EFFECTIVE USE OF ENZYMATIC PROCESSES IN BEAMHOUSE THROUGH NANOPARTICLE IMMOBILIZATION

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Abstract. One of the well-explored alternatives to the lime – sulphide approach for dehairing and fibre opening is the enzymatic approach. In the approach, using a drum method, about 2.5 – 5.0%, on the soaked weight of the skin/hide, of the protease and amylase are sequentially employed, with each operation run for about 6 h. An extensive washing between the two steps required as the activity of one enzyme may be compromised in the presence of the other, especially during the long running of the drum. Though a combination approach, through the use of a bifunctional enzyme has been reported in the past for single step dehairing and fibre opening, this process is likely to have limited applications as there are reports that the storage stability of combination enzymes comprising of protease, amylase and lipase is low, which is generally circumvented by employing higher concentration of amylase and lipase over protease. The individual enzyme activities are also compromised in the presence of detergents and chelators. A similar scenario has also been observed in other industries such as food, laundry etc. The applicability of nanoparticle-based approach to immobilization of enzymes (individual) has been reported in areas such as catalysis and our earlier work immobilization of enzymes on iron oxide nanoparticles has been well received. In this paper, the immobilization of multiple enzymes on copper oxide nanoparticle surfaces is reported. The immobilization, the stability of the enzyme immobilized nanoparticles and the activity of the enzymes present in the immobilized system has been confirmed using various analytical techniques. The extended storage stability of the protease – amylase – nanoparticle system has been studied. A comparative study between protease – amylase combination (in the absence/presence of nanoparticles) indicated that in the absence of nanoparticles, the amylase activity was reduced, possibly due to denaturation of the amylase by the protease. The mechanism by which copper oxide nanoparticles prevent the denaturation of amylase has been studied through computational methods. From the leather processing point of view, the use of protease – amylase – nanoparticle system for combined dehairing and fibre opening has been established and the intact nature of the collagen fibres confirmed through histopathological studies. A comparison between lime-sulphide, protease followed by amylase, protease-amylase-nanoparticle systems for dehairing – fibre opening has been made and the effectivity of the nanoparticle immobilization demonstrated.

1 Introduction

In biological systems, the biotransformation and bioconversion process are carried by biocatalyst called Enzymes. Enzymes are proteins with particular conformation having one or more active sites where catalysis will occur. They are being produced inside a cell of a living organism. Currently enzymes are produced, isolated, characterised and tested for carrying out green-chemistry synthesis in multiple industries.[1]

In leather industry, enzymes are being used extensively which substitutes hazardous chemicals used in the processing of leather. Leather Tanning is a series of events which converts the raw skin/hide to stable leather. There are three main stages in tanning process: pre-tanning, tanning and post-tanning. Dehairing and fibre opening are two main processes during pre-tanning of skins and hides. The conventional method for dehairing and fibre opening is through the usage of Lime and Sulphide paste. Lime and Sulphide let out hazardous solid sludge discharge and toxic wastes into the environment. In addition to harmful by-products, the chemical method consumes copious amount of water.[2] As a bioremediation solution, enzymes like Amylase, Protease, and Lipase are used as substitutes for Lime and Sulphide.[3]

However, in an Industrial application, there are few drawbacks of using enzymes. The enzymes have inferior stability. They get degraded easily. Change in temperature, pH and other factors affects the enzymatic activity. Significant issues with enzymes include poor thermal and chemical stability, high cost, requires skilled labor.[4] Enzyme immobilization is carried out to overcome these drawbacks. Enzyme Immobilization is a process where the enzyme molecules are attached or conjugated onto solid support or matrix. There are many advantages of enzyme immobilization.[5-7]

Nanoparticles have extended their application in the field of enzyme immobilization. Nanoparticles are used as a carrier molecule to immobilize the enzymes.[8] They have a high surface to volume ratio, better-withstanding capability during high pressure applications and flexible platform for surface modification. Among the nanostructures, metal oxide nanoparticles are found to be very efficient in enzyme immobilization, bio-sensing, drug delivery etc. Unique physical and chemical properties of metal oxide nanoparticles which differs from the bulk material has found its application in various facets.[9]

Cupric Oxide nanoparticle (CuO Nps) is one of the oxide compounds of Copper. Copper Oxide nanoparticles is a brownish black powder with 6.3-6.49 g/cm^3 density and melting point of 1201ºC. It is soluble in dilute acid, ammonium chloride, ammonium carbonate and potassium cyanide solution. It is insoluble in water. One of the leading property which will help in retrieving the CuO nanoparticles from the reaction system. Enzyme immobilized CuO can be easily recovered. CuO is used in the field of catalysis, superconductor, rocket fuel, active electrode potential etc. The particle size of nano copper oxide should be between 1-100 nm. CuO nanoparticles have additional peculiar properties like surface effect, Quantum size effect, optical absorption, chemical activity, thermal resistance, catalysis, and quantum tunnelling effect.[10]

2 Materials and methods

The CuO Nps were prepared using Sol-Gel method as described in the "Synthesis and Characterization of CuO Nano Particles by Novel Sol-Gel Method"[11] with slight modifications. After the synthesis of Copper Oxide nanoparticles, they were subjected to X-Ray diffraction analysis by Rigaku Mini Flux X-Ray Diffractometer to confirm the elements present are Copper and Oxygen and to determine the crystalline size and crystal system. The hydrodynamic diameter of the sample was measured by Dynamic Light Scattering (DLS) technique after dispersing the nanoparticles in water under sonication. The Zeta Potential of nanoparticles to enzyme coating using Malvern Zetasizer Nano ZS.

For immobilization studies, 1mg/mL α-Amylase-Protease solution and 1mg/mL CuO nanoparticle solution were prepared. 1mL of CuO solution was added to all the tubes. The supernatant was collected, and protein content was estimated through Lowry Protein estimation method.[12]Further, with the pellet containing the CuO and immobilized enzymes, Amylase and Protease assay were performed.

As per the industrial requirement, 4% Protease, 1% α-Amylase and 1% CuO nanoparticles were used to obtain the immobilized product. The CuO nanoparticles were dispersed in water by sonication. The enzyme mixture was added to the CuO solution drop wise. The mixture was stirred continuously at 800 rpm for 1hour. The mixture was washed for 3 times by centrifugation. The pellet was dried in vacuum to obtain the immobilized product.

For leather trials, the skin was taken from the vertebral region of the goat. The skin was washed thoroughly to remove blood, dirt and other undesirable particles. The adipose tissue layer was removed with the knife. The wet skin was cut into five pieces each weighing around 100g.

Five different samples were taken for the study – 5% of Lime and 5% of Sodium Sulphide. The second sample consisted of 5% of Immobilized product (α-Amylase+ Protease+ CuO nanoparticles). The third sample consisted of 5% of Immobilized product (α-Amylase+ Protease+ CuO nanoparticles)

and 5% of Lime. The fourth sample consisted of 5% of enzyme mixture (α-Amylase+ Protease) and 5% of Lime. The fifth sample consisted of 5% of enzyme mixture (α-Amylase+ Protease).

Each sample was made into a paste by adding 10% of distilled water. Each piece of skin was treated with the respective sample paste, by applying the paste on the flesh side of the skin. The skin pieces were incubated for 16 hrs. After the incubation, the dehairing process was carried to remove the hairs from the skin pieces. The treated skins were thoroughly washed and subjected for conventional tanning procedures.[13]

Proteoglycan Assay was carried to quantify the proteoglycan released from each piece of treated skin. After dehairing, the skin pieces were put into a respective shaker flask containing distilled and were kept in shaker at 160 rpm for 2 hours. The liquor was collected for the analysis.

Copper Oxide nanoparticles have anti-bacterial properties. Mostly Gram-negative bacteria are more susceptible to Copper Oxide nanoparticles. The mechanism is not yet known.

The anti-microbial assay was carried out using Gram-negative Escherichia coli (ATCC 8739). The Bacterial culture was purchased from CSIR-IMTech, Chandigarh. Disc diffusion method was used for this assay. Autoclaved Luria Bertani- Agar media was used for plating. The 100µL of bacterial suspension of $1x10^6$ was inoculated on the plate. Gentamycin (30µg/disc) was used as positive control for antimicrobial activity.[14] 50µL of CuO nanoparticles solution having the concentration of 1mg/mL and Immobilized CuO solution having 1mg/mL nanoparticle and 1mg/mL enzyme mixture were loaded on to the respective wells. The plate was incubated overnight in the incubator at 37°C. The zone of inhibition around the wells were measured.

3 Results and Discussion

The Figure 1. Shows the XRD pattern of Copper Oxide Nanoparticles. Sharp peaks formation exhibits crystalline structure. The peaks corresponding to the following 2θ values: 33, 36, 39, 49, 54, 62, 66, 72, 75 confirms the CuO formation.[10] TEM image indicates that the synthesized sample possess rod-shaped particles with homogeneous nature.

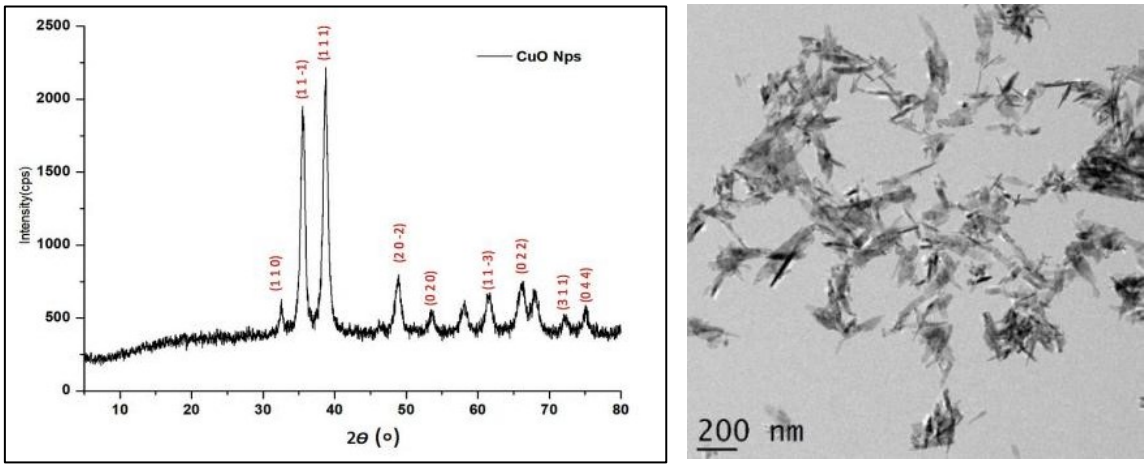

Fig. 1. XRD and TEM of synthesized CuO Nps

From Lowry, the protein content in Blank enzyme and immobilized samples were quantified. The Blank enzyme contained around 992 µg. The supernatant from the immobilized sample comprised around 943µg. From the above results, protein content in the pellet was calculated. It was 48.8µg. This shows that the protein loading capacity of CuO nanoparticles is quite weak when the concentration of the enzyme is high (i.e. around 1000µg).

But from the α-amylase activity assay, it is clear that the activity of the immobilized enzyme is retained. Whereas in Blank Enzyme sample, over the time, the amylase enzyme is getting degraded by the protease enzyme. Thus, immobilization prevents Protease from cleaving Amylase enzyme. These results thus support the fact that multiple enzymes can be loaded on to one carrier without affecting the enzymes activity.

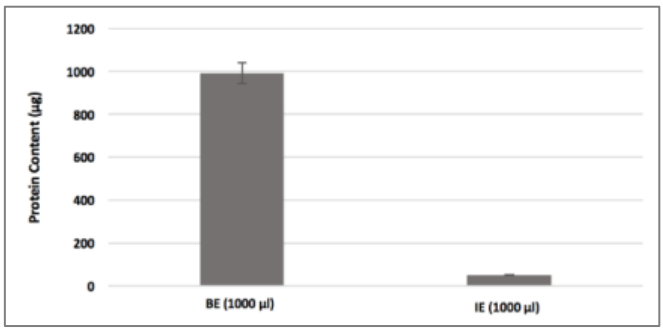

Fig. 2. Protein quantification by Lowry analysis

The Protein content in Blank Enzyme and immobilized samples are 748µg and 133.3µg respectively. The protein loading capacity of CuO nanoparticles is poor. But comparing with the 1000µg sample, the 750µg sample has got more protein immobilized on to the CuO nanoparticles. Thus, the loading efficiency of the CuO nanoparticles is better at lower enzyme concentrations.

From Protease assay, the activity of the immobilized enzyme is comparatively higher than that of blank enzyme. In immobilized enzyme sample, for 18% of protein content it is having44% of enzymatic activity. Thus, the activity of the enzyme is increased due to immobilization.[5]

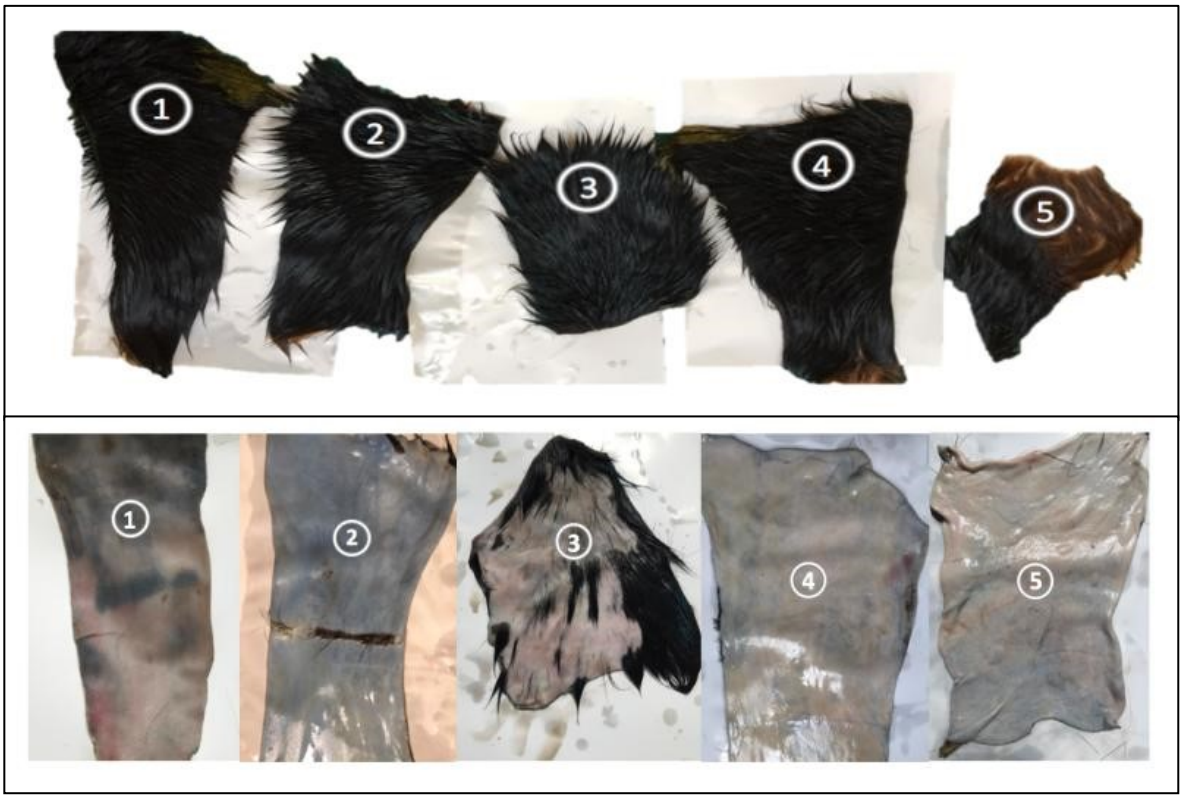

Fig. 3. Skins before and after dehairing. [5% of Lime and 5% of Sodium Sulphide- (1), 5% of Immobilized- (2), 5% of Immobilized +5% of Lime- (3), 5% of bare enzyme mixture +5% of Lime - (4), 5% of bare enzyme mixture]

From the leather dehairing studies it was clear that sample 2(Immobilized enzyme can perform proper dehairing to that of control sample (5% lime+ 5% Sodium Sulphide). Nevertheless the presence of lime in the sample 3 along with immobilized enzyme shows a phenomenal setback in the performance of the sample by incomplete dehairing. On the other hand, skins treated with sample 4 and 5 with 5% bare enzymes started to decay, which was indicated by strong putrefaction smell.

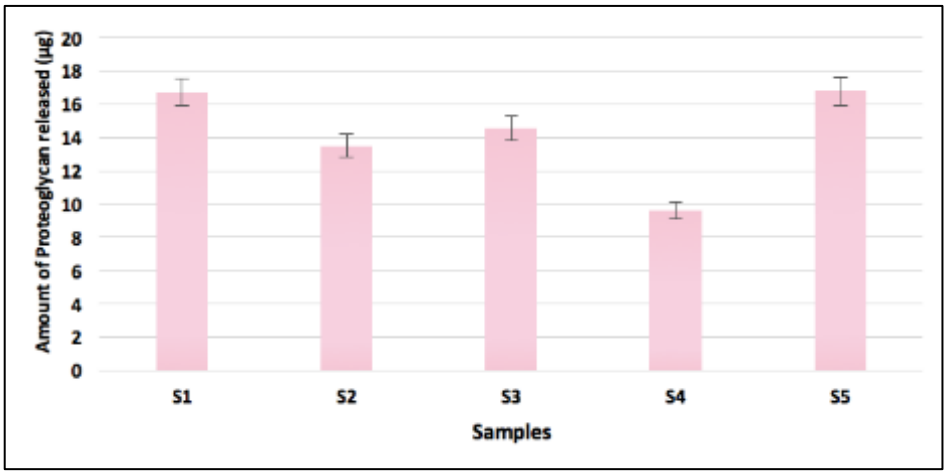

Fig. 4. Proteoglycan release assay.

The proteoglycan released were quantified through proteoglycan assay. The Figure. 4 Represent the amount of proteoglycan released from each skin pieces. The types of Proteoglycans released are Heparan Sulphate, Chondroitin and dermatan. Compared with the other samples, the Proteoglycan released is higher in Sample 1 where Lime and Sulphide was used. Comparable or similar result is observed in Sample 5 where only 5% of enzyme mixture is used. In Sample 4, the proteoglycan release is quite low due to presence of Lime along with enzyme mixture. This is because the enzymes got denatured in the presence of Lime. Although, in Sample 3 containing Immobilized enzyme and Lime, the proteoglycan released from the sample is higher than Sample 4. This shows that denaturation of enzymes by Lime is minimized due to the immobilization. Sample 2 contains only 2.5% of enzyme mixture (along with 2.5% of CuO nanoparticle). For the amount of enzyme (2.5%) provided, Proteoglycan released from Sample 2 is comparably higher. This is because, activity of the enzymes has increased significantly due to immobilization. The CuO nanoparticles because of their small size, can easily penetrate through the skin pores and acts as a vehicle to make enzymes accessible in the hair follicle regions, thus there is higher removal of proteoglycans and hairs from the skin.

Fig. 5. Anti-microbial assay.

For this study, E. coli gram negative bacterial culture was used. The zone of inhibition around the antibiotic gentamycin formed was measured to be 30mm. The zone of inhibition formed for CuO sample and Immobilized enzyme sample does not have a clear distinct boundary. For CuO nanoparticles, the zone of inhibition was measured to be 25mm. And for immobilized enzyme, the zone of inhibition was measured to be 21mm. This shows that CuO has some effect on gram negative bacteria. By increasing the concentration of the CuO nanoparticles, toxicity level will increase resulting in the formation of clear and distinct zone of inhibition.

4 Conclusion

In this study, we have successfully demonstrated that Cupric Oxide nanomaterials can be employed as a support material for enzymes. Immobilization of enzymes with nanoparticles were done through adsorption with 80% yield, which addresses the possible transition from chemical to bio-based leather processing. Also Nano delivery carriers increase the acceptability of enzymatic approaches as known lacunae are overcome. Thus Nanotechnology paves the way for sustainable beam house operations.

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