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Cold active lipase from *Pseudomonas* sp. VITCLP4 as degreasing agent in leather processing

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The potential of cold active lipase from *Pseudomonas* sp. VITCLP4 in degreasing of greasy skin has been investigated. Degreasing efficacy has been studied at four different stages (liming, deliming, pickling and depickling) of the pre-tanning process. Being alkaline, expectedly the enzyme at 10% concentration on pelt weight performed well at deliming stage, resulting in acceptable residual fat content of 3.5%. The more desired residual fat and free fatty acid content (FFA) of less than 2% and 0.5% has been achieved when the enzyme (10%) is used along with a commercial degreasing formulation (0.5%) at depickling stage, cutting short the concentration of degreasing agent from 3% to 0.5%. Scanning electron microscopic (SEM) studies and physical properties testing reveal that the enzyme treatment imparts desirable properties to finished leather. The results indicate that the enzyme alone or along with a chemical degreasing agent can be used at deliming or depickling stage to produce leathers with acceptable or desirable fat content as per the need of finished leathers.

Keywords: Alkaline lipase, Degreasing, Pre-tanning, *Pseudomonas* sp, Leather processing

Lipases (EC 3.1.1.3: Triacyl glycerol acyl hydrolases) constitute the third most important category of enzymes, next to carbohydrases and proteases. They are unique in hydrolysing and synthesizing fatty acid esters in aqueous and non-aqueous medium. In industries, enzymes are steadily replacing chemical reactions since they are greener in approach. Enzymes produce fewer by-products, consume less energy, reduce environmental pollution and add improved value to the products. Consequently it is not surprising to notice the blooming global enzyme market despite economic slowdown. According to the market research report published by BCC research LLC in 2014, the global market for industrial enzymes is expected to reach $7.1 billion by 2018, registering a five-year compound annual growth rate (CAGR) of 8.2% (BBC Research 2014, in report BIO030H- Global markets for enzymes in industrial applications). The global market size of lipases in particular is projected to reach $590.5 million by 2020, at a CAGR of 6.5% between 2015 and 2020 (Research and Markets 2015, in report- Lipase market by source, application and geography- Global forecasts to 2020 for the $590.5 Million industry).

Among the end users of technical enzymes, leather industry is on top[1]. This is due to environmental pollution by the chemicals used and stringent enforcement of environmental regulations in many countries. Pre-tanning and tanning steps contribute to 80-90% of the total pollution[2]. With respect to biological oxygen demand (BOD), chemical oxygen demand (COD) and total dissolved solids (TDS), approximately 70% of the pollutants are generated from pre-tanning operations[3]. Dehairing and chrome-tanning steps results in the release of sulphide, chromium, chloride and sulfate ions in the effluent[4,5]. Degreasing leads to discharge of solvents and surfactants[6-8].

Despite the fact that leather industry contributes significantly to economical development and employment opportunities in many developing countries it has gained a negative image due to environmental pollution and associated health hazards. In the recent years, application of various enzymes like proteases, amylases and lipases in leather industry is steadily increasing to reduce the discharge of chemical pollutants into the environment. In leather processing, lipases are screened for replacing formulations containing solvents and emulsifiers in the removal of natural fat present in animal skins as separate degreasing step is required for pelts with high fat content. Fat is present in sebaceous gland, hair follicle, between collagen fibres and connective tissue fibres in the subcutaneous layer of the skin. The level of fat in skin varies with factors like breed, age, sex, etc. It is approximately 2-4% in cattle skin, 12-15% in goat skin and 30% in sheep skin and mainly comprise of 56% triglycerol, 23% glycerol, 6% phospholipid, 5% cholesterol and 10% fatty acids[6].

Insufficient removal of natural fat during processing, prevents the chemicals from penetrating into the leather which leads to negative impacts on the quality of finished leather such as hardness without sufficient

internal softness, fatty spew formation, stained appearance due to chrome soap formation, weak bonding of the finishing layer and bad odour. Traditionally excess fat was removed using solvents and emulsifiers but they add to pollution. Alternatively, lipases of microbial origin can be used in degreasing to reduce pollution. Research on the use of lipase for degreasing dated a few decades back. Use of acid lipase of fungal origin in degreasing on pickled leather was reported in 1978[9]. Acid lipase from *Rhizopusnodosus* along with commercial degreaser was used in degreasing in 1982[10]. Recently with the availability of commercial lipases, the effectiveness of acid and alkaline lipases of commercial origin in degreasing at various stages of leather processing is also reported[11].

In this study, the efficacy of cold active lipase from *Pseudomonas* sp. VITCLP4 in degreasing of sheep skin was explored. Lipolytic enzymes with high specific activity at 0–30°C are considered as cold adapted lipases[12]. They are present in psychrotrophic and psychrophilic microbes isolated from Antarctic and Polar regions[13,14]. One of the key features of these enzymes is consumption of less energy due to low working temperatures. But these enzymes from Antarctic and Polar regions are naturally not thermostable to withstand the temperatures of tropical and temperate climates to be used in industries. They can be used only after improving their thermostability by immobilization, directed evolution, protein engineering and chemical modifications[15-18]. Therefore cold active lipases obtained from microbes native of tropical region with good thermal stability are better choice for industrial applications. We have earlier reported the isolation of *Pseudomonas* sp. VITCLP4, a new strain producing cold active lipase from tropical part of India and its purification scheme[19,20].

Both alkaline and acid lipases can be used in degreasing. Alkaline lipases are generally used on limed and delimed pelts where *p*H is about 11-12 and 8.5-9 respectively. Acid lipases are employed on pickled and depickled pelt with the corresponding *p*H of 3-3.5 and 5. With the optimum *p*H of 8 and extended lipase activity upto *p*H 9, the ability of cold active lipase from *Pseudomonas* sp. VITCLP4 in degreasing was assessed at three stages of pre tanning namely, liming, deliming and depickling sequentially. Compatibility and efficiency of lipase with commercial degreasing agent on pickled pelts was also evaluated. This was mainly done to achieve the desired residual fat content of less than 2% and to find out the % reduction in the use of commercial degreasing agent.

Experimental Section

Chemicals

p-Nitrophenyl palmitate (*p*NPP) was obtained from Sigma chemicals. Culture media ingredients were purchased from Hi-Media, Mumbai. All other chemicals used were of analytical grade commercially available in India.

Organism

Pseudomonas sp. VITCLP4 was isolated from marine samples collected from Tamilnadu coast by enrichment in olive oil containing medium followed by screening for lipolytic activity on tributyrin agar and rhodamine-B-olive oil agar. The lipase activity was determined by spectrophotometric assay using *p*NPP as substrate[21]. *p*-nitrophenol (*p*NP) released was quantified at 410 nm. One unit of lipase activity was defined as the amount of enzyme required to release one µmol of *p*NP mL^{-1} min^{-1}. Being cold active, the extra cellular enzyme from *Pseudomonas* sp. VITCLP4 manifested optimum activity at 25°C and retained 80% of activity at 15°C and 20°C and 50% at 10°C. Optimum *p*H was found to be 8. The enzyme was stable at alkaline *p*H of 8-10 and in the temperature range of 30-40°C. It was stable in presence of high and non-polar solvents as well as anionic and non-ionic detergents.

Preparation of enzyme extract for degreasing

The production medium for lipase was slightly modified to reduce the incubation time and the medium contained peptone, 0.5% (w/v), yeast extract, 0.25% (w/v), sodium chloride, 0.05% (w/v), sodium hydrogen phosphate, 0.3% (w/v), disodium hydrogen phosphate, 0.4% (w/v) and olive oil, 1% (v/v)[22]. *p*H was adjusted to 7.2 using 0.1 N NaOH and HCl. The medium was dispensed in 30 mL aliquots in 100 mL conical flasks, autoclaved and inoculated with 10% (v/v) inoculum and incubated at 25°C in orbital shaker (180 rpm) for 4 days. Supernatant obtained by centrifugation at 8000 rpm for 15 min at 4°C was concentrated and partially purified by ammonium sulphate precipitation (40% saturation) to yield more stable fraction[20]. The enzyme extract was adjusted to contain 1000 units mL^{-1} min^{-1} and used in degreasing experiments.

Degreasing at liming, deliming and depickling stages

Wool sheep (South Indian breed) skin was obtained from slaughter house, preserved with salt and stored at 4°C briefly. The skin was cut into pieces measuring

5×5 cm each mostly from butt regions and used in experiments. The cured pieces of skin were soaked in water with surfactant for 9 hr to remove salt, blood, dirt and to regain moisture. In conventional leather processing, liming of the skin with lime and sodium sulphide remove most of the natural fat present in skin. In high greasy skins, the excess fat is removed at the end of pre tanning operations using solvents and surfactants. The potential of cold active lipase from *Pseudomonas* sp. VITCLP4 in degreasing was determined at three stages of pre-tanning viz, liming, deliming and depickling. At each step, the enzyme was used at the concentration of 2.5-15% on pelt weight basis and the skin pieces were treated as per standard process recipe. At the end of every step, the treated skins were subjected to analysis for residual fat, FFA and moisture by International Union of Leather Technologists and Chemists Societies (IULTCS) official analytical protocols.

Briefly, to evaluate the degreasing efficiency at liming stage, the soaked skins were treated with different concentrations of lipase (on wet weight of wool sheep skin basis) in stainless steel laboratory drums containing 5% lime and 3% sodium sulphide. The liming operation was carried out in experimental drums with intermittent rotating (at 5 rpm) and stopping for 24 hr (running time of 5 min h^{-1}) and then the skins were dehaired and defleshed. In the second step, the defleshed pelts were delimed and bated as per standard process recipe and washed. The degreasing effectiveness of lipase on delimed pelts was studied by treating the pelts with various concentrations of lipase on fleshed weight basis in the drums for 3 h. In case of high greasy skins, degreasing is more effective after pickling process since the acid treatment is believed to rupture the fat pockets to provide better access to fat for its removal. Therefore in the third step, the bated pelts were pickled, aged for 2 days and depickled to a *p*H of 5 as per standard process recipe. The depickled pelts were then treated with different concentrations of lipase on fleshed weight basis in the rotating drums for 3 h.

Degreasing on pickled pelt

Though, there are no standards for the maximum allowable limit for the residual fat or FFA to avoid spew problems or bad odour in the final leather, based on working experience with greasy skins, it is believed that the residual fat and FFA content after degreasing and before chrome tanning should be less than 2% and 0.5% respectively. The stepwise degreasing with cold active lipase from *Pseudomonas* sp. VITCLP4 (liming, deliming and depickling) results in residual fat and FFA content of 2.6% and 0.52% respectively, very close to the desired level. Therefore possibility of using lipase along with commercial degreasing agent in degreasing to achieve the desirable residual fat and FFA levels was explored. Lipase was used at fixed concentration of 10% on pelt weight basis. The concentration of commercial degreasing formulation from a reputed multinational leather chemical house was varied in the range of 0.5–1% in order to find out reduction in the level of degreasing agent achieved by the use of lipase. To carry out the experiment, the bated pelts were pickled, aged for 2 days and treated with 10% lipase alone, 10% lipase + different concentrations of degreasing formulation and 3% degreasing formulation alone on fleshed weight basis in the rotating drums for 3 h. All the degreased pelts were subjected to further processing to convert them into dyed leathers as per standard process recipe.

Histological examination

To support the findings of chemical analysis for fat content, the degreased pelts were subjected to histological staining. Lipase from *Pseudomonas* sp. VITCLP4 manifested the appropriate degreasing levels when used on pickled leather pelts. Consequently the pickled pelts were considered for histological studies. The pelts treated with 10% lipase, 10% lipase + 0.5% degreasing agent and 3% degreasing agent were taken for tissue sectioning. After degreasing experiment, the pelts were washed and frozen. Tissue sections of 25 µm thickness were prepared using cryomicrotome and stained with Sudan Black B[23] with slight modifications where 70% isopropanol was used as vehicle for dye and differentiating agent to avoid solvent based removal of fat. Sudan Black B is an azo dye which combines with acidic groups of lipid and fat and makes them appear blue–black[24]. After staining, the tissue sections were mounted with glycerine jelly to study fat content and distribution.

SEM studies

Leather pieces measuring 5×2 mm were sliced from control (lime + sulphide) and test (10% lipase + 0.5% degreasing agent). The cut surface was mounted on aluminium slab and coated with gold (Edwards E-306). The SEM micrographs were taken using Vega 3 Tescan scanning electron microscope operated at 10 kV.

Evaluation of physical properties of dyed leather

The degreased pelts treated with 3% degreasing agent and 10% lipase + 0.5% degreasing agent were

considered as control and test respectively in this study. The dyed leathers made from them were cut into pieces and conditioned at the temperature of 20°C and relative humidity of 65% for 48 h. The tensile strength, % elongation at break and tear strength were determined as per the methods described by International Union for Physical testing for Leather (IUP).

Experimental statistics

All the experiments were done thrice. The values in the tables and graphs represent the arithmetic mean and standard deviation. The standard error was within experimental limits. For all the experiments appropriate control was in place.

Results and Discussion

Degreasing at liming, deliming and depickling stages

The results of degreasing experiment done at liming, deliming and depickling stages using cold active lipase from *Pseudomonas* sp. VITCLP4 are presented in Table 1. The enzyme treatment resulted in 180, 380 and 54% fat removal at liming, deliming and depickling stages respectively. The enzyme appeared to work well at the optimum level of 10%. Maximum fat removal efficiency (50%) was achieved at depickling stage where the *p*H of the operation was about 5. The best performance of the enzyme at the *p*H of 5 which is well below the *p*H of maximum activity of the enzyme may be explained as due to the easy access of the fat by the enzyme since the fat pockets were ruptured by the action of sulphuric acid used in pickling. For 10% offer level of the enzyme used on depickled pelt, fat and FFA content retained was estimated to be 2.6 and 0.52% respectively. According to Afsar and Cetinkaya, presence of 2-4% residual natural fat after degreasing is acceptable[11]. Novozymes, the leading enzyme maker in the world also specified 2-4% residual fat for its degreasing agent Novo Cor ADL (www.novozymes.com 2009, in report-Case study on leather tanning enzymes).

Therefore the lipase from *Pseudomonas* sp. VITCLP4 with the residual fat content of only 2.6% could be successfully used in degreasing. However the enzyme slightly fell short of desirable fat and FFA content of less than 2% and 0.5%. Nevertheless the results are equitable with the report on acid and alkaline lipases of commercial origin in which the researchers obtained 3.48% fat retention[11].

Degreasing on pickled pelt

To accomplish the desired residual fat and FFA level, the lipase was used in conjunction with a commercial degreasing agent. The results are displayed in Table 2 and Fig. 1. Commercial degreasing agent when used at the concentration of 0.5% along with 10% lipase exhibited the desirable fat and FFA content of 1.75% and 0.38% respectively. Acid lipases are commonly employed for fat removal at pickled pelt stage. Acid stable alkaline lipases with commercial degreaser can also be used at this stage. The alkaline lipase from *Pseudomonas* sp. VITCLP4 with commercial degreasing agent exhibited 75% degreasing effectiveness which is comparable to the findings of an acid lipase with commercial degreaser where 78%

Table 1 — Degreasing efficiency of lipase at different stages of leather processing

Leather processing stage	Expt. No.	Experiment	Fat Content (%)	Free fatty acid content (%)	Relative fat content (%)	Efficiency of fat removal (%)
		Lime + Sulphide (Control)	5.6 ± 0.22	1.6 ± 0.12	100	0
Liming	1	Lime + Sulphide + 2.5% Lipase	5.1 ± 0.42	1.5 ± 0.21	91	9
	2	Lime + Sulphide + 5% Lipase	5.0 ± 0.24	1.45 ± 0.24	89	11
	3	Lime + Sulphide + 7.5% Lipase	4.9 ± 0.28	1.35 ± 0.15	87	13
	4	Lime + Sulphide + 10% Lipase	4.6 ± 0.18	1.32 ± 0.12	82	18
	5	Lime + Sulphide + 15% Lipase	4.6 ± 0.12	1.32 ± 0.12	82	18
Deliming	6	2.5% Lipase	4.5 ± 0.08	1.1 ± 0.18	80	20
	7	5.0% Lipase	4.1 ± 0.18	0.7 ± 0.08	73	27
	8	7.5% Lipase	3.8 ± 0.08	0.6 ± 0.12	67	33
	9	10% Lipase	3.5 ± 0.10	0.65 ± 0.22	62	38
	10	15% Lipase	3.5 ± 0.22	0.62 ± 0.24	62	38
Depickling	11	2.5% Lipase	3.4 ± 0.08	0.6 ± 0.18	60	40
	12	5.0% Lipase	3.3 ± 0.12	0.6 ± 0.18	58	42
	13	7.5% Lipase	3.1 ± 0.14	0.6 ± 0.12	55	45
	14	10% Lipase	2.8 ± 0.22	0.58 ± 0.16	50	50
	15	15% Lipase	2.6 ± 0.12	0.52 ± 0.24	46	54

Table 2 — Results of degreasing experiment on pickled pelt

Expt. No.	Experiment	Fat Content (%)	Free fatty acid content (%)	Relative fat content (%)	Efficiency of fat removal (%)
	Control	5.6 ± 0.22	1.6 ± 0.12	100	0
1	10% Lipase+0.5% Degreasing agent	1.75 ± 0.16	0.38 ± 0.12	31	69
2	10% Lipase+0.75% Degreasing agent	1.42 ± 0.12	0.32 ± 0.16	25	75
3	10% Lipase+1% Degreasing agent	1.40 ± 0.24	0.34 ± 0.12	25	75
4	3% Degreasing agent	0.8 ± 0.22	0.28 ± 0.10	14	86
5	10% Lipase	3.0 ± 0.28	0.62 ± 0.24	53	47

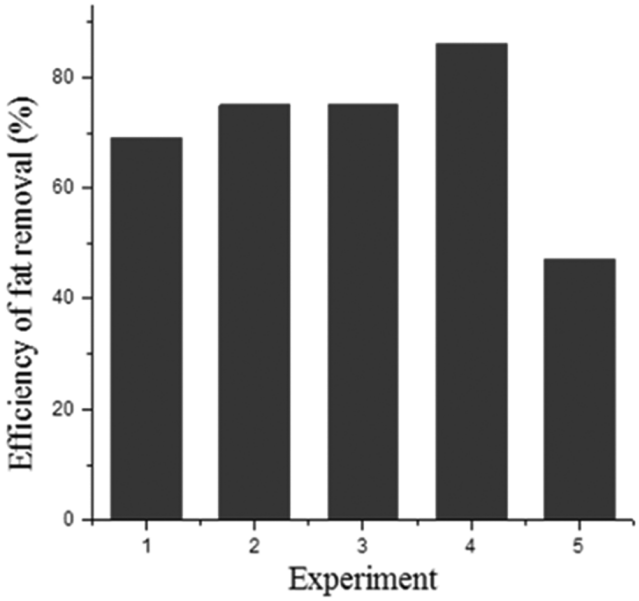

Fig. 1 — Efficiency of degreasing (Experiment No. 1, 10% lipase + 0.5% degreasing agent; Experiment No. 2, 10% lipase + 0.75% degreasing agent; Experiment No. 3, 10% lipase + 1% degreasing agent; Experiment No. 4, 3% degreasing agent; Experiment No. 5, 10% lipase).

efficiency was observed at pickling step[25]. Commercial degreasing agents are generally used at the concentration of 2-4%. While imparting perfect properties to pelts, the lipase from *Pseudomonas* sp. VITCLP4 also reduced the concentration of commercial degreaser to 0.5% which will substantially reduce the pollution loads in the effluent and also lead to reduced air pollution due to decrease in use of solvent based degreasing formulations.

Histological studies

Light microscopic images of pickled pelts treated with 10% lipase, 10% lipase + 0.5% degreasing agent and 3% degreasing agent, stained with Sudan Black B are shown in Fig. 2. Sudan Black B, a basic dye combines with acidic groups of fat and appears blue-black under microscope revealing fat content and distribution. The image of control pelt displayed actual

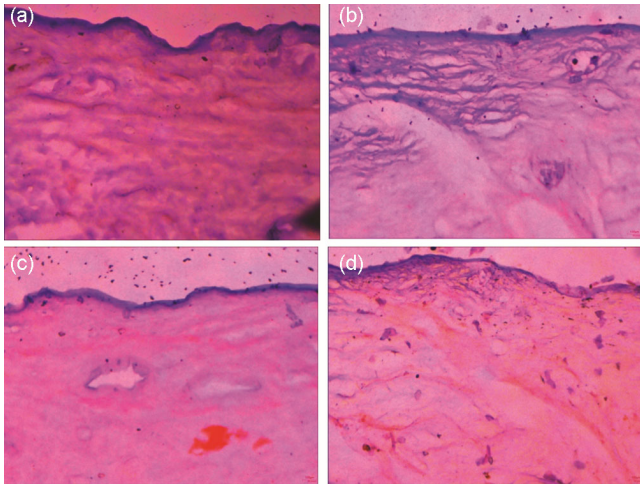

Fig. 2 — Photographs of pickled pelts representing fat content and distribution (a, control; b, 10% lipase; c, 3% degreasing agent; d, 10% lipase + 0.5% degreasing agent).

fat content of dermis which contains sebum secreted by sebaceous gland and that of subcutaneous layer which is richin adipose (fat)tissue (Fig. 2a). The pictures of pelts treated with enzyme alone and degreasing agent alone exhibited substantial removal and almost complete removal of fat respectively (Fig. 2b and c). The photograph of pelt treated with 10% lipase + 0.5% degreasing agent also revealed near total removal of fat (Fig. 2d). The findings of histological studies strongly support the results of chemical testing.

SEM studies

The cross sectional view of SEM pictures taken from control (lime + sulphide) and experiment (10% lipase + 0.5% degreasing agent) are presented in Fig. 3. The collagen and elastinfibre bundles present in dermis are well separated in enzyme treated leather compared to control due to optimal dissolution of fat. The results support the findings of optical studies.

Physical properties of dyed leathers

The results of physical properties testing for the dyed leathers are given in Table 3. For all three parameters the values obtained for experiment (10%

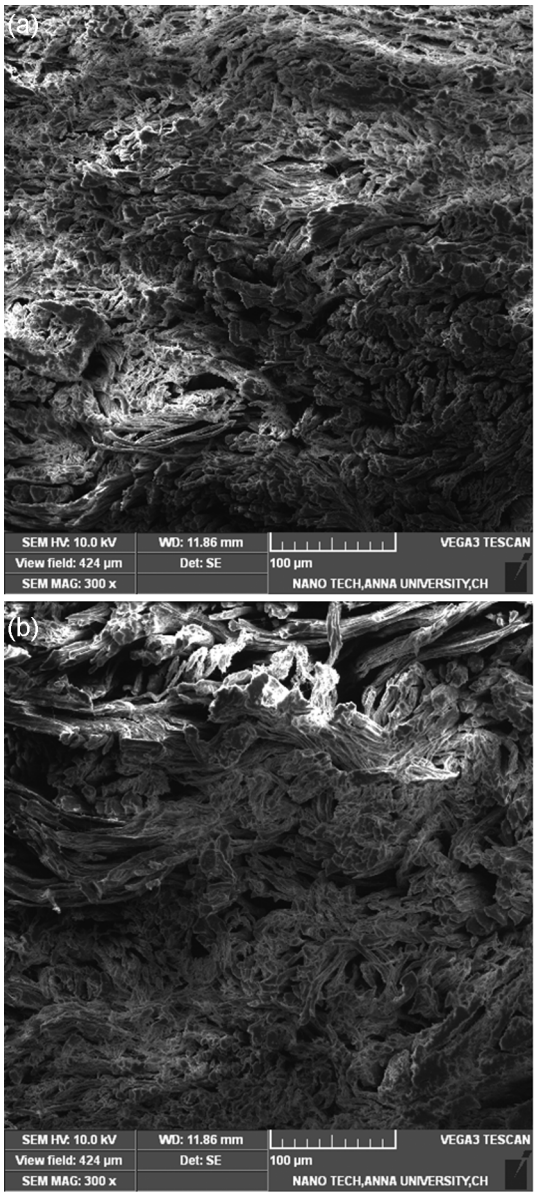

Fig. 3 — SEM pictures of control treated with lime + sulphide (a) and experiment treated with 10% lipase + 0.5% degreasing agent (b).

Lipase + 0.5% degreasing agent) were very close to that of standard (3% Degreasing agent). The results are comparable to the observations noted for *Bacillus subtilis* lipase in a similar study[26]. It has been widely accepted that the strength properties of the leather obtained from the enzyme mediated process were better compared with conventionally (chemical) processed leathers[26].

Conclusion

The above study demonstrated the promising potential of cold active lipase from *Pseudomonas* sp. VITCLP4 for degreasing in leather processing. Being cold active, the enzyme worked well at the ambient temperatures prevailed in leather industry. With optimum *p*H of 8, the enzyme expectedly contributed to acceptable fat content of 3.5% at deliming stage. The desirable fat content of less than 2% was achieved at pickling stage along with commercial degreaser. Use of enzyme resulted in significant reduction in the level of commercial degreaser to 0.5% from 2-4%. Therefore the enzyme could be successfully used in degreasing either alone at deliming step or with commercial degreaser at pickling step in order to minimize the chemical pollution load on environment and also to cut short the cost of effluent treatment.

Table 3 — Results of physical properties testing

Parameter	Standard (3% Degreasing agent)	Experiment (10% Lipase + 0.5% Degreasing agent)
Tensile Strength (kg/cm^2)	121.9 ± 3.9	118.5 ± 11.1
Elongation at Break (%)	68.6 ± 5.0	65.1 ± 7.9
Tear Strength (kg/mm)	38.5 ± 12.1	36.6 ± 7.8

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