0.9	1.2 ± 0.7	1.6 ± 0.8
pH	10.7 ± 0.1	8.32 ± 0.04	8.52 ± 0.01
Chemical characteristics			
Total carbon (% db)	57.2 ± 0.9	42.0 ± 0.1	68.3 ± 0.4
Total nitrogen (% db)	12.1 ± 0.1	7.2 ± 0.6	6.9 ± 0.1
C/N ratio	4.7	5.9	11

db: dry basis; wb: wet basis; OM: organic matter; N.D.: not determined; *ratio 1: 2 (sludge:hair waste)

Table 2. Summary of the replicates of solid state fermentation (SSF) using 10-L air tight reactors with hair waste and anaerobically digested sludge as substrates

	Initial water content of mixture (%)	Bulk density (kg L^{-1})	Process parameters during SSF				Max PA[a] (U g^{-1} DM)	sPA[a] (U mg^{-1} soluble protein)	Dry mass reduction[a] (%)	Stability after SSF
			Area T[a] (^oC day^{-1})	sOUR max (g O2 kg^{-1} DM h^{-1})	Area sOUR[a] (g O2 kg^{-1} DM)	Time sOUR max (h)				DRI (gO_2 kg^{-1} OMh^{-1})
Experiment performed with in-situ enzyme extraction										
R1	76.1	0.5	513.4	3.9	387	11.7	52230 ± 1601	12615 ± 111	10	0.39±0.04
R2			475.8	3.1	337	11.3	37732 ± 1608	15403 ± 909	7	
R3			500.3	5.3	538	10.9	37782 ± 1514	13547 ± 626	10	
Control experiment										
C1	73.3	0.67	497.0	2.8	220	48.4	48354 ± 78	3454 ± 21	11	0.87±0.01
C2			507.9	2.3	165	71.8	49587 ± 324	4157 ± 86	17	

R1, R2 and R3: replicates SSF experiments; C1and C2: control SSF experiments.

PA: protease activity; sPA: specific protease activity; OUR: oxygen uptake rate; DRI: dynamic respiration index; DM: dry matter; OM: organic matter; values are the average of three replicates of experiments ± standard deviation of triplicates.

[a]The parameters were calculated after 14 days of SSF (maximum protease production).

Table 3. Partial purification of protease enzyme from extraction

Sample	Purification step	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	Recovery (%)	Purification fold
R1	Crude extract	124410	9.91 ± 0.04	12560 ± 111	100	1
	Lyophilization	94408	9.86 ± 0.03	9571 ± 240	76	0.76
	Ultrafiltration	85180	4.10 ± 0.72	20796 ± 631	68	1.66
R2	Crude extract	85830	5.83 ± 0.01	14715 ± 909	100	1
	Lyophilization	62180	5.34 ± 0.20	11646 ± 244	72	0.79
	Ultrafiltration	60230	3.39 ± 0.16	17788 ± 163	70	1.21
R3	Crude extract	102600	6.43 ± 0.01	15059 ± 625	100	1
	Lyophilization	90180	6.37 ± 0.28	14148 ± 375	88	0.89
	Ultrafiltration	86340	3.34 ± 0.38	25843 ± 288	84	1.62

R1, R2 and R3: triplicates SSF experiments. Samples were taken after 14 days of SSF (maximum protease production).
Values are the average of three replicates in the experiments ± standard deviation.

Table 4. Analysis of variance (ANOVA) for the response surface quadratic model.

Source of variations	Sums of squares	Degrees of freedom	Mean square	F-value	(P value)
Regression	184.36	5	1236.87	242.97	< 0.0001
Residual	35.64	7	5.09		
Pure error	24.8	4	6.2		
Lack of fit	10.84	3	3.61	0.58	
Total	6220	12			

R^2 0.9943; adjusted R^2 0.9902; predicted R^2 0.9797

Table 5. Effect of protease inhibitors on protease activity from different replicates

Inhibitor	Concentration (mM)	Residual activity (%)		
		R1	R2	R3
Control	-	100	100	100
Pepstatin A	1	98	94	99
	10	97	91	96
PMSF	1	36	47	49
	10	3.8	5.1	6
e64	1	99	98	98
	10	93	87	89
EDTA	1	98.7	94	99.4
	10	81	78	86

The residual activity was assayed under the standard assay conditions in the presence of various inhibitors. 1mM is the initial concentration of the inhibitors and 10 mM is the final concentration of the inhibitors. Enzyme activities measured in the absence of any inhibitor were taken as 100%

Figure captions

Fig. 1 Solid state fermentation profiles (one replicate is shown) for 23 days of SSF: Temperature (—— , solid line), oxygen content in exhausts gas (····, dotted line), protease activity profile (—●—, long dash) from several SSF.

Fig. 2 Response surface of residual protease activity (%) on stability of enzymes with respect to pH and temperature.

Fig. 3 Zymogram of partially purified enzyme on 12% polyacrylamide gel after SDS-PAGE under non-reducing condition (Lane M1: standard molecular mass marker proteins in kDa; Lane M2: egg albumin (45 kDa); bovine serum albumin (66 kDa); Lane 1: crude extracts; Lane 2, 3, 4: partially purified enzymes from R1, R2 and R3).

Fig. 4 Percentage of enzymatic dehairing with respect to chemical treatment; hair removal of cowhide using the chemical treatment was considered 100% of dehairing. Percentage of hair removal for each treatment was calculated as the hair removed using enzymatic dehairing with respect to chemical dehairing on a dry matter basis.

Fig. 1

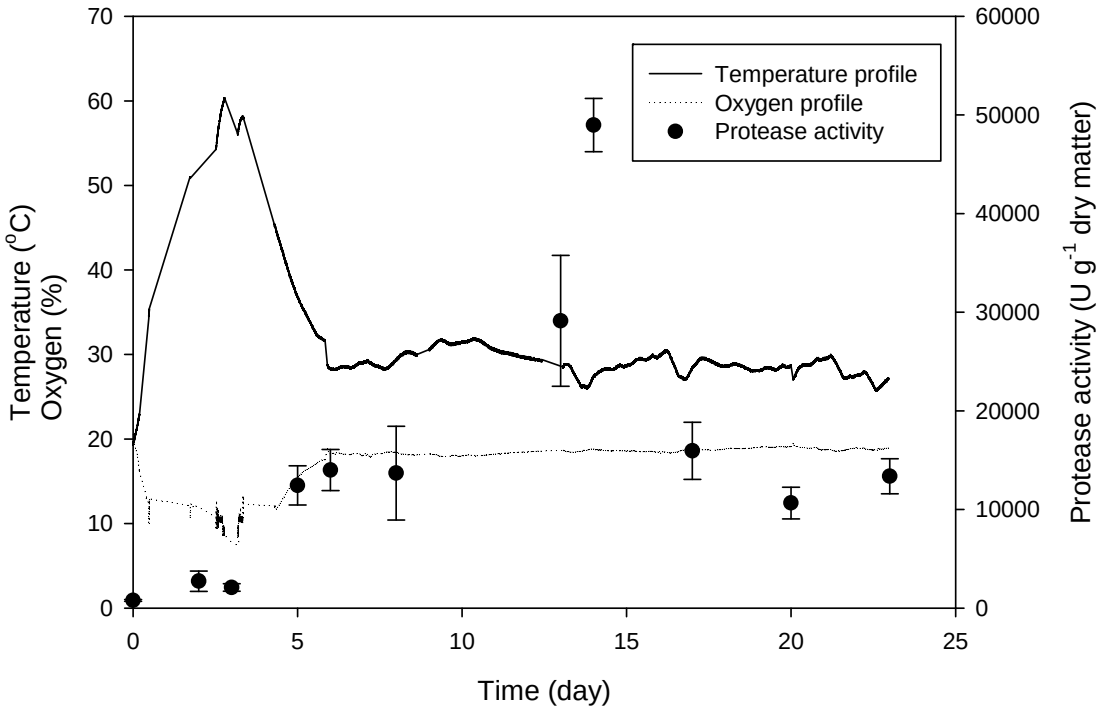

Temperature profile
Oxygen profile
Protease activity
Temperature (°C)
Oxygen (%)
Protease activity (U g-1 dry matter)
Time (day)
0
10
20
30
40
50
60
70
0
10000
20000
30000
40000
50000
60000
0
5
10
15
20
25

Fig. 2

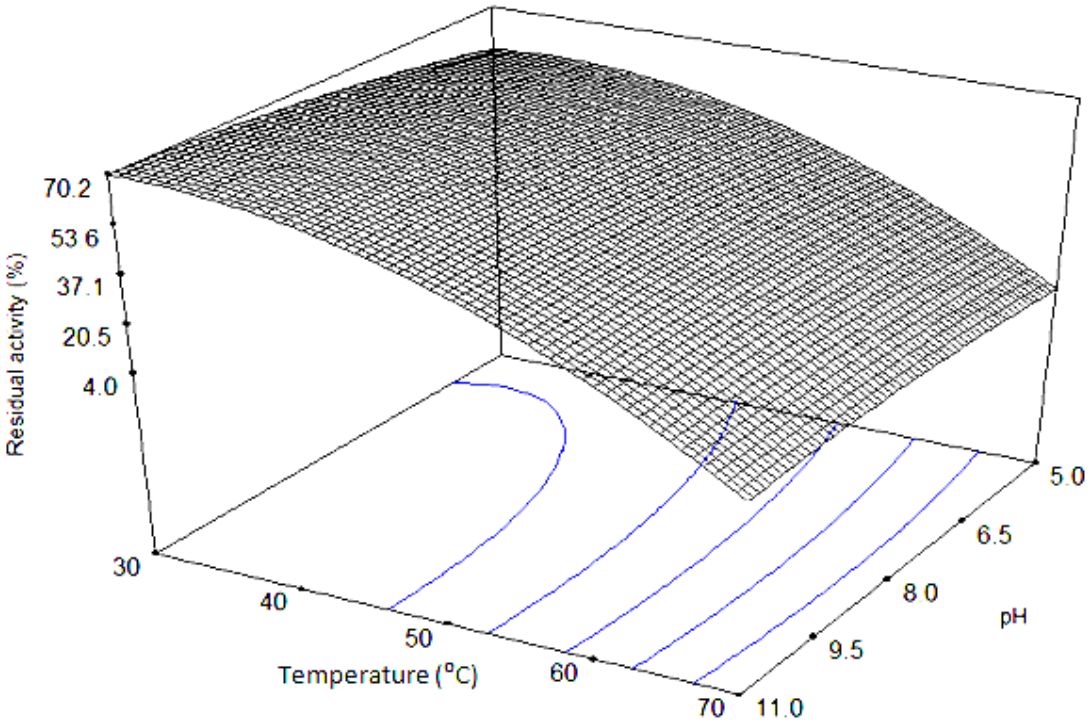

70.2
53.6
37.1
20.5
4.0
Residual activity (%)
30
40
50
60
70
Temperature (°C)
11.0
9.5
8.0
6.5
5.0
pH

Fig. 3

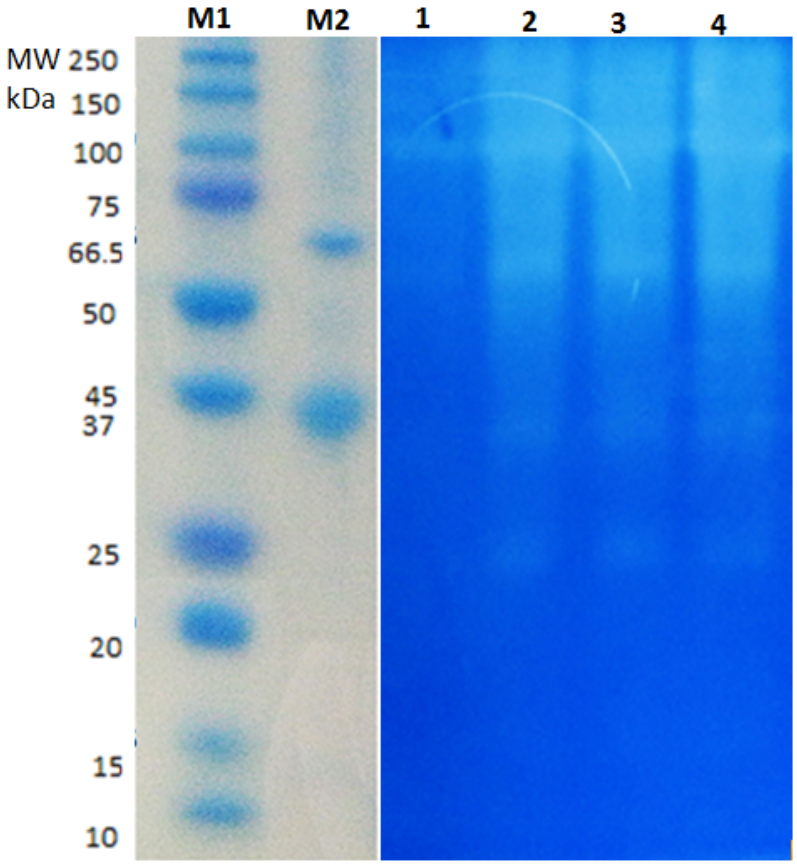
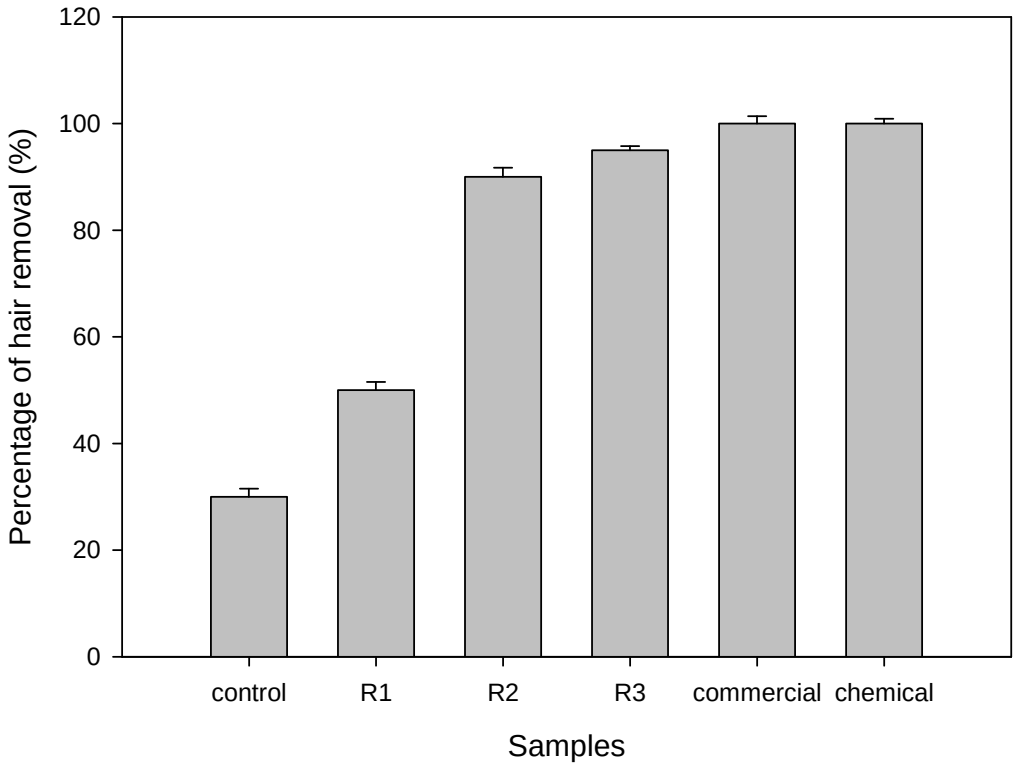


Fig. 4


120
100
80
60
40
20
0
Percentage of hair removal (%)
control
R1
R2
R3
commercial
chemical
Samples