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Wealth from waste: Recovery of the commercially important waxy ester from enzymatic dehaired sheep wool

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ABSTRACT

In the present study, the *Aspergillus flavus* were screened to produce multienzyme containing amylase and protease using starch agar and skimmed milk agar plate assay. The fungus was cultivated by solid state fermentation using wheat bran as substrate. The parameters such as pH, temperature, incubation time, and moisture content were optimized. The maximum production was achieved by amylase with a yield of 55 U/mL and followed by protease yielding to 37 U/mL at 70% moisture (Room temperature), pH of 9.5, at 28 °C and 96 h of incubation. Application studies were carried out for dehairing and extraction of lanolin, using sheep wool. Results suggested that use of multienzyme substantially replaces the usage of chemicals and will be considered as a suitable bio-agent in the current scenario on green process technology development.

1. Introduction

Enzymes production is carried out by using diversified microbial sources. In particular, aerobic fungi have opted for the production of enzymes owing to high growth as well as high protein secretion rates. Development of a multienzyme system is an exciting research field with high industrial potentials such as food processing, therapeutic protein production, persistent organic pollutant remediation and paper recycling (Lynd et al., 2002; Wilson et al., 2009; Saranya et al., 2017). Multienzymes have broad applications in all industries to the household sector, biotechnological, medicinal, and hold a significant share in the global enzyme market. In the leather processing industry Proteases, Lipases and Amylases have an important role in the soaking, dehairing, degreasing and bating operations of leather manufacturing (Dayanandan et al., 2003, 2012). The leather industry in developing countries like India, China, and Brazil make enormous profits while also bringing significant environmental pollution (Dayanandan et al., 2003, 2012). The fungal pectinase from *A. tamarii* has also reported for the bioscouring of cotton, and extraction of biopigment such as lycopene and anthocyanin (Shanmugavel et al., 2018). Therefore, it is urgent that clean and eco-friendly technologies are to be developed for leather processing and effluent treatment. Use of multienzyme in places of hazardous chemicals at different stages of leather processing, especially in dehairing. *Bacillus subtilis* was also reported for the production of amylase and protease together in the same culture medium in submerged fermentation (Bhange et al., 2016 and Blanco et al., 2016) and Qureshi et al., 2016 also reported this similar enzyme production in *Bacillus* sp. by solid state fermentation. Compared with traditional chemical methods, enzymatic processes not only yield quality-improved products but also reduce the use of hazardous and polluting chemicals (Lee et al., 2003; Saravanabhavan et al., 2003). Dehairing is the significant step of tanning operation wherein the hair, epidermis, some portion of non-collagenous proteins and other cementing materials are removed from the skin (Sivasubramanian et al., 2008). Dehairing of the skin using protease will reduce the sulfide contents in the effluent and recovery of the hair/wool, which in good quality. It is an increased yield of leather area, handling of the pelts by workers will make accessible, the production of a good quality pelts/leather by simplifying the pretreatment elimination of bate in the deliming stage finally (Sandhya et al., 2005).

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A New Zealand sheepskin have been collected and used for the enzymatic method of dehairing by using the enzymes protease and amylase produced by *Aspergillus flavus* (*A. flavus*). Then the wool was further used for lanolin extraction. Lanolin is a greasy yellow substance made by secretion of sheepskin from the sebaceous gland which containing wool and stored in wool fiber. It is a long chain of fatty acid containing cholesterol with a different composition than human sebum. Because of its high-fat content, lanolin is occlusive; it forms of protection against nature and insects; which used for ointments, cosmetics, emollient, moisturizer, stabilizer, anti-corrosive agent and, metal cutting fluid. Lanolin has various applications apart from cosmetics, pharmaceuticals, personal and health care, such as lubricant and in production of anti-corrosion paints for ferrous material (Industry analysis Industrial analysis report, 2019). It is used to treat diaper rashes, chapped lips and skin eczema, due to Lanolin's healing effect and moisturizing nature on tender and sensitive skins (Persistence market research, 2019). The growing global concern of environmental pollution is forcing all the processing industries to adopt greener and cleaner manufacturing practices, so the enzymatic process reduces the effluent, avoids the use of toxic chemicals, and simplifies the procedures and complete recovery of hair/wool and smooth handling without any discomfort. Since lanolin is biodegradable, nontoxic, biocompatible, and has a potential alternative for mineral oil, thus pave the way for market growth promotion (Global Market Insights, 2018). The global lanolin market report shows growth of CAGR of 15% from 2017 to 2021 growth prospective (Research and market, 2017). Finding suitable biocompatible polymer systems (Ranganathan et al., 2018) to be used as a nano carrier for lanolin controlled release can pave way for nano medicine applications for cancer therapy and potential cure (Khargonekar et al., 2017).

2. Materials and methods

The sheepskin pieces were obtained from CSIR-CLRI tannery and used for processing. The *A. flavus* was isolated from soil, and it was maintained in Czapek Dox's agar medium (Verma et al., 2011). The enzyme consortium produced by fungus *A. flavus* was used for dehairing of sheepskins. All other chemicals used were of commercial grade. All the experimental procedures was done as per Fig. 1 and Fig. 2.

2.1. Maintenance of culture

The fungus was maintained on Czapek Dox's agar slants and stored in the 25 °C and sub-cultured at monthly intervals. Stock culture of

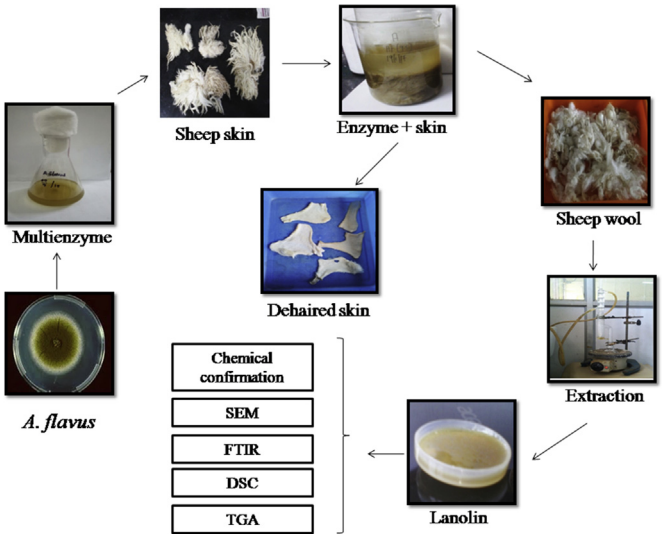

Fig. 1. Pictorial representation of the developed technology

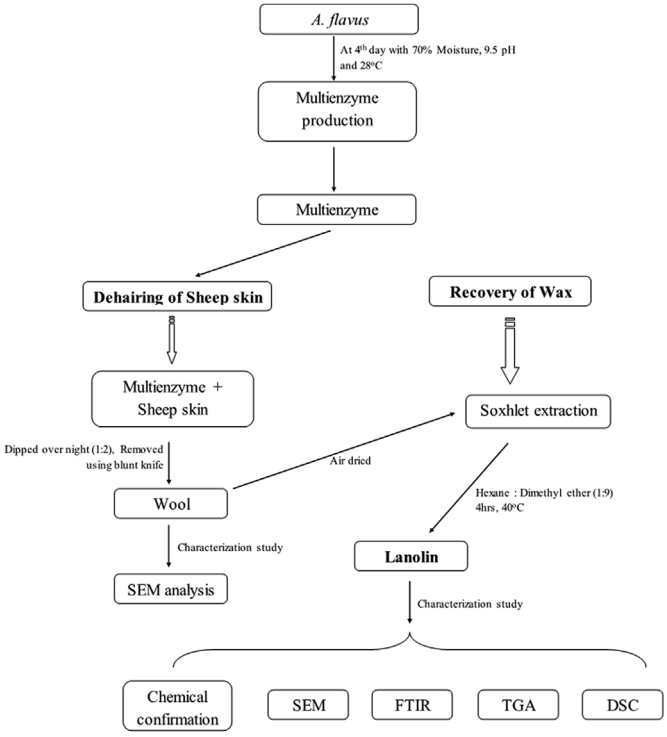

Fig. 2. Schematic flow diagram.

fungal spore suspensions stored at −20 °C. The spore suspension was filtered aseptically, and appropriate volumes were used (Verma et al., 2011).

2.2. Solid state fermentation

Wheat bran used as a substrate in solid state fermentation. 25 g of wheat bran was taken into each sterile conical flask and mixed with water to maintain 70% moisture. The wheat bran flasks sterilized (Shanmugavel et al., 2016). The inoculum ($1X10^{-6}$ spores/ml) inoculated at the aseptic condition. After inoculation, the flask was incubated for four days (Shanmugavel et al., 2016). The containers were shaken thoroughly to disperse the spores (Kranthi et al., 2012) and incubated at 25–35 °C for 1–7 days. The culture was mixed with Borate buffer (pH 9.5), and the pH was altered by using 0.2N NaOH and 0.2N HCl respectively and was kept in the shaker for another 30 min. The crude enzyme extract filtered through nylon mesh; centrifugation was carried out at 7826 'g' (10,000 rpm) for 20 min at 4 °C. The collected supernatant was stored at 4 °C and used as a crude enzyme. Sampling was done at 24hrs intervals to ascertain fungal growth and enzyme activity (Shanmugavel et al., 2011; Oyeleke et al., 2010).

2.3. Plate assay

The plate assay qualitatively analyzed the ability of this fungal organism producing a particular enzyme. The *A. flavus* and their enzyme were tested for amylase production by starch hydrolysis (Suganthi et al., 2011). Flooding plates detected the zone with a solution of iodine (Brown et al., 2001). The protease production was also confirmed in the organism as well as in the enzyme using 1% agar with skimmed milk powder (w/v) was poured in Petri dishes then BCG reagent was flooded and incubated for 20–30 min at room temperature (25–30 °C) (Vijayaraghavan and Vincent, 2013).

2.4. Enzyme assay

The enzyme amylase activity assay was carried out by the method of

Miller (1959). Dinitrosalicylate (DNSA) using 0.3% starch as the substrate. The amylase activity was determined using a standard graph prepared from maltose. The amylase activity defines by calculating the amount of enzyme required to liberate 1 mg of maltose/ml/min at 55 °C (Bhange et al., 2016; Blanco et al., 2016; Miller, 1959; Saxena et al., 2003; Shanmugavel et al., 2011).

Protease activity determined by the method of Anson (1938) with slight modification using casein as the substrate. The enzyme powder was dissolved in different concentration in 20 mM borate buffer (pH 9.0) containing 2 mM $CaCl_2$. The protease activity amount defines the amount of enzyme required to produce 1 mg of tyrosine per ml of the enzyme in 30 min at 55 °C (Anson, 1938; Anuradha et al., 2014; Bhange et al., 2016; Lim et al., 2019; Sandhya et al., 2005; Shanmugavel et al., 2011; Qureshi et al., 2016).

2.5. Effect of different parameters on the enzyme production

Optimization of various parameters such as incubation period (1–7 days), moisture content (50%–90%), pH (8.5, 9.5, 10 and 10.5) and temperature (25 °C, 28 °C, 30 °C and 35 °C) was done to attain maximum enzyme production. The extracted enzyme assayed with these parameters and the optimal condition for maximum production and calculated (Sandhya et al., 2005; Suganthi et al., 2011).

2.6. Statistical analysis

Each different parameters studies were performed in triplicates and repeated thrice. The samples collected from each replicate were tested for amylase and protease production. Means of these enzymes production were calculated, and significant differences were calculated by determining standard error.

2.7. Application of crude enzyme

The New Zealand sheepskins were washed and cut into small pieces (10 × 20 cm). The dehairing was done by dipping the skin into the enzyme solution (Madhavi et al., 2011; Mehtani et al., 2013; Mortuza et al., 2017). Dehairing trials were conducted by following sulfide free method (using enzyme alone). The experiment conducted in 1:2 (Enzyme: skin) for volume by weight. The surfaces, after application were kept overnight at room temperature and assessed for dehairing. The hair was removed using a blunt knife (Khandelwal et al., 2015). The efficacy of the dehairing enzyme studied by comparing the dehaired skin with control (Dayanandan et al., 2003; Verma et al., 2011).

2.8. Recovery of lanolin

2.8.1. Extraction method

The dehaired wool was washed and dried for further use. The extraction was carried out by the traditional method. Lanolin has extracted by using Soxhlet apparatus using solvents in the ratio of (1:9) hexane and dimethyl ether, for 4 h, at 40 °C (Jones, 1996; Lopez-Mesas et al., 2005; Zhou, 2012). The lanolin compound was confirmed by using confirmatory analysis. The treated and untreated wool were further characterized using Scanning Electron Microscope (SEM), Fourier-transform infrared spectroscopy (FTIR), Differential scanning calorimetry (DSC) and Thermal gravimetric analysis or Thermogravimetric analysis (TGA).

2.8.2. Characterization studies

The characterization of the wool samples and lanolin have characterized by SEM, (TESCAN VEGA-3 SBU) (Senthilvelan et al., 2012; Shanmugavel et al., 2018; Sundararajan et al., 2011) FT-IR (JASCO FT-IR 4700) (Shanmugavel et al., 2018), DSC (TA Instruments, model Q200) (Marti et al., 2007) and TGA (SDT Q600, TA Instruments, New Castle, DE, USA) (Marti et al., 2007) performed.

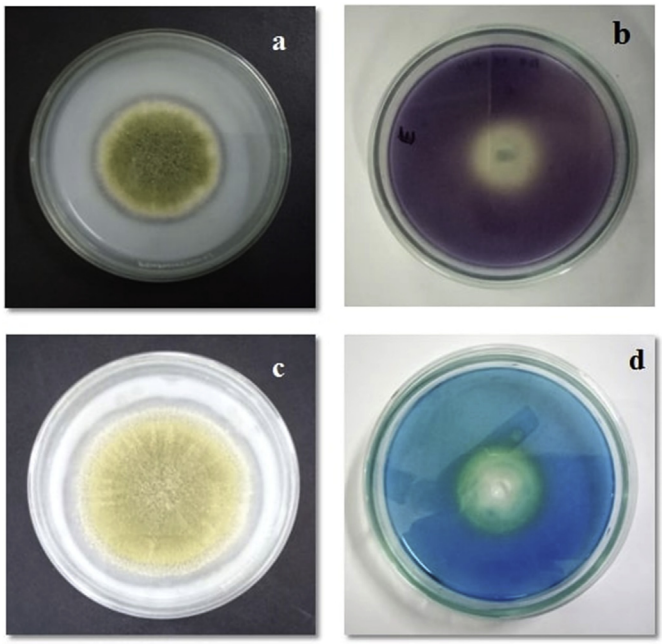

Fig. 3. Starch agar plate amylase (a. organism & b. enzyme), Skimmed milk agar plate protease (c. organism & d. enzyme).

3. Results and discussions

3.1. Plate assay

Fig. 3a, and Fig. 3c shows that organism *A. flavus* were producing enzyme while growing by hydrolyzing the substrate. This result confirms the production of both amylase and protease. The zone of clearance was observed both in starch agar plate (Fig. 3b) as well as in skimmed milk agar plates (Fig. 3d) which indicates the production of amylase (Suganthi et al., 2011) and protease production (Kranthi et al., 2012; Malathi and Chakraborty, 1991; Muthulakshmi et al., 2011; Vijayaraghavan and Vincent, 2013).

3.2. Effect of different parameters on enzyme production

The optimization of various parameters for the multienzyme (amylase and protease) performed, and graphical representation of their activity was plotted respectively and shown in Fig. 4a, b, c & d. The highest activity of 62 U/mL and 36 U/mL observed at the period of 96days (4days) whereas 3rd, 5th, 6th and 7th day (Fig. 4a) show less amount of activity of enzyme while comparing to the 4th day. The moisture content during fermentation was maintained, the maximum activity at 70% results for both enzyme as 49 U/mL and 36 U/mL, respectively (Fig. 2b). The pH plays a vital role in the production process 50 U/mL and 39 U/mL at pH 9.5, where other shows less activity while comparing this (Fig. 2c). The temperature at 28 °C showed maximum enzyme production (59 U/mL and 37 U/mL respectively) (Fig. 2d). The optimal condition for multienzyme production was optimized at 4th day with 70% moisture at pH 9.5 and 28 °C. The similar type of work was carried out by Oyeleke et al. (2010) and Kranthi et al. (2012) for protease and Shanmugavel et al. (2018) for pectinase production.

3.3. Enzymatic dehairing of skin

Enzymatic dehairing was carried out with crude enzyme. If the crude enzyme containing collagenase activity, then it attacks the collagen of the grain layer leading to damage of grain structure and its destruction, which has a significant impact on the final quality of the leather (Choudhary et al., 2004; Khandelwal et al., 2015) but it doesn't

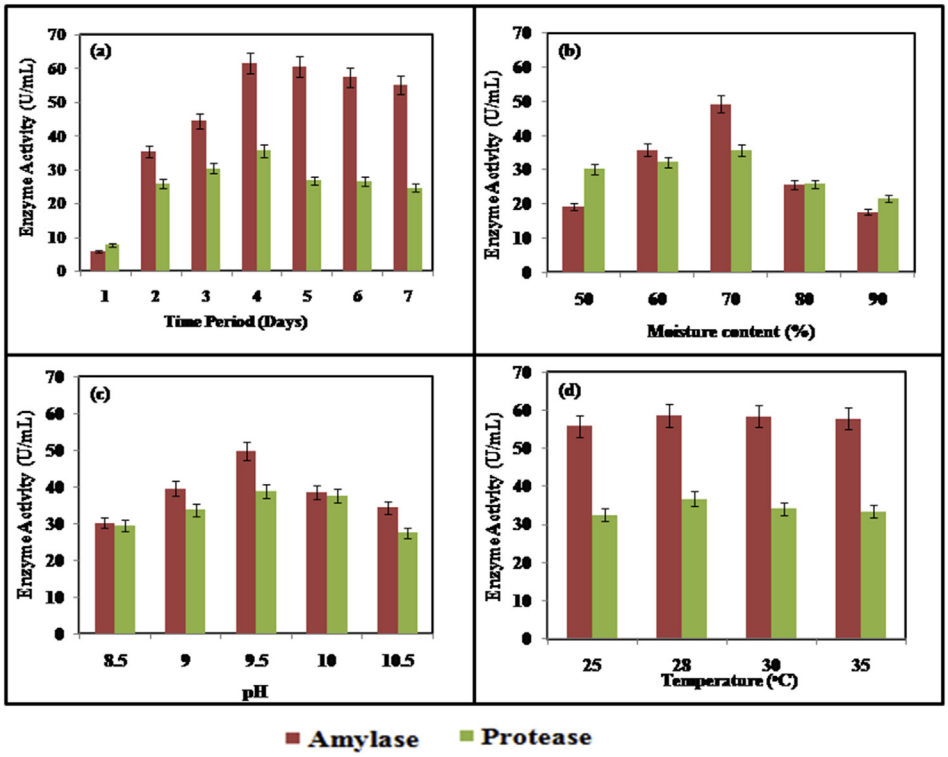

Fig. 4. Effect of (a) Incubation period, (b) Moisture content, (c) pH and (d) Temperature for enzyme production.

have collagenase.

Enzymatic dehairing based on the epidermis basal layer in which the hair is bound with proteins. The hair root is weekend by hydrolysis of protein by protease, and the hairs have removed along with root (Fig. 5c). The enzyme soaked sheep skins were showed excellent dehairing effect after overnight incubation. It can be seen from all the experimental results (Fig. 5a and b) that the enzymatic dehairing of skins a potential for reduction of pollution considerably and the hair can also be saved without any degradation (Arunachalam and Saritha, 2009) and used for further process. After dehairing the wool further processed for extraction of lanolin.

3.4. Recovery of lanolin

3.4.1. Lanolin extraction and confirmation

The lanolin has extracted by treating it with solvents, after 4 h of extraction process the greasy yellow colored waxy substance collected of about 10% from the wool sample. The extracted compounds were taken and dried and used for the analysis (Jones, 1996; Lopez-Mesas et al., 2005; Zhou, 2012). 1 g of extracted wax mixed with chloroform (10 ml), acetic anhydride (2 ml) and few drops of sulphuric acid added. The dark green color indicates the presence of lanolin (Fig. 7a).

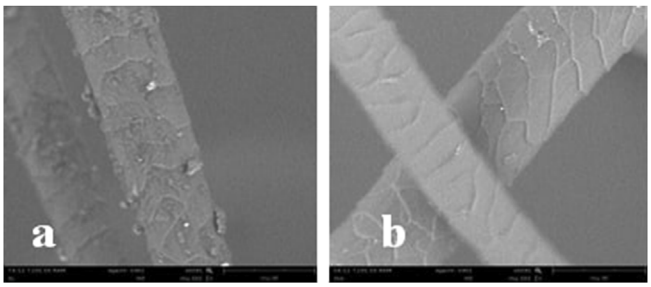

Fig. 6. SEM image of Zealand Sheep wool (a) Before and (b) After extraction of lanolin.

3.4.2. Characterization studies

The surface morphological change of wool untreated and treated, as shown in the SEM image Fig. 6 (a & b). The untreated wool has rough surfaces and ridges (Wang et al., 2006). Whereas the treated wool after extraction of lanolin, the surface becomes smoother, which indicates the lanolin extracted from the wool and this compared with the untreated wool sample which has rough surface morphology. This conforms the complete recovery of lanolin (Senthilvelan et al., 2012;

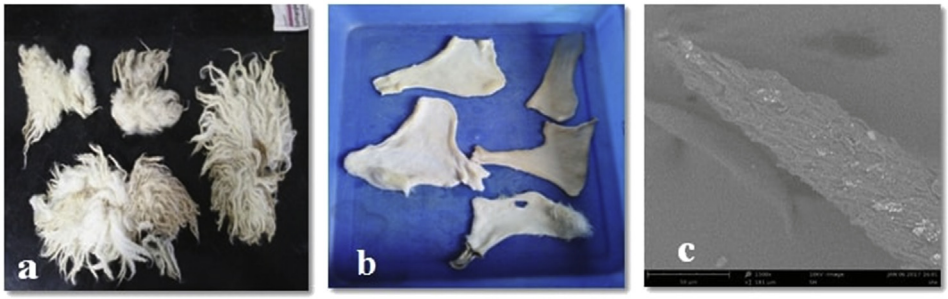

Fig. 5. (a) New Zealand Sheep skin, (b) Enzymatic dehaired skin and (c) Enzymatic dehaired wool along with root hair.

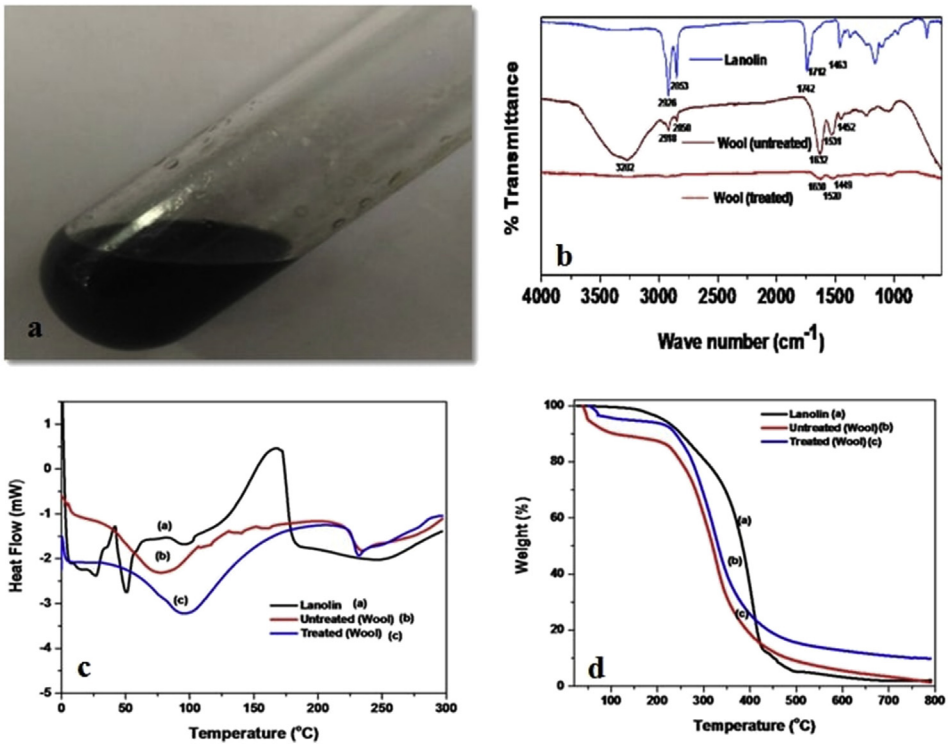

Fig. 7. Characterization studies of lanolin (a) Chemical confirmatory test, (b) FTIR, (c) DSC and (d) TGA.

Shanmugavel et al., 2018). Presence of functional group in the compound was analyzed using FT-IR spectrum (Fig. 7b). The peaks observed in these results were similar to the FT-IR of Sagiri et al. (2013) study. The lanolin shows the peak at 1712 cm^{-1} which corresponds to C═O stretching, moreover that incorporated wool (untreated) shows the carbonyl stretching at 1632 cm^{-1} finally the treated sample showed in the region of 1630 cm^{-1} due to less carbonyl stretching (Sagiri et al., 2013). DSC shows the phase changing the (thermal) property of the lanolin, wool untreated, and treated in Fig. 7c (Marti et al., 2007) the graph shows exothermic and endothermic curve, the result obtained mainly as a melting curve which in turns gives or identifies the thermal stability. The untreated and treated wool are maximum temperatures correlated, and the lanolin has different phase change. TGA analysis gives the material change as a function to temperature or time. From the graph Fig. 7d, the untreated and treated wool have single step degradation whereas the lanolin curve undergoes multi-step degradation which appeared in the thermogram. Most of the material starts to degrade from 200 °C to 400 °C, and weight loss occurs; this observation was similar to previously reported results (Marti et al., 2007).

4. Conclusion

From the above experimental study, the multi-enzyme has high effective on dehairing of sheep wool and can use as an alternative for the chemical method of dehairing of skins. From the dehaired wool, the lanolin has extracted, and the experimental data shows the excellent support of the lanolin compound.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101255.

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