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Dehairing of animal hides and skins by alkaline proteases of *Aspergillus oryzae* for efficient processing to leather products in Tanzania

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The leather industry is one among the most vital sectors for economic development contributing to high earnings. However, tanning of hides and skins is constrained by primitive technology and the use of hazardous chemicals that contribute to environmental pollution. This study was conducted to evaluate the efficacy of alkaline protease on dehairing animal hides and skins under different conditions. Alkaline protease was extracted from *Aspergillus oryzae* MG429773 cultured by solid state fermentation with a medium containing rice husks supplemented with 1% mineral solution incubated at 30 to 35°C for 7 days at pHs ranging from 7.5 to 9.5. The filtrate was used as a crude enzyme solution and also partially purified for a dehairing bioassay. Four pieces of cattle hide and goat skin (10 cm × 10 cm) were soaked or sprayed with enzyme solutions for a maximum of 5 days. Percentages of depilation of the hides and skins were recorded at 6 and 12 h intervals. Enzyme produced by *A. oryzae* showed the highest dehairing activity from 15 to 20% concentration of partially purified enzyme whereby 100% of goat and cattle hairs were removed within 24 and 48 h, respectively. For 5 and 10% enzyme concentration, 93.75 and 68.75% of hide and skin depilated in 72 h, whereas for the control 0.0 to 6.5% depilation was achieved in 72 h in hide and skins, respectively. Application of the *A. oryzae* protease by spray method was superior over a dipping method that released hair in effluent and caused skin decay after 5 days of exposure. This study indicates that enzyme extracted from cheaply available resources is efficient for dehairing by a spray method could be a potential technology for application by local tanners to improve the leather industry in Tanzania.

Key words: Alkaline protease, enzymatic dehairing, hides and skins, leather industry, fungal biotechnology.

INTRODUCTION

The leather industry is an important segment of the evolving industry in developing countries including Tanzania. The leather industry contributes to growth of the domestic production and adds to foreign currency reserves (China and Ndaro, 2015; Humphrey, 2003). Several countries including China, India and Ethiopia

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improve their economies through export of leather products such as coaches, clothes and shoes although the later faced a number of challenges (Mahmud, 2000 (Fan and Scott, 2003; Amiti and Freund, 2010). Tanzania is endowed with large number of cattle and goats, but animal by-products are underutilized for economic purposes. Cattle hides, sheep and goat skins are poorly handled and exploited due to the low level of processing technologies (China and Ndaro, 2015; Eifert et al., 2005; Murphy, 2002; Mahmud, 2000) including application of hazardous chemical such as sodium sulphide and chromium that are toxic and cause pollution of water bodies and are hazardous for all kinds of organisms. All these processes release waste and render leather as one of the most polluting industries due to high amount of chemical and solid wastes especially hair and chrome released into environment (China and Ndaro, 2015; Jian et al., 2011; Ozgunay et al., 2007). Leather processing involves multiple processes such as soaking, liming, deliming, tanning bating and degreasing and use of a considerable amount of chemicals and water to produce leather (Dixit et al., 2015; Thanikaivelan et al., 2004). Soaking hides or skin to soften and remove hair is the most important step in leather processing requiring proper performance to facilitate the proceeding steps (Thanikaivelan et al., 2004). Such processes are dehairing, liming, with use of alkaline sodium sulphide to complete hair removal which in turn accumulate toxic sulphide and chrome to environment (Sawant and Nagendran, 2014). Use of these hazardous chemicals releases toxic substance to environment. This has raised concern among environmental activists and has hindered growth of the tanning industry in Tanzania (Kumar et al., 2008; Perkins, 1983). Enzymatic processing of leather is gaining popularity worldwide due to safety and quality of leather products (Fathima et al., 2010; Thanikaivelan et al., 2004). Enzyme biotechnology is an environmentally friendly alternative for some processes such as deliming, bating and degreasing and enzyme treatment can be used for removal of unwanted protein materials with the exception of the pickling process which is an acidic treatment of the hides/skins to increase shelf life of leather (Saran et al., 2013; Thanikaivelan et al., 2002). Tanning is the process using complex chemicals such as chromium VI (CrVI) to change the skins and hides into colourful and flexible materials. Biotechnological tanning reduces the effects of chromium in effluents (Jenitta et al., 2013; Suresh et al., 2001). Neutralization, dyeing and degreasing are the final processes to improve the structure, texture and appearance of leather. But the common dye used is an azo dye compound that persists in sewage and might harm the environment. The application of alternative technologies such as enzymic dehairing would minimize application of toxic chemicals and their effects on humans and other organisms. Microbial processing is a good alternative to simplify leather processing by reducing processing steps into a few important ones and making the products more safe, attractive and durable (Dayanandan et al., 2003). Several proteases including keratinase from filamentous fungi have been reported as effective in leather for soaking, dehairing and degreasing (Choudhary et al., 2004; Gupta and Ramnani 2006). *Aspergillus* species is one of the most studied genera for production of industrial enzymes (Chellapandi, 2010) whereby *Aspergillus oryzae* has been reported as effective fungus for removal of chromium from tanning effluent (Nouri et al., 2005). In this study, local isolates of *A. oryzae* (MG429773) from previous study (Zekeya et al., 2019) were used for production of alkaline protease using rice husks as medium. Use of rice husk supplemented with nitrogen source (Pedri et al., 2015) is reported to add value to over 4.1 million tons of rice husks produced annually in Tanzania with scarce utilization (Said et al., 2014). Furthermore, it replaces use of food stuff like sugar cane, corn wheat in solid state fermentation, which is threatening food security (Pensupa et al., 2013). Then crude enzyme was evaluated for its efficacy for dehairing of cattle hides and goat skins. The overall aim is to reduce economic and environmental costs encountered with conventional tanning in Tanzania.

MATERIALS AND METHODS

The fungus was collected from Nelson Mandela African Institution of Science and Technology laboratory. Rice husks were supplied from Moshi rice milling and the brown sugar (sucrose) purchased at local shops in Arusha. The mineral solution included sodium chloride (NaCl), ammonium sulphate ($(NH_4)_2SO_4$ and manganese (II) sulphate ($MnSO_4.H_2O$) and agar supplied by Sigma Aldrich, Chemie Gmbh, Germany whereas Ammonium sulphate ($(NH_4)_2SO_4$) from LOBA and Trichloroacetic acid AR (TCCA) from LOBA Chemie Pvt, Mumbai, India. Yeast extract from SIGMA ALDRICH, Louis, USA and Tris Hydrochloric acid ($C_4H_{11}NO_3.HCl$) and Casein was supplied by Duchefa Biochemie, Haarlem, The Netherlands. Growth media, potato dextrose agar (PDA), potato dextrose broth (PDB), tween 80 and tween 20 were supplied by HiMEDIA Laboratories Pvt, Mumbai India and sunflower oil from Singida Sunflower (local).

Collection and isolation of fungal isolate

Two fungal isolates were supplied by Nelson Mandela African Institution of Science and Technology and sub-cultured in-potato dextrose broth (PDB) followed by serial dilution in a 96 well microplate preloaded with antibiotic (Ciprofloxacin) to prevent bacterial growth in the well. When a pure colony grew in a well it was isolated using a needle and incubated on potato dextrose agar (PDA).

Enzyme production and fermentation conditions

Enzymes from *A. oryzae*; MG429773 and MG429774 were produced in solid state fermentation on 10 g of broken rice husk mixed with 1% w/v of casein, 5% sugar, 1% yeast extract all together moistened with 20 mL of mineral salt solution (0.15%) containing (0.05 g NH_4NO_3, 0.025 g NaCl, 0.05 g $(NH_4)_2SO_4$) dissolved in 1000 ml of distilled water at a pH adjusted to 8.5. The

mixture was sterilized by autoclaving at 121°C for 15 min and inoculated with 1 mL of 1×10^7 conidia accessed by a haemocytometer and incubated at 35°C for 7 days.

Protease hydrolysis assay

The proteolytic activity of the crude enzyme solution was determined by using casein as a substrate. 450 mL of 1% (w/v) of casein was mixed in 50 mL of Tris-HCl buffer at 8.5 pH followed by addition of 50 ml of the crude enzyme extract and incubated in water bath at 40°C for 20 min. After 20 min, enzyme reaction was determined by addition of 500 ml of Trichloroacetic acid and incubated at 35°C for 12 h. After 12 h, enzyme activity was visualized as clear zones around the petri dishes due to hydrolysis of media (PDA containing casein) in the after addition of indicator solution.

Enzyme isolation

Ten milliliters of 0.1% triton X-100 was added to 2 g of fermented rice husk mixed and homogenized by using magnetic stirrer 100 rpm for 1 h. Solids were removed by using Whatman no. 1 filter paper followed by centrifugation of solution at 8000 rpm for 15 min. The resulting supernatant was used as the crude enzyme for hair removal bioassay.

Partial purification of enzyme

The most effective enzyme solution from isolate *A. oryzae*, MG429773 from the cell free supernatant portion of the culture was precipitated by ammonium sulphate to a saturation point of 60 to 80%. The salt was added slowly in small quantities under constant stirring by a magnetic stirrer to equilibrate the salt for maintaining equal concentration throughout the culture filtrate. After 1 h, the solids were collected by centrifugation at 10,000 g for 15 min at 4°C and the solids of different fractions was suspended (re-dissolved) separately in Tris-HCl buffer (pH 8.5). The re-suspended pellets were dialyzed against Tris-HCl buffer (pH-8.5) by placing them into a visking dialysis membrane. Salt was removed by dialysis with constant stirring for 12 h and periodic changeover of buffer 7 times. The dialyzed fraction was used as the enzyme for bioassay and samples with high activity were lyophilized and stored as solid pellets at -4°C in freezer.

Skin and hide dehairing bioassay

The dehairing bioassay was conducted on pieces of cattle hide with an area of 10 cm × 10 cm with a weight of 13.81±0.64 g and 0.41±02 cm thick whereas goat skin measured (10 cm ×10 cm) area with weight of 4.82±0.22 g and 0.21±0.02 cm thick. Hide and skin samples were separately placed into beakers and soaked in distilled water overnight and left to dry at room temperature (29.5±2.4°C) for 2 h and then soaked into 2, 5, 10, 15 and 20% of crude enzyme solutions of *A. oryzae* MG429773 with a control of 0.1% triton X-100. The fungal isolate was grown in larger volumes testing the effects of different parameters including substrate (rice, wheat, maize grain), nitrogen source, carbon source, pH effect, surfactant (triton x-100, tween 80, tween 20 and vegetable oil), temperature and partially purified.

To evaluate the effect of dipping against spray method on dehairing activity 2, 5, 10, 15, and 20% of partial purified enzyme and control (0.1% triton X-100) was applied. Degree of hair removal was calculated by subtracting dehaired portion over total skin/hide portion. The experiment was replicated four times and data was recorded after every 12 h.

Data analysis

Data on enzymic dehairing of cattle hide and goat skin was determined by assessing effect of fungal isolates in different conditions. Substrate, carbon source, nitrogen source, temperature, pH and surfactant were the factors for analysis of degree of hair removal. In each treatment, pieces of hides and skins were subdivided into ten portions each measuring 1 cm. Portion of hair removed over total area of skin exposed to treatment times 100% was calculated. Scores were graded as: 0=skin or hide portions without depilation, 1-3/10=25% portions of skin or hide depilated (partial dehairing), 5-6/10=50% portions of skin or hide depilated (moderate dehairing), 7-9/10=75% portions of skin or hide depilated (incomplete dehairing), and 10/10 portions of skin or hide depilated=100% (complete dehairing).

RESULTS

The results obtained showed that dehairing activity of enzyme was time and concentration dependent. 15% of crude enzyme exhibited high activity of depilation compared to 10%, 5%, and for the control no hair was removed after 48 h (Figure 1). Dehairing activity was fast on goat skin compared to cattle hide after 48 h (Figure 2).

The effect of substrate and carbon source on enzyme activity was different between treatment where rice husks showed high activity of up 100% dehairing compared to cotton seeds, wheat grain and PDA where the last showed only 56.25% dehairing after 48 h (Figure 3).

The effect of the nitrogen source on enzyme activity varied between substrates with the use of collagen resulting in the highest enzymic dehairing activity compared to casein, yeast extract, ammonium nitrate and ammonium sulphate (Figure 4).

pH also affected enzyme activity were alkaline pH in the range of 8.5 to 9.5 favoured enzyme activity, and low enzyme activity was observed in acidic and neutral conditions (Figure 5). The effect of temperature on enzyme production was high at temperature range of 35 to 40°C whereas enzyme activity slowed down at temperature below 30°C and above 40°C (Figure 6). Results also showed that surfactant application enhanced enzyme activity in which triton x-100 showed high enzymic dehairing than tween 80, tween 20 and sunflower oil (Figure 7).

The dehairing method for enzymic depilation of cattle hide and goat skin was more effective using the spray method which induced dehairing within a short time as compared with the dipping method depending on concentration of enzyme and time (Figure 8). An increase in incubation time improved the dehairing activity and a high percentage of hair removal was observed after exposure for 48 h indicating that enzyme activity was time dependent (Figure 9). Enzymic dehairing also varied for type of animal hide or skin and the method used. For goat skin depilation was faster than for cattle hide and the spray

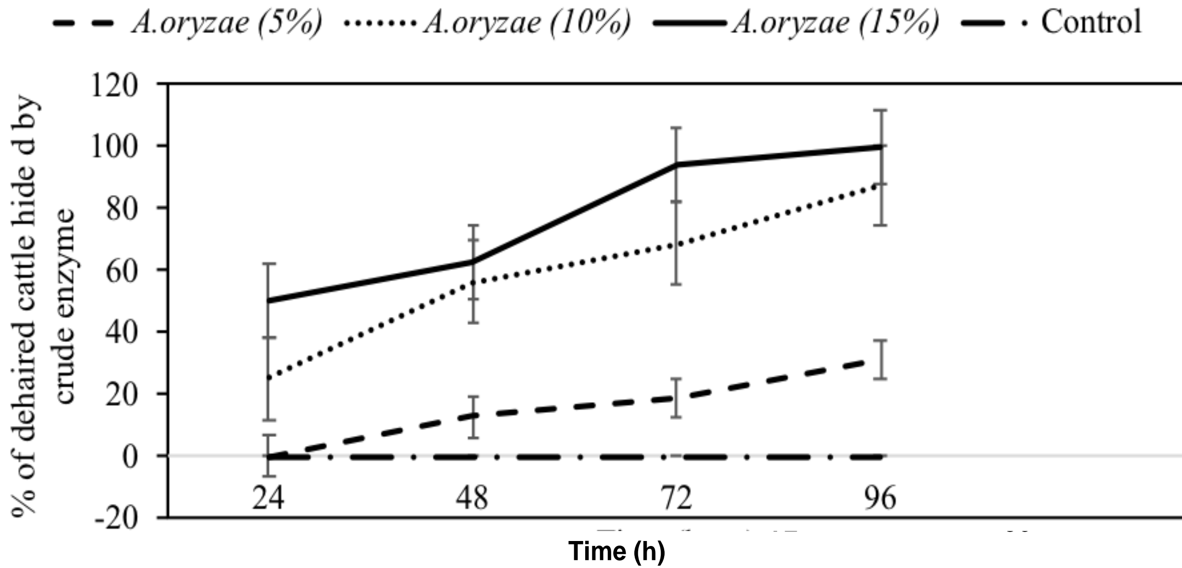

Figure 1. Percentage of dehaired cattle hide after exposure to various concentrations of crude protease at maximum of 96 h.

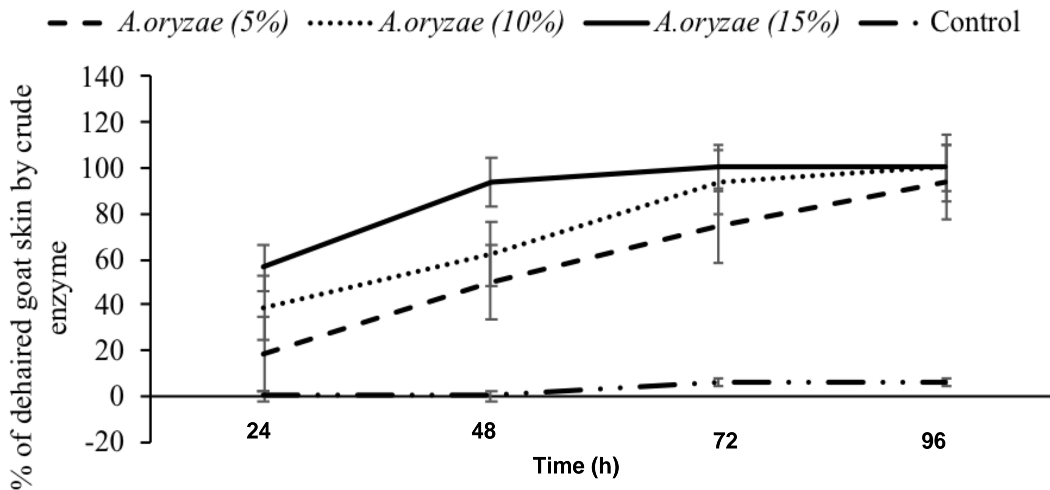

Figure 2. Percentage of dehaired goat skin after exposure to various concentrations of crude protease at maximum of 96 h.

method produced good quality of skins and hides (Figure 10).

DISCUSSION

Enzymic dehairing of hides and skins have been reported as effective and safe for both human and environment (Choudhary et al., 2004). Recently isolated fungus from Tanzania was revealed to depilate goat and cattle hair within 48 h with quick activity in goat skin compared to cattle hide. The enzyme activity was time and dose dependent in which more activity using higher enzyme concentration that lower doses even when exposed for a long time for cattle hide and less significant for the goat skin.

The fermentation conditions are essential to obtain high enzyme activity. Various conditions were investigated for their influence on enzyme activity. The solid substrate is a key factor for enzyme production and maximum enzyme activity was obtained for rice husk as compared to wheat grain, cotton seed flower and PDA indicating that rice was superior source of carbon for fermentation of *A. oryzae* enzyme, cheap and easy in downstream

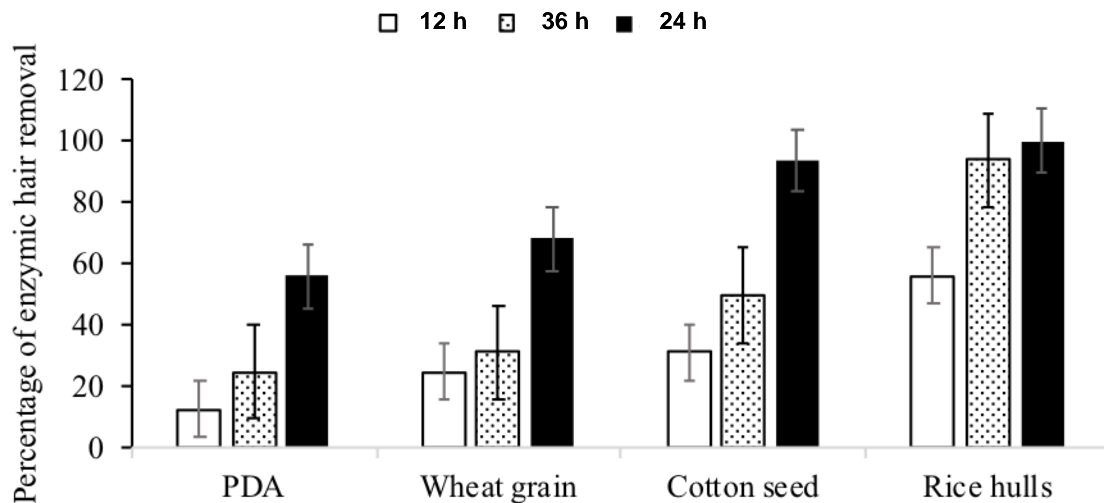

Figure 3. Effect of carbon source on dehairing hide by crude alkaline protease over time.

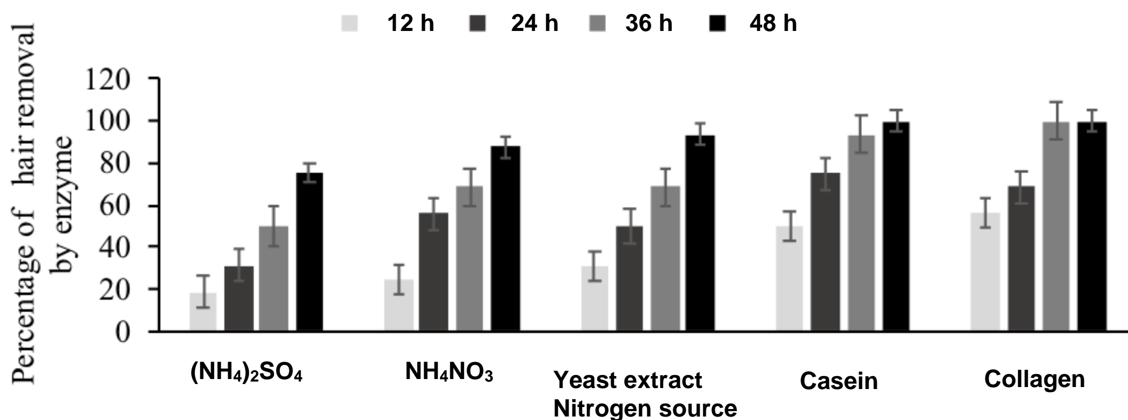

Figure 4. Effect of Nitrogen source on dehairing hide by crude alkaline protease over time.

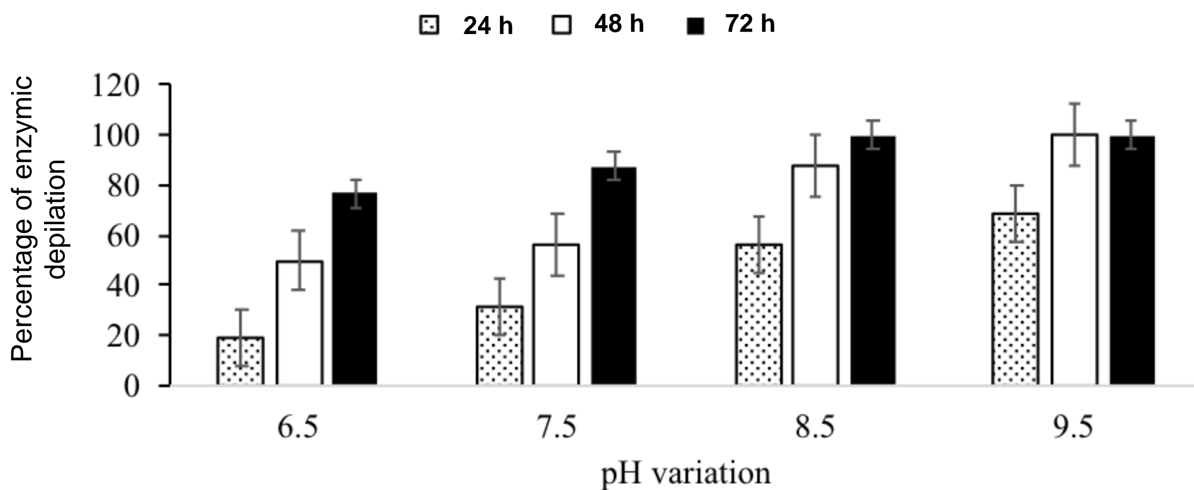

Figure 5. Effect of pH on dehairing of cattle hide by crude alkaline protease of *A. oryzae.*

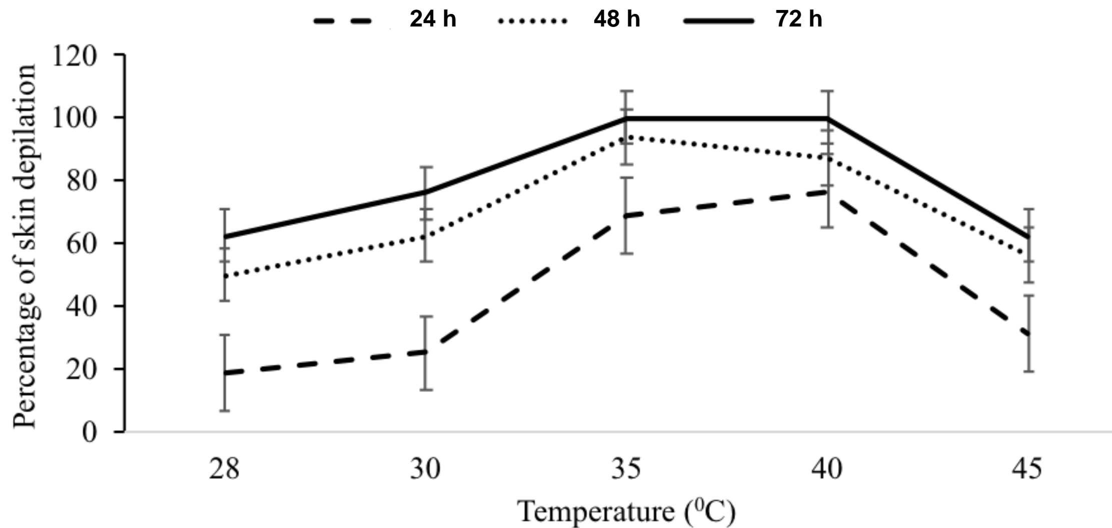

Figure 6. Effect of temperature on alkaline protease in removal of hide and skin hair.

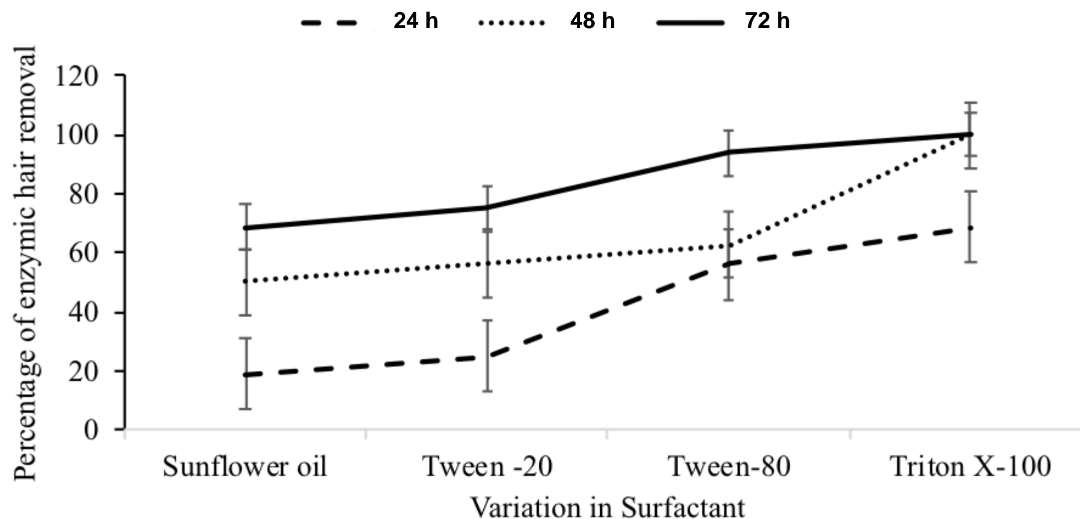

Figure 7. Effect of surfactant on alkaline protease activity in dehairing cattle hide over time.

process compared to special media including PDA (Sandhya et al., 2005). Good performance of rice husks entails value addition to this abundantly available agricultural waste to replace use of cotton seed and wheat grain, which are food, thereby ensuring food security while protecting the environment.

Nitrogen is another important fermentation medium ingredient and the best source of nitrogen was casein yielding a high amount of enzyme compared to ammonium sulphate, ammonium nitrate and yeast extract when used solely. Earlier studies reported that organic sources of nitrogen greatly improve the production of enzymes (Dong et al., 2005). This might be the reason for good performance observed with collagen and casein. Temperature influences enzyme activity and also fungal enzymes have minimum, maximum and optimal temperature for specific activity. This study showed that the optimum temperature for production of alkaline protease from *A. oryzae* is 35°C grown for 5 days under static conditions in an incubator followed by 3 days on an incubator shaker. At temperatures below 30°C and above 40°C, enzyme activity declined, and more incubation time was required to reach optimal activity of the enzyme due to sensitivity and specificity of microbes and enzyme on temperature.

It is well established that enzymes are most active in a pH range of 5 to 10, varying from one type to another. Protease enzymes are active at pH rage of 7.5 to 8.5. A

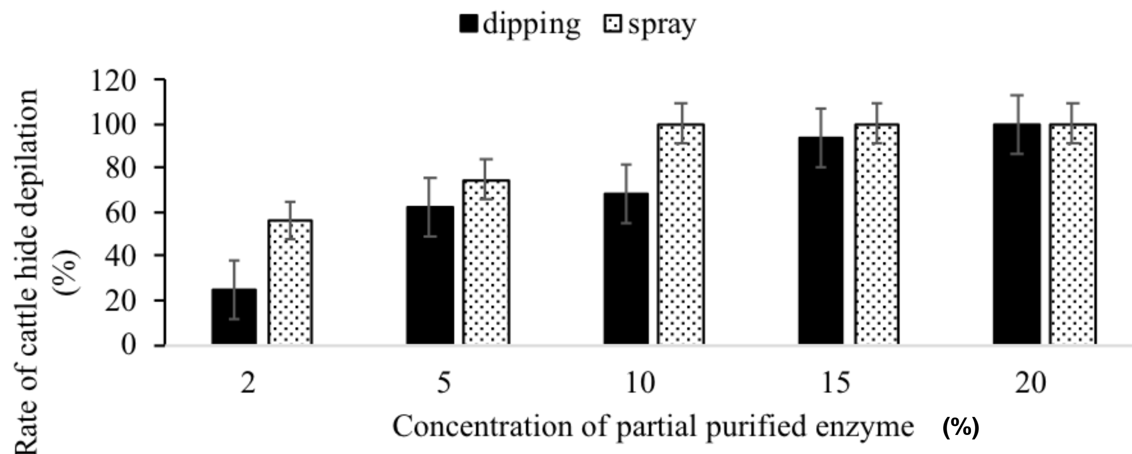

Figure 8. Dipping and spraying as application methods for enzymic dehairing of cattle hide. 24 h of exposure to partially purified *A.oryzae* enzyme at different concentrations.

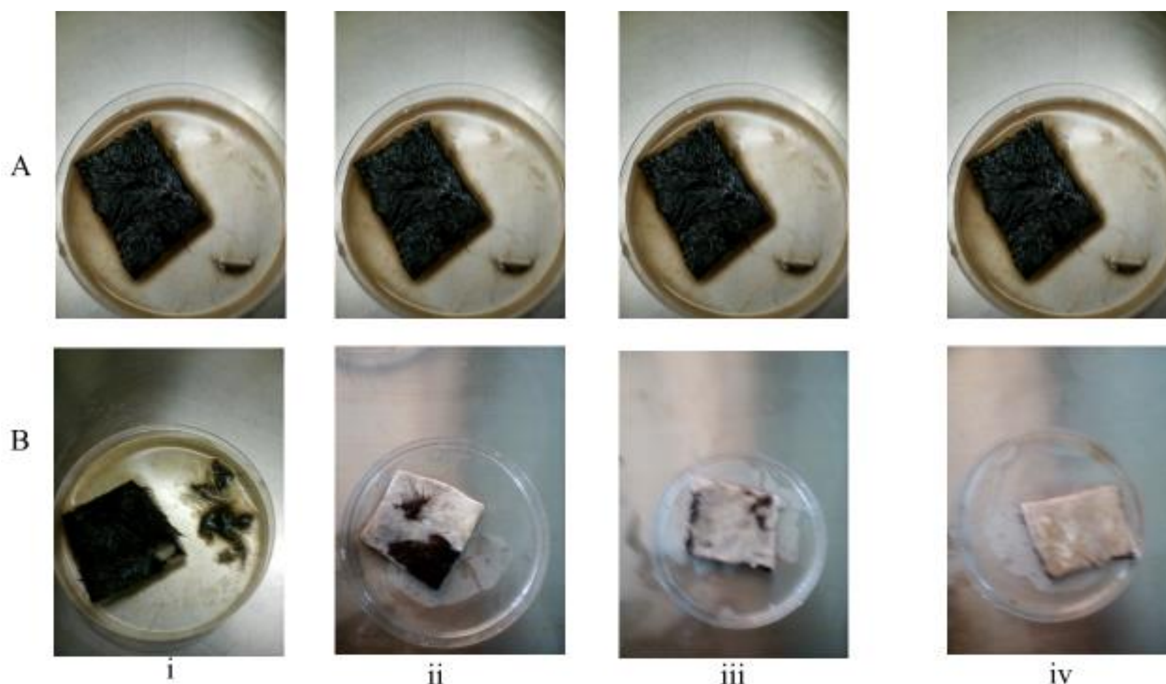

Figure 9. A picture showing pieces of hide after soaking for 3 h and air dry before dehairing activity (A), and variation in depilation activity from none, slight, moderate, partial to complete depilation after exposure to control and 10% of *A.oryzae* enzyme solution by dipping method into control for 48 h (Bi), *A.oryzae* after 12 h (Bii) *A.oryzae* after 24 h (Biii), and *A.oryzae* after 48 h (Biv), respectively.

change in pH can protonate or deprotonate a side group (carboxyl and amino termini), thereby changing its chemical features and conformation, resulting into loss of interaction with an adjacent subunit, hence decrease in substrate affinity. Alteration of pH can further lead to protein un-folding, thereby completely deactivating the enzyme. Higher protease activity was observed at alkaline pH at the range of 8.5 to 9.5 than in neutral and acidic pH. This shows that alkalinity favor the dehairing activity of *A. oryzae* protease similar to in chemical dehairing where lime and sodium sulphide exhibit dehairing activity at alkaline pH above 8.5.

Surfactants are important for emulsification and of hydrophobic molecules in water and play role as

Figure 10. Difference in application of dehairing method on quality of hide and skin.

dispersant and wetting agent. Triton X-100 was the best surfactant in this study. It enhanced extraction and emulsification of enzyme during incubation and in water solution (Rastogi et al., 2008). Triton X-100 has advantage over tween 80 and tween 20 as it is heat tolerant and tolerant on wide range of parameter when included in culture media (Chen et al., 2005).

Enzymic dehairing was effective and faster for goat skin compared to cattle hide probably due to its thicker layer with more proteinous material than for goat skin. The pieces with similar area had different weight and volume as described earlier. The spray method exhibited higher dehairing activity for goat skin compared to dipping method especially in cattle hide. The thickness of hide prevented quick penetration of enzyme into animal hide. The skin depilation by spray method was the best as no hairs remained in solution whereas in dipping method high amount of hair was retained in solution which might lead to environmental pollution. This study suggests that, enzymic dehairing by spray method could save dual purposes; efficient depilation and dry/firm hair could be processed for making other products such as painting brushes than being waste in dipping method.

Conclusion

Alkaline protease produced by *A. oryzae* (MG429773) was effective in dehairing goat skin and cattle hides when rice husk was used as the solid substrate and source of carbon and casein as source of nitrogen at temperature range of 35 to 40°C and pH range of 8.5 to 9.5. In addition, 0.1% of triton X-100 was found to be good surfactant during enzyme extraction. This study found that an alkaline protease has potential activity for dehairing hides and skins. However, further experiments including purification and trials by local tanners should be conducted to evaluate cost and environmental benefits in comparison to conventional for validating its application in processing leather products in Tanzania.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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