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Optimization of Inducer Concentration to Enhance Leather Degrading Enzymes of Bacillus Sp Isolated from Different Environmental Samples

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Abstract
Leather being an organic material from animal hides are resistant to be decayed. It contains protein that are challenging to be decayed as it contains complex collagen along with toxic dyes. The degradation of collagen includes the breaking down of sulfuric acid and redox reaction to produce ammonia. For this reaction to occur it takes about a time period of 25 to 50 years. Through bioremediation, the process can be fastened. Bacillus subtilis produce laccases and peroxidases (oxido reductases) in a minimum quantity. This study isolated a Bacillus sp. from soil and optimized its production of laccase and peroxidase enzymes for leather waste bioremediation. The bacteria demonstrated collagen-degrading ability, forming a clear hydrolysis zone. Enzyme production was maximized at 1.5mM Cu_2SO_4 (laccase) and 2mM $MnSO_4$ (peroxidase). Following partial purification and SDS-PAGE characterization, these induced enzymes showed high activity, presenting an effective, eco-friendly method for accelerating the degradation of tough leather proteins.
KEYWORDS: Leather, Collagen degradation, Laccase, Peroxidase, Bacillus sp., Inducer.

1. Introduction

The leather industry generates significant environmental pollution through its solid and liquid wastes. Leather, a chemically stabilized collagen-based material, is slow to degrade, taking 25-50 years, while its processing consumes large volumes of water and produces effluents containing hard-to-degrade proteins, synthetic dyes, and hazardous chemicals like chromium. These pollutants, including sulfides and acidic effluents with high BOD, threaten aquatic ecosystems by blocking sunlight, corroding infrastructure, and being toxic to life. Historically, solid tannery wastes like trimmings were land filled, but restrictions and high incineration costs have prompted the search for alternative treatments. Bioremediation presents a promising solution by using microorganisms and their enzymes to break down these contaminants. Specifically, the enzymes laccase and peroxidase, produced by the non-pathogenic soil bacterium Bacillus subtilis, show great potential in degrading leather wastes and dyes. Laccases oxidize a wide range of substrates using molecular oxygen, while peroxidases use hydrogen peroxide to catalyze oxidative reactions. The activity of these enzymes can be optimized using inducers like copper and manganese salts ($CuSO_4$ and $MnSO_4$), with the goal of maximizing the degradation of leather under controlled growth conditions, offering an efficient and low-cost alternative to conventional disposal methods.

In addition to the severe environmental burden caused by tannery effluents, the complex composition of leather wastes makes their treatment particularly challenging. Leather processing involves multiple chemical-intensive steps such as soaking, liming, dehairing, pickling, tanning, dyeing, and finishing. Each stage contributes distinct pollutants, including proteins, fats, lime, sulfides, chlorides, chromium salts, synthetic dyes, surfactants, and organic solvents, leading to wastewater with high chemical oxygen demand (COD), biological oxygen demand (BOD), total dissolved solids (TDS), and extreme pH values. Conventional physicochemical treatment methods—such as coagulation–flocculation, chemical oxidation, and membrane filtration—are often costly, energy-intensive, and generate secondary sludge requiring further disposal.

Solid tannery wastes, including fleshings, shavings, trimmings, and buffing dust, are rich in collagen and chromium. Chrome-tanned leather wastes are particularly problematic due to the risk of chromium(III) oxidation to the more toxic chromium(VI) under environmental conditions. These wastes pose long-term risks to soil and groundwater when disposed of in landfills, highlighting the urgent need for eco-friendly and sustainable waste management strategies.

Bioremediation exploits the metabolic capabilities of microorganisms to detoxify, transform, or mineralize pollutants into less harmful forms. Compared to conventional treatments, microbial approaches are cost-effective, environmentally benign, and adaptable to large-scale applications. Microorganisms such as bacteria and fungi produce extracellular enzymes capable of degrading complex organic compounds, including recalcitrant proteins, phenols, and synthetic dyes commonly found in tannery wastes.

Among bacterial species, Bacillus subtilis has gained considerable attention due to its non-pathogenic nature, rapid growth, ability to survive under harsh environmental conditions, and high enzyme secretion capacity. This bacterium produces oxidative enzymes such as laccases and peroxidases, which play a crucial role in the breakdown of aromatic and high-molecular-weight compounds present in leather effluents.

Laccases (EC 1.10.3.2) are multicopper oxidases that catalyze the oxidation of phenolic and non-phenolic compounds using molecular oxygen as the final electron acceptor, producing water as the only by-product. Their broad substrate specificity enables them to degrade azo dyes, phenolic tanning agents, lignin-like structures, and protein-bound chromophores, making them highly suitable for tannery wastewater treatment.

Peroxidases (EC 1.11.1.x), on the other hand, catalyze oxidative reactions in the presence of hydrogen peroxide. These enzymes are particularly effective in breaking down complex aromatic rings and recalcitrant dye molecules, thereby reducing color, toxicity, and COD levels in effluents. The combined action of laccase and peroxidase often results in synergistic degradation, improving overall treatment efficiency. The production and activity of laccase and peroxidase can be significantly enhanced through medium optimization and the use of suitable inducers. Metal ions such as copper (Cu^{2+}) and manganese (Mn^{2+}) act as cofactors and transcriptional inducers for oxidative enzymes. Supplementation with $CuSO_4$ and $MnSO_4$ has been shown to increase enzyme expression and catalytic efficiency in Bacillus species. Additionally, parameters such as pH, temperature, agitation speed, carbon and nitrogen sources, and incubation time play critical roles in maximizing enzyme yield.

1. **Objectives**

Under optimized conditions, these enzymes can efficiently hydrolyze collagenous materials, decolorize dyes, and reduce organic load, thereby accelerating the biodegradation of leather wastes. Enzyme-assisted processes also minimize the formation of secondary pollutants and allow for potential resource recovery, such as the production of amino acids or biofertilizers from treated wastes. The application of Bacillus subtilis–derived laccase and peroxidase offers a sustainable alternative for tannery waste management, aligning with the principles of green chemistry and circular economy. By reducing toxicity, color, and organic load in tannery effluents, this approach protects aquatic ecosystems and public health while lowering treatment costs for the leather industry. Furthermore, the scalability and adaptability of microbial enzyme systems make them suitable for integration into existing wastewater treatment plants. Overall, enzyme-based bioremediation represents a promising, low-cost, and environmentally friendly strategy for addressing the persistent pollution problems associated with the leather industry, paving the way for cleaner production and sustainable industrial development.

2. Methods

3.1. MATERIALS USED

Glassware: Conical flasks, Petri dishes, Glass beakers, Measuring cylinder, Test tubes with stand, Boiling tubes with stand, Centrifuge tube.

Chemicals and Reagents: Nutrient agar, Nutrient broth, Simmon's citrate agar, Mannitol motility agar, TSI Agar, Urease agar, Safranin, Crystal violet, Ethanol, Malachite green, Gram’s Iodine, Hydrogen peroxide, Tryptic soy broth, Guaiacol, Collagen

Apparatus: Autoclave, Incubator, Laminar air flow chamber, Orbital shaker, Refrigerator, Hot air oven, Centrifuge, Calorimeter, Microscope, Weighing balance.

3.2. FROM SAMPLE COLLECTION TO ISOLATION OF COLONIES:

Soil samples were collected from waste disposal areas at Koladi (near Thiruverkadu) and Ayyapakkam (near Ultramarine pigments Ltd.) using sterile polythene bags and a spatula, and transported to the laboratory. Glassware was soaked overnight in a cleaning solution, washed thoroughly with tap water, cleaned with a detergent solution, rinsed with tap water and distilled water, and air-dried. Final sterilization of glassware

and media was performed in an autoclave at 15 psi and 120°C for 20 minutes. All necessary glassware and media were autoclaved. One gram of soil sample was added to 9 ml of distilled water to create a 10^{-1} dilution. A serial dilution was performed up to 10^{-9}. Samples from the 10^{-2}, 10^{-4}, and 10^{-6} dilutions were inoculated onto freshly prepared nutrient agar plates using both spread and pour plate techniques. The plates were incubated for 24 hours to observe viable colonies.

Composition of Nutrient Agar: 0.5% Peptone, 0.3% Beef extract/Yeast extract, 1.5% Agar, 0.5% Sodium Chloride, Distilled water; pH adjusted to 6.8 at 25°C. Spread Plate Method: 0.1 ml of sample from the 10^{-2}, 10^{-4}, and 10^{-6} dilutions was transferred onto the surface of solidified nutrient agar plates. Pour Plate Method: 1 ml of culture from the same dilutions was poured into petri dishes, followed by the addition of molten nutrient agar. All plates were incubated for 48 hours. Colonies from the spread plate were streaked onto Trypticase SoyAgar (TSA) plates using the quadrant streak method to obtain isolated colonies. The TSA plates were incubated at 37°C for 24 hours.· Composition of TSA: Pancreatic Digest of Casein (15.0 g), Peptic Digest of Soybean Meal (5.0 g), Sodium Chloride (5.0 g), Agar (15.0 g).Isolates were inoculated into 20 ml of Nutrient Broth. A control flask without inoculum was also prepared. The optical density (OD) of the culture media was measured at 540 nm every hour for 48 hours. After each measurement, the culture flask was incubated in an orbital shaker at 37°C.

A heat-fixed bacterial smear was prepared. The slide was sequentially flooded with crystal violet (1 minute), Gram's iodine (1 minute), a decolorizer (1-5 seconds), and safranin (30 seconds), with rinsing between each step. The stained slide was dried and observed under a microscope at 10x and 40x magnification.Malachite green applied with heat (5 min), then safranin counter stain which was then observed under microscope (10X–100X). This test was done to stain endospores.Biochemical tests were performed such as Urease Test, Citrate Test, catalase test, and the Oxidase Test.

3.3. ENZYME PRODUCTION:

Bacillus was streaked on collagen-nutrient agar; zone formation indicated degradation. Agar plates with/without guaiacol was streaked to observe color change after 7 days which indicated laccase.Optimization of Enzyme Production was done using the media (Nutrient broth + 0.5% collagen) and inducers [$CuSO_4$ (0.5–2.5 mM) for laccase; $MnSO_4$ (0.5–2.5 mM) for peroxidase].It was then stored throughnutrient agar slants streaked with isolates, incubated 24h, and stored at 4°C.The process began with the optimization of production media for peroxidase using different concentrations of an inducer, later specified as Manganese Sulphate.

Approximately 120 ml of Nutrient Broth media, supplemented with 0.5% collagen, was prepared and divided into six 20 ml aliquots. Each boiling tube, except for one which served as a positive control without the inducer, was inoculated with about 1 ml of Bacillus sp. seed culture. These tubes were then incubated at 37°C for seven days. Following incubation, 5 ml samples from each tube were centrifuged at 4000 rpm for 20 minutes. The resulting supernatant was analyzed for its optical density at 420 nm to gauge enzyme production. A parallel procedure was followed for the peroxidase enzyme, confirming the standardized protocol.

Subsequently, mass production of the enzymes was carried out at the previously identified maximum inducer concentration. For laccase, the inducer was Copper Sulphate ($CuSO_4$). The analysis focused on comparing the rate of collagen degradation in production media with and without the optimized inducer concentration. The production media for laccase was composed of Glucose (0.3%), Peptone (1%), KH_2PO_4 (0.06%), $ZnSO_4$ (0.0001%), K_2HPO_4 (0.04%), $FeSO_4$ (0.00005%), $MnSO_4$ (0.005%), $MgSO_4$ (0.05%), and Collagen (0.5%). About 200 ml of this media was prepared in distilled water, autoclaved, and divided into two aliquots. Both were supplemented with 0.5% collagen and inoculated with Bacillus sp., but one contained the maximum optimized concentration of inducer while the other had none. Similarly, for peroxidase, the production media was prepared using Nutrient Broth (2.8%) and Collagen (0.5%) in distilled water. After autoclaving, it was also divided into two aliquots, one with and one without its respective inducer (Manganese Sulphate). Both sets of production media were incubated for 24-48 hours at room temperature. The cultures were then centrifuged to obtain the crude enzyme, and the optical density of the supernatant was measured at 420 nm, marking the first step in enzyme recovery.

The crude enzyme extract then underwent a purification process, starting with partial purification via Ammonium Sulphate Precipitation. A five-day-old production culture was centrifuged at 4000 rpm for 15 minutes. The collected supernatant was treated with 70g of Ammonium Sulphate dissolved in 100 ml of the

crude extract. The mixture was gently shaken until the salt dissolved completely and then stored overnight at 4°C to allow for protein precipitation. The following day, it was centrifuged at 4000 rpm and 4°C for 30 minutes.

The supernatant was discarded, and the pellet containing the enzyme was resuspended in sodium acetate buffer. To further purify the enzyme and remove the salt, dialysis was performed. The ammonium sulphate-precipitated fraction was placed in a dialysis membrane and immersed in a beaker containing 50 ml of sodium phosphate buffer. This setup was placed on a stirrer at 37°C. The dialysis buffer was changed at regular intervals, with the process continuing for a total of approximately 27 hours, including an overnight step, to achieve equilibrium and effectively desalt the sample.Following purification, the protein concentration of the enzyme samples was estimated using Lowry's method, with Bovine Serum Albumin (BSA) as the standard.

A stock BSA solution was prepared, and a working standard was diluted accordingly. Key reagents included Reagent A (2% Sodium Carbonate in 0.1N NaOH), Reagent B (0.5% Copper Sulphate in 1% Potassium sodium tartrate), Reagent C (a mixture of A and B), and Reagent D (diluted Folin's reagent). For the standard curve, different volumes of the protein solution were mixed with Reagent C and D, incubated in the dark for 30 minutes, and the absorbance was measured at 540 nm. For the working enzyme sample, 100 µl was mixed with 5 ml of Reagent C and 0.5 ml of Reagent D, incubated similarly, and its absorbance was measured to determine the unknown protein concentration by comparison with the standard graph.

Enzyme activity was determined spectrometrically by preparing specific reaction mixtures. For laccase, the mixture contained 5mM Guaiacol in 50mM Phosphate buffer and 100µl of laccase enzyme. This mixture was pre-treated by heating at 50-60°C in a water bath, incubated for 20 minutes, and then the absorbance was measured at 470 nm every minute. For peroxidase, the reaction mixture was similar but included an additional 0.6mM of 3% Hydrogen Peroxide with 100µl of peroxidase enzyme. After incubation, its absorbance was measured at 436 nm every minute. A specific table outlined the composition of the reaction mixtures for laccase and peroxidase controls and their respective inducer-containing samples, detailing the volumes of Guaiacol, phosphate buffer, enzyme, and Hydrogen Peroxide used for each.

Finally, the molecular weight of the purified enzymes was determined using SDS-PAGE (Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis). Detailed recipes were provided for both the stacking gel and the separating gel (15%), which included components like acrylamide/bisacrylamide, Tris-HCl buffers, SDS, Ammonium Persulfate, and TEMED. A 5x sample loading buffer was also prepared. The gel was cast between two glass plates sealed with spacers. After polymerizing the stacking and separating gels, the samples, mixed with loading buffer and heated, were loaded into the wells. Electrophoresis was performed using a 1x running buffer at 100V until the tracking dye migrated sufficiently. Post-electrophoresis, the gel was carefully removed and stained overnight with a Coomassie Brilliant Blue solution. The gel was then destained with a solution of acetic acid and methanol until clear protein bands were visible against the background, allowing for molecular weight analysis.

3. Results

4.1.1. Sample Collection and Initial Isolation:

Samples were collected under sterilized conditions from two distinct locations: the Koladi and Ayyapakkamdumpyards. Initial microbiological analysis was performed using the serial dilution and spread plate technique on nutrient agar to isolate viable bacterial colonies. The colony counts at various dilutions are summarized in Table 4.1.

TABLE 4.1: BACTERIAL LOAD VIA SERIAL DILUTION OF SOIL SAMPLES

Location	Serial Dilution Count
Location I (Koladi)	10^{-2}
	10^{-4}
	10^{-6}
Location II (Ayyapakkam)	10^{-2}
	10^{-4}
	10^{-6}

From the numerous colonies obtained, three stable and distinct isolates were selected and purified for further analysis. These were designated as JKST1, JKST2, and JKST3.

Diagram 4.1: Isolation of Bacterial Colonies

The preliminary morphological characteristics of these three isolates were documented and are presented in Table 4.2.

Table 4.2: Morphological characteristics of the isolates

S.No	Colony	Colour	Shape	Consistency
1	JKST1	White	Regular	Mucoid
2	JKST2	White	Regular	Mucoid
3	JKST3	Pale Yellow	Mucoid	Mucoid

To classify the isolates based on cell wall structure and survival mechanisms, Gram's staining and Endospore staining were performed.

Gram's Staining: This differential staining technique categorizes bacteria as Gram-positive or Gram-negative based on their cell wall's ability to retain crystal violet dye. Isolates JKST1 and JKST2 appeared purple under the microscope, confirming them as Gram-positive bacteria. Isolate JKST3 appeared pink, identifying it as a Gram-negative bacterium.

Endospore Staining: This special stain identifies the presence of metabolically dormant endospores, a feature of certain genera like Bacillus. The staining revealed that only JKST2 formed endospores, visible as dark green structures within the pinkish-red vegetative cells.

Based on the results of the staining procedures, the isolates were clearly differentiated. Gram's staining revealed that isolates JKST1 and JKST2 were Gram-positive, as their cells retained the crystal violet dye and appeared purple. In contrast, isolate JKST3 was identified as Gram-negative, with its cells appearing pink after counterstaining. The endospore staining further characterized the isolates, showing that only JKST2 was a spore-forming organism, as indicated by the presence of dark green endospores. JKST1 and JKST3 both tested negative for endospore formation.

4.1.2. Biochemical Characterization of JKST2

Based on the promising results from the staining and growth curve analysis, isolate JKST2 was subjected to a series of biochemical tests for definitive genus-level identification. The results consistently pointed towards the genus Bacillus

1. Triple Sugar Iron (TSI) Agar Test

Both the slant and the butt of the agar turned yellow, indicating acid production from glucose, lactose, and/or sucrose. Gas formation was also observed, visible as cracking or lifting of the agar.

Interpretation: Acid/Acid with Gas production.

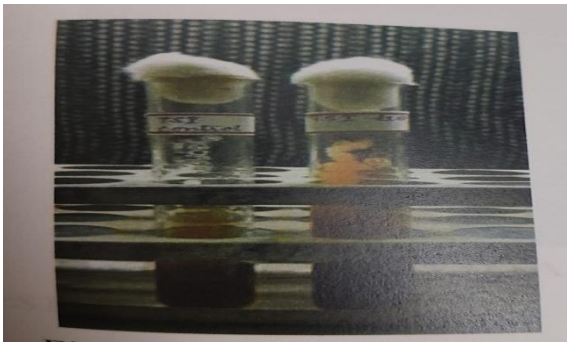

Fig 4.4.1: Triple Sugar Iron (TSI) Test

2. Urease Test

Positive. JKST2 produces the urease enzyme.

3. Citrate Utilization Test

The green Simmons' Citrate agar slant turned a distinct royal blue.· Interpretation: Positive. JKST2 can utilize citrate.

4. Catalase Test

Upon adding H_2O_2 , immediate and vigorous bubbling occurred. Positive. JKST2 produces catalase.

The study commenced with the screening of a bacterial strain, Bacillus sp. JKST2, for its potential to produce collagen-degrading enzymes. The qualitative screening on nutrient agar supplemented with collagen revealed the strain's capability, as evidenced by the formation of a large, transparent hydrolysis zone measuring 3mm in diameter around the bacterial This clear zone indicated the extracellular degradation of collagen, confirming the production of relevant enzymes. Concurrently, the strain was screened for laccase production on a specific medium containing guaiacol as a substrate. A positive result was confirmed by the development of a distinct reddish-brown zone around the colony, a characteristic color change

resulting from the oxidation of guaiacol by laccase enzymes

Following the successful screening, the production of both laccase and peroxidase was optimized by testing various concentrations of inducers. The inducer for laccase was Copper Sulfate ($CuSO_4$), while for peroxidase, it was Hydrogen Peroxide (H_2O_2). The optical density (OD) at 420 nm was used to measure enzyme production. The data revealed that 1.5 mM $CuSO_4$ yielded the maximum production for laccase (OD: 1.284), whereas 2.0 mM H_2O_2 was optimal for peroxidase (OD: 0.732) (Table 4.7 & Fig. 4.7: Optimization of inducer concentration). This graph clearly illustrates the specific inducer concentration that triggers peak enzyme yield for each respective enzyme.

The efficacy of these enzymes in collagen degradation was quantitatively assessed. When the bacterium was supplemented with collagen, the induced enzymes showed significantly higher degradation efficiency compared to the control (non-induced) enzymes. For laccase, the percentage degradation increased dramatically from 27.5% in the control to 80% when induced with 1.5 mM $CuSO_4$. Similarly, peroxidase-mediated degradation was enhanced from 57.4% in the control to 73.7% when induced with 2.0 mM H_2O_2. Collagen degradation by laccase and peroxidase enzymes).

These results underscore the critical role of inducer optimization in maximizing the enzymatic breakdown of collagen.To obtain a purer enzyme preparation for further characterization, the crude enzyme extracts were subjected to partial purification. The first step involved ammonium sulfate precipitation, a common technique to salt out proteins from a solution. The subsequent step was dialysis, which utilized a semi-permeable membrane to separate molecules based on size and diffusivity. Over 48 hours, low molecular weight impurities and residual salts diffused out into the surrounding buffer, leaving the purified enzymes retained inside the membrane. At the end of this process, the dialyzed, crude enzymes—laccase (control and induced) and peroxidase (control and induced)—were collected in clean.

The protein concentration of these purified enzyme samples was quantitatively determined using the established Lowry's method. A standard curve was first constructed using known concentrations of Bovine Serum Albumin (BSA) to correlate OD at 620 nm with protein concentration. The analysis of the enzyme samples revealed a substantial increase in protein yield upon induction. The concentration of laccase nearly doubled from 0.8 mg/ml (control) to 0.9 mg/ml (induced). A more pronounced effect was observed for peroxidase, where the protein concentration increased more than threefold, from 3.6 mg/ml in the control to 10.6 mg/ml with the inducer.

The functional activity of the enzymes was then determined by measuring the rate of guaiacol oxidation. Enzyme activity, defined as the number of units (U) per milliliter, where one unit typically corresponds to the amount of enzyme that converts one micromole of substrate per minute, was calculated. The results demonstrated a significant boost in activity for the induced enzymes. Laccase activity increased approximately six-fold, from 0.003 U/ml (control) to 0.020 U/ml (induced). Peroxidase activity also showed a marked increase of about 1.7-fold, from 0.027 U/ml to 0.046 U/ml upon induction with H_2O_2.

Finally, the molecular weights of the purified laccase and peroxidase enzymes were determined using Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). This technique separates proteins based on their molecular size under an electric field. The migration distances of the unknown enzyme proteins were compared to a standard protein ladder with known molecular weights. The analysis confirmed that laccase is a larger protein than peroxidase. The molecular weight of laccase was determined to be 52 kDa, and this size remained consistent between the control and induced samples. For peroxidase, the molecular weight was found to be 45 kDa for the control and 46 kDa for the induced sample, indicating a very similar size, with the minor difference likely falling within the margin of error for the technique or suggesting a slight modification. This characterization provides essential physical data for identifying and comparing these enzymes.

4. Discussion

Based on the provided data, the study successfully demonstrates the potential of a specific bacterial strain, Bacillus sp. JKST2, for the eco-friendly bioremediation of leather waste. Leather waste, which is approximately 80% protein, poses a significant environmental challenge. This research focused on using bacterial enzymes to degrade collagen, the primary protein in leather, as a sustainable alternative to conventional methods.

The bacterium was isolated from soil samples and confirmed to be a Gram-positive, spore-forming

Bacillus species through staining and biochemical tests. Initial screening confirmed its capability to degrade collagen, evidenced by a clear hydrolysis zone on collagen-supplemented agar. The organism was also confirmed to produce the oxidoreductase enzymes laccase and peroxidase, indicated by the formation of a reddish-brown zone when grown with specific substrates.

A key finding was that the production of these enzymes could be significantly enhanced by optimizing inducer concentrations. The study found that 1.5 mM copper sulphate ($CuSO_4$) and 2 mM manganese sulphate ($MnSO_4$) were the optimal concentrations for maximizing laccase and peroxidase production, respectively. This optimization had a direct and substantial impact on collagen degradation efficiency. While control samples (without inducers) showed degradation rates of 27.5% for laccase and 57.4% for peroxidase, the induced enzymes achieved markedly higher rates of 80% and 73.7%.

Following production, the enzymes were partially purified through ammonium sulphate precipitation and dialysis. Protein estimation using Lowry's method revealed that induction tripled the peroxidase yield (from 3.6 mg/ml to 10.6 mg/ml) and increased laccase yield. Furthermore, enzyme activity assays showed a six-fold increase in laccase activity and a 1.7-fold increase in peroxidase activity upon induction. Finally, molecular characterization via SDS-PAGE determined the molecular weights of the enzymes to be approximately 52 kDa for laccase and 46 kDa for peroxidase, which aligns with existing literature.This work establishes that Bacillus sp. JKST2 is a highly promising candidate for the biotechnological treatment of leather waste. By using simple inducers, the production and efficacy of its collagen-degrading enzymes can be significantly boosted, offering an effective, non-toxic, and environmentally friendly solution for managing this industrial pollutant.Future studies should focus on scaling the process by fully purifying and kinetically characterizing the enzymes, then optimizing large-scale production in bioreactors. Employing genetic engineering to enhance microbial strain efficiency and enzyme stability, while testing synergistic enzyme cocktails on real leather waste streams, will be crucial for developing a viable, eco-friendly industrial application for collagen degradation.

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