Eco-friendly Enzymatic Dehairing of Cowhide Using Thermostable Alkaline Serine Protease 50a

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Abstract. The leather goods sector faces ecological issues caused by typical dehairing technique, which requires an environmentally friendly solution. The incorporation of synthetic compounds in the conventional dehairing practice could pose an important contribution to the water pollution. The study analyses enzymatic and conventional treatment, examines at the implications of different enzyme concentration and measures the effluent quality of wastewater. A range of dehairing treatment including conventional, enzyme assisted and single enzyme. The performance of dehairing treatments were observed using scanning electron microscopy (SEM) and tensile tests. The effluent from dehairing process was focused on pH and chemical oxygen demand (COD) level. The findings demonstrate that enzyme assisted methods, particularly with a 20% thermostable alkaline serine protease 50a (TAP50a) showed superior dehairing efficiency. SEM results from enzymatic method indicated improved grains surface quality of cowhides. The mechanical tests demonstrated that the cowhides dehaired using enzyme dehairing exhibited greater tear resistance compared to conventional methods. Water quality assessment indicates pH level under acceptable ranges and able to reduce COD level compared to conventional method. Thus, the developed enzymatic treatment using the TAP50a offers an alternative dehairing method to replace a conventional method by improved leather properties and decreased ecological implications.

1 Introduction

The leather industry is one of the most significant sectors in many countries due to its economic and cultural significance. The global market for leather products, according to a report by Grand View Research was estimated to be valued USD 242.85 billion in 2022 and is expected to grow at a CAGR of 6.6% from 2023 to 2030 [1]. There are several steps

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involved in the production of leather, including curing, soaking, dehairing, fleshing, deliming, pickling, and tanning. Among these steps, dehairing is an important step that removes hair and other unwanted substances from the skin. However, conventional leather processing methods, especially dehairing, involve the use of harsh chemicals that not only pollute the environment but also cause health hazards to workers [2]. The most common chemicals used include sodium sulphide, calcium hydroxide, sodium hydrosulphide and sodium carbonate. Eco-friendly enzymatic dehairing has become a viable alternative to conventional chemical dehairing to solve these issues.

Enzymatic dehairing is a more eco-friendly and effective technique that utilizes enzymes to break down and dissolve the proteins in the hair, leaving the hides clean and ready for tanning. Enzymes are biodegradable, and their use in the leather industry can reduce pollution caused using harsh chemicals [3]. The most used dehairing enzymes are proteases because they can catalyse the hydrolysis of peptide bonds in proteins, including keratin, the major structural protein in animal hair [4]. Proteases are found in many different origins, such as in plants, animals, and microbes. Bacteria are the most common among the several sources, and the most typical genus employed in the synthesis of commercial protease is *Bacillus subtilis* [5].

Escherichia coli BL21 (DE3) pLysS, the recombinant host carrying the protease 50a gene, was able to effectively generate thermostable alkaline protease 50a (TAP50a). The aforementioned enzyme is also identified as an alkaline protease [6]. Because alkaline proteases, such as TAP50a, can work efficiently at higher pH values, they are frequently employed in dehairing procedures and are hence ideally adapted to degrading the keratin in animal hair. This quality functions exceptionally well for dehairing animal hides, such as cowhides. Briki et al. [7] stated that alkaline proteases work best in a pH range of 8.0 to 11.0, which makes them ideal for dehairing. It has been found that such types of enzymes work better than other proteases to dehair animal hides at lower temperatures and in shorter treatment durations [8]. By lowering the quantity of chemicals utilised and using these proteases in a process, worker safety and the environment can be improved and sustained. Furthermore, by shielding the hide from the degradation caused by abrasive compounds, enzymatic dehairing can raise the grade of the leather [9, 10].

The leather sector is important to the global economy, but the hazards to the environment and human health caused by conventional methods of leather processing, especially dehairing, must be considered. Due to its effectiveness and environmental sustainability, using enzymes as an alternative dehairing agent for cowhides and other animal hides is becoming increasingly common. Thus, TAP50a has been used in enzymatic dehairing to improve leather quality and use less chemicals. In order to fulfil the increasing demand for eco-friendly and sustainable leather production, it is crucial to develop and optimize enzyme-based dehairing methods.

2 Materials and methods

The thermostable alkaline protease 50a (TAP50a) was extracted from intracellular expression of *Escherichia coli* BL21 (DE3) pLysS/pET22b(+)/50a protease gene (Microbial Technology Lab, UMK). The cowhides obtained from the local slaughterhouse at Pasir Mas, Kelantan. Analytical grade calcium oxide and sodium sulphide powder purchased from HmbG and Merck, respectively.

2.1 Cow hide sample preparation

First, cowhide was subjected to a curing and drying process based on a technique proposed by Ibrahim et al. (2019) [11]. Then, the cowhide was cut in size of 5 cm × 5 cm.

2.2 Dehairing with Different Treatments

The first treatment is a conventional method. In this process, a piece of hide was immersed in a solution containing distilled water, 5% calcium oxide (CaO) (w/w), and 2% sodium sulphide (Na_2S) (w/w).

The second treatment is an enzyme-assisted treatment. In this process, a piece of hide was dipped in a solution containing 5% calcium oxide for 6 h at 37 °C. Then the piece of hide was transferred into a solution containing 10% (1200 U/mL) and 20% (2400 U/mL) of TAP50a, respectively. The third treatment is a single enzyme treatment. In this process, a piece of hide was dipped in a solution containing 10% (1200 U/mL) and 20% (2400 U/mL) of TAP50a.

For the control, a piece of hide was dipped in distilled water. All samples were then treated at 37 °C for 24h at 150 rpm. Next, the hair from the hide was removed by using a blunt knife [12]. Each treatment was performed in duplicate.

2.3 Physical and Mechanical Properties of Treated Cowhides

2.3.1 Morphology of the Treated Cowhides

Morphology of treated cowhides was observed using Scanning Electron Microscopic (SEM) JSM-IT100 In Touch Scope. Each sample from the control and treated samples was cut from the official sampling position. Samples were first washed in water. Subsequently, the samples were gradually dehydrated using acetone and methanol per standard procedures. The micrographs for the grain surface were obtained by operating the SEM at an accelerating voltage of 20 kV with different magnification levels [14].

2.3.2 Tensile Properties of the Treated Cowhides and Hand Evaluation

Each sample was taken approximately in size of 5 cm × 5 cm for testing in the testometric machine. The samples were dried in an oven. Prior to the tensile test, the width, thickness, distance between the jaws, and initial length of the cowhides were measured. For the tensile test, the testometric machine was designed with jaws that held the hide and prevented it from slipping off the sample. Tear resistance was measured based on tear initiation and tear propagation. A 1.000 N tensile force was applied at a speed of 5 mm/min. The samples for the tensile test had the following physical properties: Young's Modulus (N/mm^2), tensile strength (N/mm^2) and strain at break (%) as per standard procedures [13]. Each sample was assessed for softness and thickness and general appearance by hand and visual examination. The samples were rated (+, ++, and +++, where + is the poorest and +++ is the best) based on the modification method by Kanth et al. [14].

2.4 Analysis of effluent from dehairing process

Within 24 hours of collection, an effluent sample from each dehairing process was subjected to measure pH values and chemical oxygen demand (COD). A high-range COD kit (20-1500 mg/L) was employed using the DRB 200. 100 mL of the sample was blended for 30 seconds for homogenization. The DRB200 Reactor was then set to a preheat of 150 °C. Following preheating, a COD vial kit was prepared by adding 2.00 mL of the blended sample for testing. A blank COD vial kit was also prepared by adding 2.0 mL of deionized water. After cooling, the COD reading was measured using a spectrophotometer with suitable wavelengths for high-range COD (20-1500 mg/L) [15].

3 Results and discussion

3.1 The Effectiveness of Different Dehairing Treatments on Cow Hides

The study investigated the effectiveness of various dehairing treatments on cow hides, employing the dip method for a comprehensive and universally applicable approach. The treatments included conventional treatment (5% CaO and 2% Na_2S), enzyme-assisted treatment with different concentrations of TAP50a solution, single enzyme treatment with different concentrations of TAP50a solution, and control (distilled water). The results, as presented in the Figure 1, highlighted the dehaired area obtained and the corresponding area yield for each treatment.

The conventional treatment exhibited a 100% area yield, indicating complete hair removal. In contrast, the control treatment (distilled water) demonstrated a significantly lower area yield of 14.7%, confirming the ineffectiveness of water alone for dehairing. The combined action of calcium oxide and sodium sulphide ensures effective dehairing by first preparing the hide with calcium oxide to enhance penetration and then using sodium sulphide to break down the keratin in the hair [16].

The enzyme-assisted treatments showed varying degrees of effectiveness, with the best result achieved using a 20% TAP50a solution, producing an impressive 81.9% area yield. While a 10% concentration resulted in a moderate 21% area yield. This suggested that enzymatic action played a crucial role in enhancing the dehairing process. Surprisingly, the 20% concentration, which contained 2400 U/mL of enzyme activity, outperformed all others, emphasizing the importance of optimizing enzyme concentrations for maximum efficiency. Similar patterns happen when the treatment using single enzyme, 20% TAP50a solution exhibited an 81.4% area yield compared to the other single enzyme treatment.

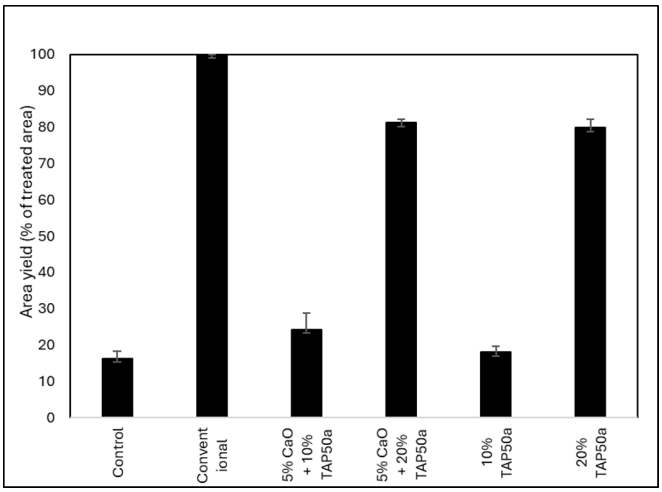

Fig. 1. Effect of different treatments on dehaired area yield of the cowhides

The superior performance of the 20% TAP50a solution can be attributed to the delicate balance between enzyme activity and substrate interaction (collagen and keratin protein are found in cowhide). Higher concentrations may inhibit saturation or substrate, hindering the enzymatic dehairing process [7]. Conversely, lower concentrations may not provide sufficient enzymatic activity for effective hair removal. To promote eco-friendly dehairing methods, it was essential to consider alternatives to conventional treatments. Enzyme-assisted and single enzyme treatments, particularly with the optimal 20% TAP50a solution,

showcased promising results and presented a sustainable and environmentally friendly option. Enzymatic dehairing demonstrated efficacy and aligned with the growing emphasis on green technologies and reduced chemical usage in industrial processes.

3.2 Physical and Mechanical Properties of Treated Cowhides

Scanning Electron Microscopy (SEM) was used to examine the morphology of the grain surface of dehaired hides, which resulted from the removal of hair from the epidermis. It also showed the undamaged grained structure of hides. In the conventional dehairing treatment of cowhides, the use of hydrogen sulphide, classified as highly alkaline, raised concerns about environmental impact due to the release of toxic chemicals such as calcium oxide (lime) and sodium sulphide into the effluent [17]. This not only posed a threat to aquatic ecosystems but also presented a significant health hazard to tannery workers. The conventional treatment, using 2% Na_2S and 5% CaO, successfully achieved 100% dehaired area for cowhides, measured at 25 cm^2. However, this treatment resulted in low-quality leather as the method denatured and coagulated hide proteins at a high pH, causing the hide's surface structure to become wrinkled and less smooth, as shown in Figure 2 (a). The high pH denaturation and coagulation of hide proteins induced changes in the collagen fibre structure, impacting the leather's texture [18]. Increasing the pH of the soak can aid the removal of non-structural proteins as it causes the hide or skin to absorb more water. However, it is very important that the pH does not exceed 10.5 since this could cause immunisation and subsequently poor removal of the hair [19]. Conventional treatment, also known as chemical treatment, produces significant hazardous waste, contributing to environmental concerns [20].

In the control treatment using distilled water, Figure 2 (b) clearly showed hair remaining on the hide, indicating ineffective dehairing. The hide surface exhibited slight wrinkling, possibly due to changes in collagen hydration and structure caused by distilled water. Hair follicles remained intact and of regular size, suggesting that distilled water alone is not effective in removing or loosening hair. Specific dehairing treatments typically involve chemicals or enzymes to break down the bonding structures between hair and hide.

The combined use of calcium oxide with TAP50a at concentrations of 10% and 20% exhibited varying effects on the efficiency of dehairing. The most favourable outcome was observed with a 20% TAP50a solution with a pre-treatment 5% calcium oxide. Observations made through SEM revealed that the enzyme-assisted treatment, as depicted in Figure 2 (c, d), prevented damage to the grain surface compared to conventional treatments using Na_2S and CaO. Within the optimal enzyme concentration, hair removal was more effective. White residue was observed on cowhide, as a result of cowhides being soaked in the CaO solution. When CaO reacts with water, it forms a new substance called as calcium hydroxide. Thus, the white precipitation on cowhides after CaO treatment is due to the formation of $Ca(OH)_2$ during the dehairing process. The enzyme-assisted treatment demonstrated a clear opening of hair pores on the cowhide. This effect was facilitated by the action of CaO, which played a crucial role in opening up collagen fiber bundles. As a result, water absorption was enhanced during the treatment. In Figure 2 (d), despite the pores on the cowhide opening, the hair on the cowhide remained intact, but most of the hair had fallen out compared to Figure 2 (c). At the same time the surface of the hide appeared smooth.

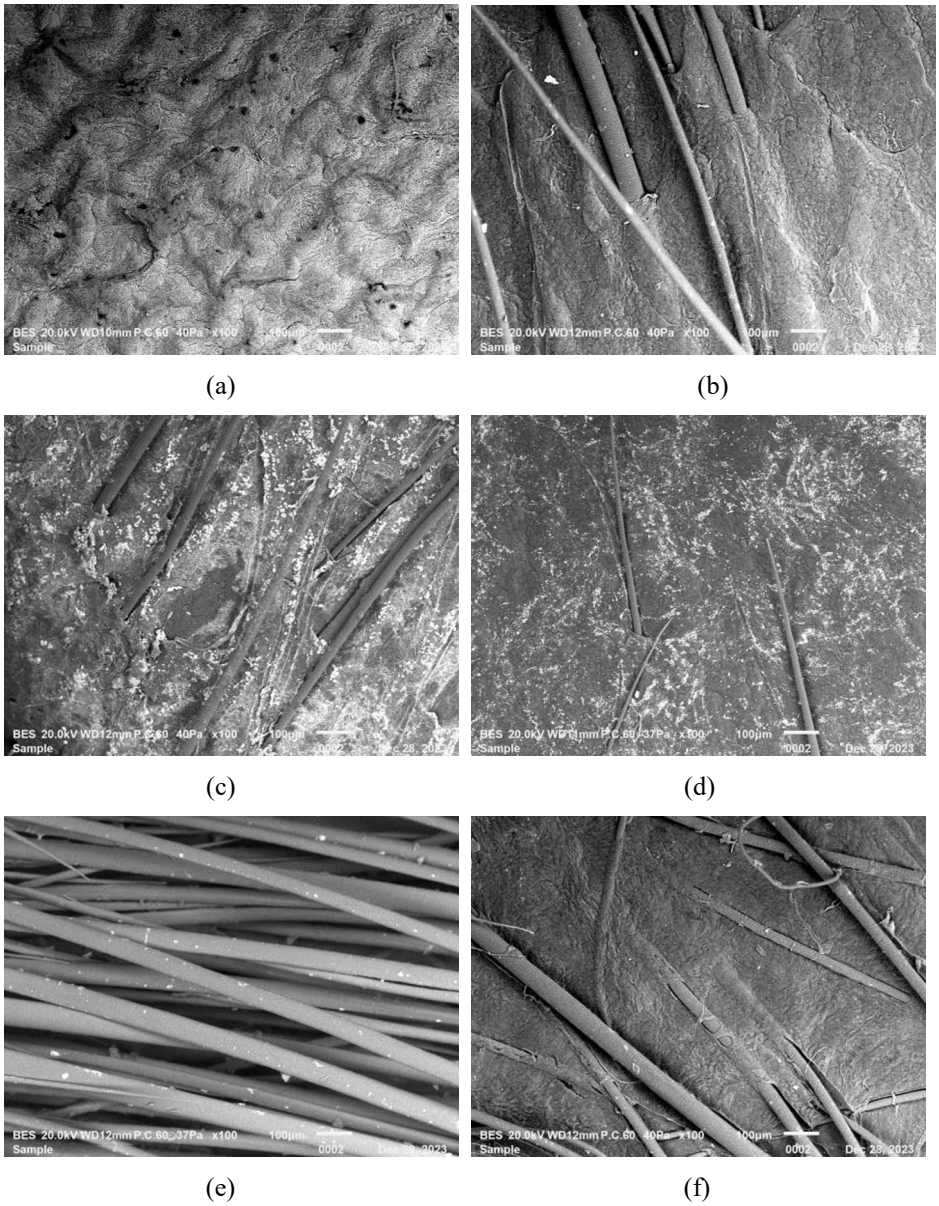

Fig. 2. SEM at magnification x100 showing the grain surface of cowhides from (a) Conventional (b) Control (c) CaO + 10% TAP50a (d) CaO + 20% TAP50a (e) 10% TAP50a (f) 20% TAP50a

The application of single enzyme in dehairing treatment is to reduce utilization of chemical. Observations made through SEM revealed that the single enzyme treatment, as depicted in Figure 2 (e and f), prevented damage to the grain surface compared to conventional treatments using CaO and Na_2S. As in Figure 2 (e), the hair structure on the cowhide remained intact, and most of the hair did not fall out, resulting in a very dense appearance. However, in Figure 2 (f), the hair on the cowhide remained intact, but most of the hair had fallen out compared to Figure 2 (e). The application of a single enzyme treatment for dehairing cowhides proved to be a viable alternative to traditional methods involving lime and sulphide. The enzymatic dehairing exhibited nearly the same

effectiveness in hair removal while mitigating the use of sulphide. The enzyme-based dehairing process showcased environmental benefits through reduced chemical effluent decomposition and improved wastewater quality. TAP50a emerged as a promising dehairing agent due to its stability, straightforward production process, and cost-effectiveness. However, it's essential to note that the enzymatic dehairing process necessitated a longer processing time compared to the conventional combination of sodium sulphide and lime.

While the SEM results indicated that the single enzyme treatment did not as efficient as chemical treatment in remove hair, the organoleptic properties, such as softness and grain surface, favoured the enzyme-treated hides over conventional methods. The enzyme-treated hides exhibited a smoother texture and better overall quality, despite some hair remaining intact at the hair root. The clear visibility of the grain surface, particularly with alkaline serine protease, further highlighted the potential of enzyme-assisted dehairing in producing high-quality leather. The role proteases selectively break down hair proteins without damaging collagen fibers and preserving hide quality was reported by some researcher [7, 16].

Other than SEM, general properties have been compared between the treatments of cowhides as shown in Table 1. The pieces of cowhide were evaluated for various properties by hand and general visual examination. In general, on visual assessment, it can conclude that by using single enzyme with 20% of the enzyme showed very good grain smoothness, softness and less thickness.

The results of mechanical tests demonstrated that the cowhides dehaired using enzyme dehairing exhibited greater tear resistance compared to leather treated using conventional methods. Additionally, the cowhides treated with single enzyme showed higher elongation, tensile strength and elasticity percentages than the control. Mechanical tests showed that cowhides dehaired with enzymes had higher Young modulus than those treated with conventional methods and enzyme assisted. Enzyme dehairing appears to enhance the tear resistance of cowhides. This indicated that the use of TAP50a enzyme for dehairing of cowhide has persevered the quality of the leather. This might be due to the more selective and less harsh nature of TAP50a treatments compared to traditional chemical methods. In this study, protease enzymes target specific proteins without causing as much collateral damage to the collagen fibres, which are crucial for the leather's strength. Besides, the use enzyme in mild condition i.e. lower pH and temperature during the treatment also preserved the strength of the leather. In addition, the tensile strength is also higher for single enzyme treatment compared to that of conventional and enzyme assisted treatment. In Table 1, it is also worth noting that strain at break (%) is highest for conventional treatment. This indicated that the cowhide that underwent conventional treatment is more flexible and can stretch considerable before break. It might be due to the partial hydrolysis or break down of non-collagenous proteins, such as keratin and other structural proteins that results in more flexible cowhide. These findings conclusively indicate that the dehairing protease enhances the quality of semi-finished leather, making it more suitable for clothing applications. These characteristics similar to the study on dehairing of goat skin using protease SB1 which are their result have been confirmed by an experienced technologist from National Leathers and Shoes Center Ben Arous-Tunisia [7].

Table 1. Comparison test between control, conventional, enzyme assisted and single enzyme dehairing

Comparison tests		Control	Conventional	Enzyme assisted (10% TAP50a)	Enzyme assisted (20% TAP50a)	Single enzyme (10% TAP50a)	Single enzyme (20% TAP50a)
General properties	Grain smoothness	+++	+	++	++	+++	+++
	Softness	++	+	++	++	+++	+++
	Thickness	+++	+	++	++	++	+
Mechanical test	Young modulus (N/mm^2)	50	4.9	1.9	22.5	105	97.2
	Tensile strength ((N/mm^2)	2.3	2.9	0.7	0.9	2.7	3.4
	Strain at break (%)	47	70	41	36	29	48
Chemical tests	pH	7.7	12.6	10.5	9.8	8.5	8.5
	COD of effluent (mg/L)	610	7380	2480	3890	3310	3970

3.3 Analysis of Effluent from Dehairing Process

Table 1 also provide the data related to environmental impact of effluent from different dehairing processes based on pH and Chemical Oxygen Demand (COD) values. The control sample exhibits a neutral pH of 7.7, indicating minimal impact on water acidity/alkalinity. In contrast, the conventional treatment yields a highly alkaline pH of 12.6, which poses a potential risk to aquatic ecosystems. Enzyme-assisted treatment, specifically both the 10% and 20% TAP50a treatments, demonstrate lower pH values compared to the conventional, suggesting reduced alkalinity. The elevated pH observed in these treatments is attributed to the presence of CaO, which is inherently more alkaline. Meanwhile, the effluent from the single enzyme treatment (both 10% and 20% TAP50a) shows pH values closer to neutral, highlighting their environmentally friendly nature.

For COD results, the control sample has the lowest COD value, indicating minimal organic pollution and serving as the baseline for comparison. The conventional dehairing treatment has the highest COD value, indicating a significant amount of organic pollutants in the wastewater. The enzyme-assisted treatment shows a 66 to 47% reduction in COD compared to the conventional method, demonstrating that using enzymes substantially decreases the amount of organic pollutants. The single enzyme treatment results in a 46 to 45% reduction in COD compared to the conventional method, offering a marked improvement and presenting a viable alternative for reducing environmental impact. The data clearly shows that enzyme-assisted and single enzyme dehairing processes are much more environmentally friendly than conventional methods. Although the enzyme-assisted process with 10% TAP50a is the most effective in reducing COD levels, it is inefficient in removing hair from cowhides. Meanwhile, the enzyme-assisted and single enzyme treatments using 20% TAP50a are the best treatments for both reducing COD and dehairing, making them the best options for minimizing the environmental impact of dehairing effluent. These enzyme-based methods significantly lower the organic pollutant load in wastewater, thereby helping to protect aquatic ecosystems and improve water quality [14, 21].

4. Conclusion

The treatment of cowhides using enzyme-assisted treatments and single enzymes showed promising results, with enzymatic dehairing methods, particularly the 20% TAP50a solution, outperforming conventional treatments. Scanning electron microscopy revealed the preservation of the grain surface and undamaged grained structure of hides in enzyme-assisted and single enzyme treatments. Meanwhile the tensile strength analysis indicated a significant influence of enzymatic treatments on the mechanical properties of cowhides, presenting potential enhancements in tensile strength. Water quality assessment demonstrated that enzyme-assisted and single treatments-maintained pH levels within acceptable ranges and reduced COD levels compared to conventional treatments, aligning with the objective of developing an eco-friendly dehairing process. Overall, the study concludes that enzymatic dehairing methods, particularly utilizing TAP50a offer a promising avenue for sustainable and eco-friendly cowhide processing, with positive implications for both the leather industry and environmental conservation.

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