ISSN: 2633-4828 Vol. 8 No.1, January, 2026

International Journal of Applied Engineering & Technology

OPTIMIZATION OF INDUCER CONCENTRATION TO ENHANCE LEATHER DEGRADING ENZYMES OF BACILLUS SP ISOLATED FROM DIFFERENT ENVIRONMENTAL SAMPLES

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1. 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.*

2. 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 landfilled, but restrictions and high incineration costs have prompted the search for alternative treatments. The leather industry is recognized as one of the most polluting agro-based industries due to the large quantities of solid and liquid wastes generated during various stages of processing such as soaking, liming, deliming, pickling, tanning, and finishing. Leather, being a chemically stabilized collagen matrix, is intentionally made resistant to microbial degradation, which makes its disposal a major environmental concern. Chrome tanning, the most widely used method, introduces significant amounts of chromium salts into effluents, particularly hexavalent chromium, which is toxic, mutagenic, and carcinogenic. In addition, tannery effluents contain sulfides, chlorides, lime, synthetic dyes, and high concentrations of organic matter reflected in elevated Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD). When discharged untreated into water bodies, these effluents reduce dissolved oxygen levels, block sunlight penetration, disrupt photosynthesis, and cause severe harm to aquatic organisms. Solid wastes such as fleshings, trimmings, and buffing dust further contribute to land pollution when disposed of in landfills, where their slow degradation leads to long-term environmental contamination.

In response to increasing environmental regulations and the high costs associated with incineration and chemical treatments, bioremediation has emerged as a sustainable and eco-friendly alternative for managing leather industry waste. Bioremediation employs microorganisms or their enzymatic systems to detoxify, transform, or mineralize pollutants into less harmful substances. Among the promising microbial candidates, Bacillus subtilis, a non-pathogenic and widely studied soil bacterium, has shown considerable potential. This bacterium produces extracellular oxidative enzymes such as laccase and peroxidase, which are capable of breaking down complex organic molecules present in tannery waste. Laccases are multicopper oxidases that catalyze the oxidation of phenolic and non-phenolic substrates using molecular oxygen as the electron acceptor, producing water as a by-product. Peroxidases, on the other hand, utilize hydrogen peroxide to oxidize a broad range of organic compounds, including dyes and recalcitrant proteins, thereby facilitating the degradation of persistent pollutants.

The efficiency of enzymatic bioremediation can be significantly enhanced by optimizing growth and induction conditions. Metal ions such as copper sulfate ($CuSO_4$) and manganese sulfate ($MnSO_4$) act as inducers, stimulating the production and activity of laccase and peroxidase enzymes. Controlled parameters including pH, temperature, aeration, and nutrient availability further influence enzyme yield and degradation efficiency. Under optimized conditions, these enzymes can effectively degrade synthetic dyes, reduce BOD and COD levels, and facilitate the breakdown of collagen-based leather waste. Compared to conventional physical and chemical methods, enzyme-based treatment is cost-effective, energy-efficient, and environmentally benign.

Therefore, exploiting Bacillus subtilis-derived oxidative enzymes represents a promising biotechnological strategy for sustainable management of leather industry waste and mitigation of its environmental impact.

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.

3. MATERIALS AND 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. METHODOLOGY

3.2.1. 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 TrypticaseSoyAgar(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/withoutguaiacolwas 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.

4. RESULTS

4.1. SAMPLE COLLECTION AND INITIAL ISOLATION

Samples were collected under sterilized conditions from two distinct locations: the Koladi and Ayyapakkam dump yards. 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 4.1.1: BACTERIAL LOAD VIA SERIAL DILUTION OF SOIL SAMPLES

From - the

LOCATIONS	SERIAL DILUTIONS	COUNTS
LOCATION 1 (KOLADI SAMPLE)	10^{-2}	TNTC
	10^{-4}	TNTC
	10^{-6}	72CFU
LOCATION 2 (AYYAPAKKAM SAMPLE)	10^{-2}	TNTC
	10^{-4}	TNTC
	10^{-6}	45CFU

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

Fig 4.1.1: Isolation of Bacterial Colonies

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

TABLE 4.1.2: MORPHOLOGICAL CHARACTERISTICS OF THE ISOLATES

s.no	COLONY	COLOR	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.

TABLE 4.1.3: GROWTH CURVE OF ISOLATES:

TIME (hr)	CONTROL	JKST 1	JKST 2	JKST3
0	0	0.04	0.04	0.04
2	0	0.04	0.05	0.04
4	0	0.051	0.07	0.052
6	0	0.069	0.09	0.055

8	0	0.073	0.11	0.63
10	0	0.94	0.841	0.64
12	0	0.95	0.86	0.65
14	0	0.95	0.86	0.68
16	0	0.95	0.87	0.76
18	0	0.7	0.86	0.80
20	0	0.97	0.86	0.80
22	0	0.96	0.84	0.73
24	0	0.94	0.81	0.69

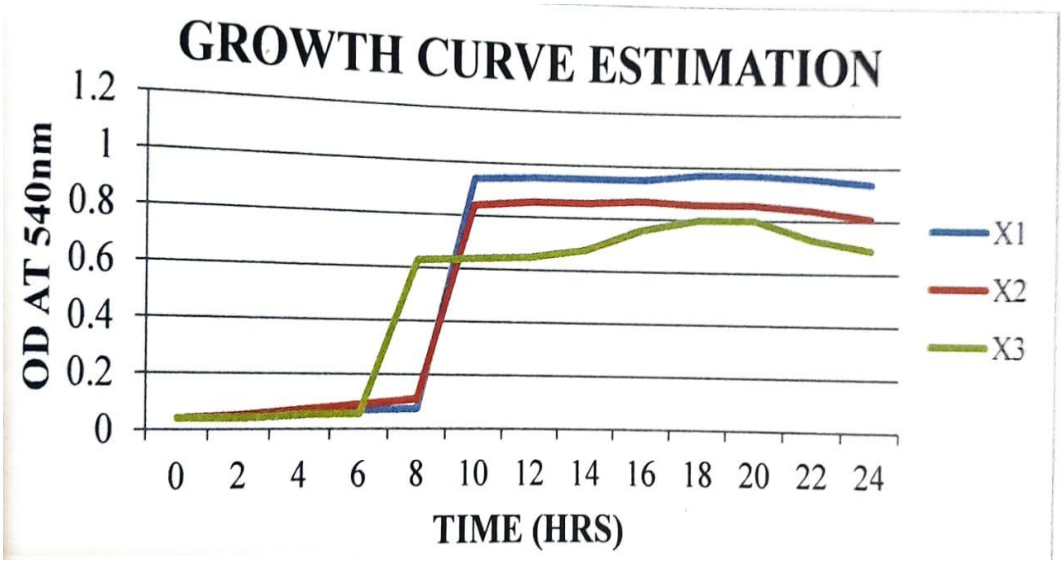

Fig 4.1.2 Growth Curve Estimation

4.2. BIOCHEMICAL TESTS:

Certain specific properties of Bacterial species were analyzed through Biochemical tests. Colony JKST2 showed positive results with all the biochemical tests mentioned below proving that JKST2 belongs to a Bacillus sp. From triple sugar ion test, it was confirmed that JKST2 was both slant and butt acidic with gas formation. Urease test results confirms that JKST2 could produce urease to hydrolyze urea. The color change indicates that JKST2 could utilize citrate as carbon source. Catalase test confirms the production of catalase enzyme. JKST2 showed positive results in oxidase test stating that it can produce cytochrome oxidase. Mannitol mobility test showed characteristic color change that confirms the bacteria was motile

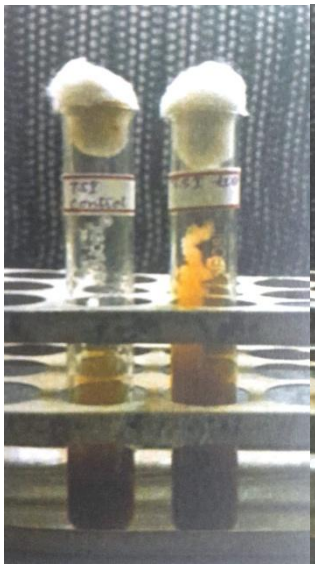

Fig 4.2.1 Triple Sugar Ion

Fig 4.2.2 Urease Test

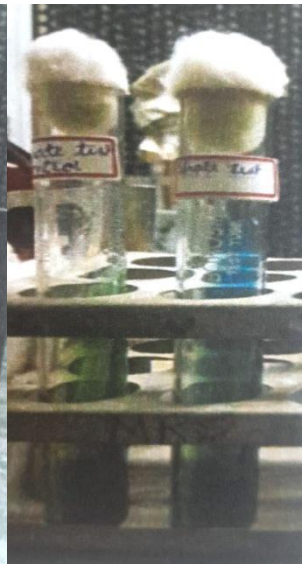

Fig 4.2.3 Citrate Test

Fig 4.2.4 Catalase Test

Fig 4.2.5 Mannitol Test

Fig 4.2.6 Oxidase Test

4.3. SCREENING OF COLLAGEN AND DEGRADATION (QUALITATIVE):

JKST2 was inoculated on Nutrient agar plate supplemented with collagen. After incubation, large transparent circle was found with 3mm of diameter.

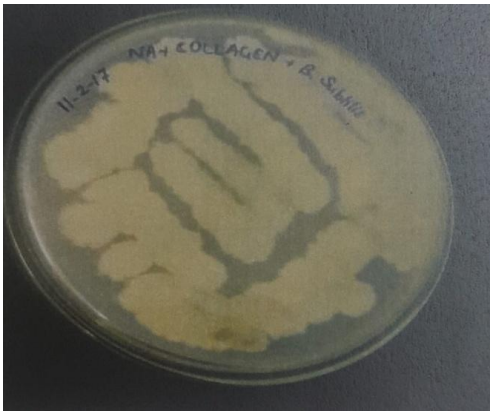

Fig 4.3 Transparent Zone Formation

4.4. SCREENING TEST FOR LACCASE PRODUCTION:

Under specific media for laccase, it could convert Guaiacol substrate which was observed by reddish brown zone formation.

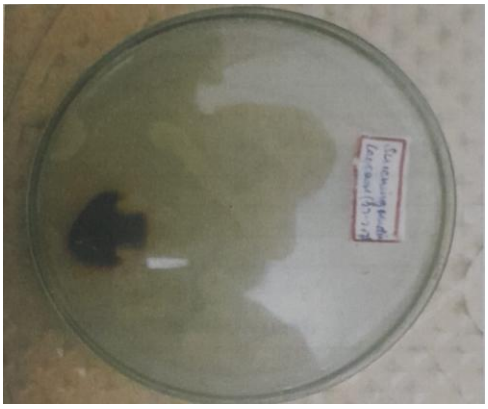

Fig 4.4 Formation of Reddish Brown Zone

4.5. OPTIMIZATION OF INDUCER CONCENTRATION FOR LACCASE AND PEROXIDASE:

With different inducer concentration given to the enzymes. 1.5mM of CuSO4 and 2mM of H2O2 was found to have maximum production.

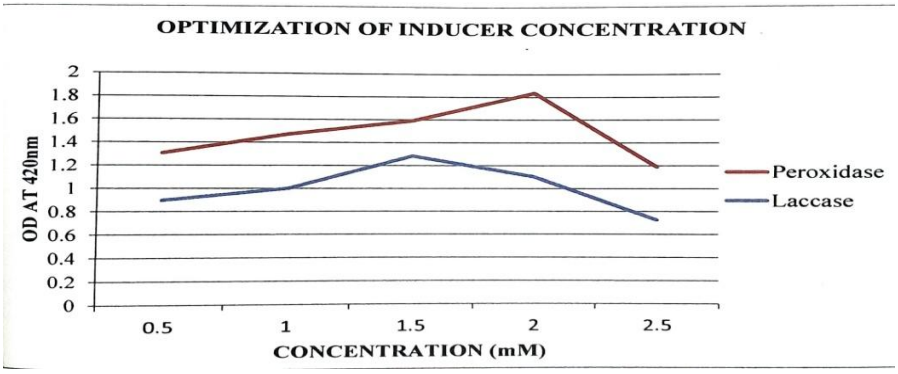

Fig 4.5 Optimization of inducer concentration

TABLE 4.5 OPTIMIZATION OF INDUCER

s.no	CONCENTRATION (mM)	LACCASE OD 420 nm	PEROXIDASE OD 420 nm
1.	0.5	0.895	0.412
2.	1.0	1.001	0.471
3.	1.5	1.284	0.308
4.	2.0	1.104	0.732
5.	2.5	0.725	0.465

4.6. PERCENTAGE DEGRADATION OF COLLAGEN:

When Bacillus sp was supplemented with 0.5 g (100%) Collagen, it showed 27% and 57% degradation by controlled enzymes respectively, whereas it was 80% and 73% by induced enzymes.

TABLE 4.6 COLLAGEN DEGRADATION BY LACCASE AND PEROXIDASE

FEATURES	LACCASE ENZYME		PEROXIDASE ENZYME	
	CONTROL	LACCASE INDUCER	CONTROL	PEROXIDASE INDUCER
INITIAL	0.24	0.151	0.792	0.881
FINAL	0.175	0.030	0.377	0.231
PERCENTAGE DEGRADATION	27.5%	80%	57.4%	73.7%

4.7. PARTIAL PURIFICATION AND DIALYSIS OF ENZYMES:

Following production, the enzymes underwent partial purification via ammonium sulphate precipitation, where the target crude enzymes, being charged proteins, combined with the salts and formed a pellet, leaving a supernatant containing cell organelles and other impurities. This crude enzyme pellet was then subjected to dialysis for 48 hours to remove small impurities like salts, leveraging differences in molecular diffusivity.

The actual enzymes were retained inside the dialysis membrane while impurities diffused out into the buffer, resulting in a purified enzyme preparation collected in a clean centrifuge tube.

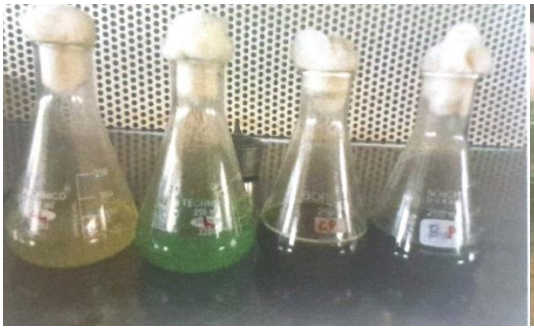

Fig 4.7.1 Supernatant (After

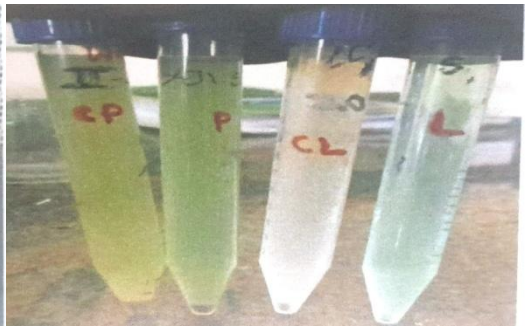

Fig4.7.2 Pellet (After ammonium salt precipitation) salt precipitation)

4.8. PROTEIN ESTIMATION BY LOWRY'S METHOD:

Quantitative measurement of enzymes were done by protein estimation (Lowry, et al, 1951 method). It showed that laccase produced by control was doubled with inducer and per-oxidase was increased more than 3 folds than the control.

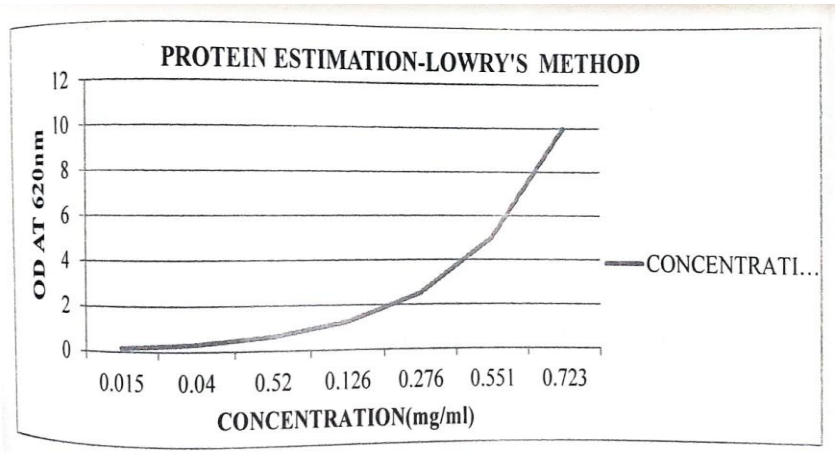

Fig 4.8 Protein estimation by Lowry's method

4.9. DETERMINATION OF ENZYME ACTIVITY

Based on the enzyme activity assay, the inducer caused a substantial increase in the activity of both enzymes. Laccase activity rose dramatically from 0.003 U/ml in the control to 0.020 U/ml in the induced sample, representing a six-fold enhancement. Similarly, peroxidase activity increased from 0.027 U/ml to 0.046 U/ml, showing a 1.7-fold increase upon induction.

4.10. MOLECULAR WEIGHT DETERMINATION BY SDS-PAGE

SDS-PAGE helps to identify the molecular weight of biomolecules based on diffusion under electric field. Molecular weight of unknown proteins can be determined by comparing with standard protein ladder . Laccase are larger than the peroxidases

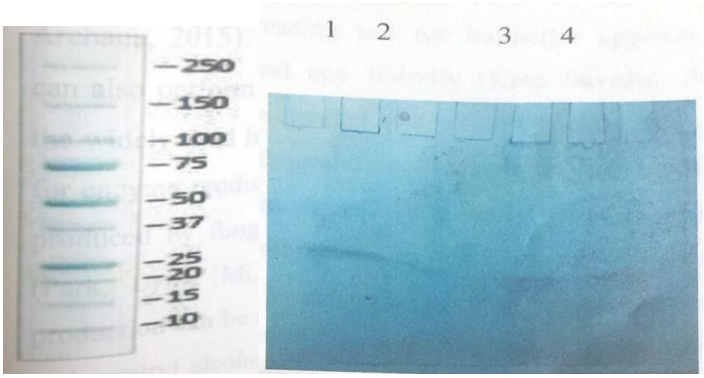

Fig 4.10 SDS-PAGE

1. Molecular weight of laccase in control: 52kDa
2. Molecular weight of laccase with 1.5mM inducer: 52kDa
3. Molecular weight of peroxidase in control: 45kDa
4. Molecular weight of peroxidase with 2mM inducer: 46kDa

.5. DISCUSSION

The study successfully demonstrates the potential of the bacterial strain Bacillus sp. JKST2 for the eco-friendly bioremediation of leather waste. Isolated from soil, this Gram-positive bacterium was confirmed to degrade collagen—the primary protein in leather—and produce the key oxidoreductase enzymes laccase and peroxidase.

A pivotal finding was that enzyme production could be significantly enhanced by optimizing inducer concentrations, specifically 1.5 mM copper sulphate for laccase and 2 mM manganese sulphate for peroxidase.

This optimization had a direct and substantial impact on the process. While control samples showed collagen degradation rates of 27.5% for laccase and 57.4% for peroxidase, the induced enzymes achieved markedly higher rates of 80% and 73.7%, respectively. Following production, partial purification revealed that induction tripled the peroxidase yield and led to a six-fold increase in laccase activity. Molecular characterization confirmed the enzymes' molecular weights to be approximately 52 kDa for laccase and 46 kDa for peroxidase, aligning with known literature.

6. CONCLUSION

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.

7. FUTURE STUDIES

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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