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Research Article

Molecular Engineering of the Thioredoxin (*trxA*) Gene in *Chryseobacterium proteolyticum* Enhanced its Keratinase Bioconversion of Chicken Feathers

¹Lindelwa Mpaka, ¹Nonso E. Nnolim and ¹Uchechukwu U. Nwodo

¹Patho-Biocatalysis Group (PBG), Department of Biotechnology and Biological Sciences, University of Fort Hare, Private Bag X1314, Alice 5700, South Africa

Abstract

Background and Objective: Keratinases are gaining traction in green technology and the bio-economy due to their ability to convert keratinous biomass into valuable products, such as animal feed. However, improving their output remains challenging. This study investigated genetic engineering approaches to increase keratinase production. **Materials and Methods:** The genome of the keratinolytic bacterium, *C. proteolyticum*, was sequenced using the Ion GeneStudio™ S5 Prime System, covering 2,484,843 reads, and sequence reads generated were assembled using SPAdes 3.13.0. The quality of the reads was assessed with QUAST V4.1, and the obtained sequences were annotated with Prokka 1.14.6, which revealed that *trxA* is associated with keratinolytic activity. The nucleotide sequence obtained was uploaded to the NCBI database and was assigned the accession number JBJLWK000000000. The *trxA* gene was amplified with primers from NCBI Primer-BLAST, cloned into a p-GEM®-T Easy vector, and transformed into *E. coli* JM109, and expressed using IPTG and SDS-PAGE analysis. The effects of pH and temperature on enzyme production were studied, and the recombinant strain's thermostability and tolerance to commercial laundry detergents were assessed. **Results:** The gene was successfully cloned and expressed, with SDS-PAGE showing a clear band at 11 kDa, matching *trxA*'s molecular weight. The pGEM-*trxA* construct demonstrated high keratinase activity across various pH and temperature conditions. Additionally, the recombinant strain showed excellent thermostability and detergent tolerance. **Conclusion:** These findings highlight the potential of recombinant technology to boost keratinase yields and support the sustainable recycling of keratinous waste.

Key words: Keratinases, *C. proteolyticum*, *E. coli* JM109, recombinant enzyme, proteolysis, sulfitolysis

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Corresponding Author: Lindelwa Mpaka, Patho-Biocatalysis Group (PBG), Department of Biotechnology and Biological Sciences, University of Fort Hare, Private Bag X1314, Alice 5700, South Africa

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The Large amounts of environmental waste generated by various agricultural sectors are rich in proteins and diverse carbon compounds. Because only minimal recycling of these wastes occurs, they accumulate in the environment and contribute to pollution, posing significant health risks. Additionally, these wastes contain high levels of keratin, an insoluble and resistant protein stabilised by disulfide bonds, hydrogen bonds, and hydrophobic interactions, making it resistant to traditional proteolytic enzymes¹. A group of proteolytic enzymes known as keratinases effectively degrade keratin-rich biomass, such as feathers, wool, nails, hair, and hooves².

Keratinases are inducible enzymes that have been reported in several microbial species, including *Bacillus licheniformis*³, *Bacillus pumilus* A1⁴, *Stenotrophomonas maltophilia* BBE11-1⁵, *Fervidobacterium* sp.⁶, and *Chryseobacterium* sp.⁷, to mention a few. The high specificity of keratinases for keratin biomass has attracted considerable attention to these enzymes for eco-friendly biological keratin recycling. Keratinases biologically convert keratin biomass into valuable compounds such as animal feed, detergents, textiles, fertilisers, and cosmetics².

The benefits of keratinases for keratin recycling have increased demand for these enzymes and driven efforts to increase enzyme production. Many studies have concentrated on isolating keratinolytic strains and on purifying and characterising keratinases^{1,8,9}, but few have focused on molecular cloning and keratinolytic genes to boost enzyme yields^{10,11}. Recently, molecular cloning and heterologous expression have been considered promising approaches for enhancing keratinase production. This study reports the molecular cloning of the *trxA* gene from *Chryseobacterium proteolyticum*, which exhibited keratinolytic activity by completely degrading chicken feathers as the sole carbon source, and employed the recombinant gene to increase enzyme yields.

MATERIALS AND METHODS

Study area and duration: The *C. proteolyticum* used to extract the *trxA* gene was previously isolated from the Fort Cox poultry slaughter facility located in the Eastern Cape, South Africa, in 2021. The isolate was stored in a -80°C freezer at the Patho-Biocatalysis laboratory (PBG) in the Department of Biotechnology and Biological Sciences at the University of Fort Hare, Alice campus in the Eastern Cape, South Africa. The

isolate was selected for this study based on its knack for feather degradation and extracellular keratinase production compared to other bacterial strains. The study was conducted from February 2023 to September 2024. Whole-genome sequencing was done at Central Analytical Facilities of Stellenbosch University, Stellenbosch, South Africa, and all other experiments in this study were carried out in PBG laboratory at the University of Fort Hare, Alice.

Media and plasmids: *Escherichia coli* JM109 cells, the p-GEM®-T Easy vector with multiple cloning sites, isopropyl β-d-1-thiogalactopyranoside (IPTG), X-gal, and the Wizzard® SV Gel and PCR Clean-up System were purchased from Promega, Madison, Wisconsin, United States. Ampicillin (50 µg) and other chemicals used in the study were purchased from Merck Life Science Limited (Johannesburg, South Africa) and were of analytical grade. All reagents used in the polymerase chain reaction (PCR), including target primers, master mix, and nuclease-free water, were procured from Inqaba Biotechnical Industries (Pty) Ltd. (Pretoria, South Africa). The gel electrophoresis reagents were obtained from Lasac (Pty) Ltd. (Port Elizabeth, South Africa).

Bacterial strain passaging: The previously isolated keratinolytic *C. proteolyticum*, maintained on a feather-agar slant, served as the wild bacterial strain for this study. The strain was incubated at 30°C for 48 hrs in media containing the following basal salts (K₂HPO₄; 0.3 g/L, KH₂PO₄; 0.4 g/L, MgCl₂; 0.2 g/L, CaCl₂; 0.22 g/L) and pulverised chicken feather (10 g/L) as the sole carbon and nitrogen source¹². This procedure was repeated 3-4 times to reacclimatize the strain for enhanced keratinous feather utilisation.

Validation of the enzyme activity from the previously isolated *C. proteolyticum*: The strain was evaluated for its ability to secrete keratinase-associated enzymes by running a keratinase enzyme assay and determining the thiol concentration.

Keratinase assay: Briefly, after strain passaging, about 2 mL of the inoculum from the fermentation broth was suspended in a microcentrifuge tube. The crude enzyme was extracted from the cells by centrifuging the inoculum at 15,000 × g for 10 min. The crude enzyme was subjected to a keratinase assay by suspending 14 mg of keratin azure in 0.5 mL of the crude enzyme and 0.1 M Tris-HCl buffer (pH 8). The reaction mixture was then incubated at 37°C and 220 rpm for 1 hr¹³. The reaction was terminated by placing the mixture on ice, and

the mixture was filtered through a 0.45 µm Millipore cellulose membrane filter (Merck (Pty) Ltd, Germany). Subsequently, absorbance was measured at 595 nm using a SYNERGY Mx 96-well microplate reader (Biotech Instrument Inc., Winooski, VT, USA) to determine the free azo dye released from the reaction mixture, together with a control. The control was prepared using the same method, except that the crude enzyme was not added to the reaction mixture. One keratinase unit (KU) was defined as the amount of enzyme causing an increase of 0.01 in absorbance per minute at 595 nm under the standard assay conditions described. Keratinase activity was calculated using the following formula:

$$\text{Keratinase activity (U/mL)} = \frac{\text{OD}_{595} \times \text{VET} \times d}{t \times \text{VE}}$$

where, OD is the optical density, VET is the total volume of the reaction mixture, d is the dilution factor, t is the incubation time, and VE is the enzyme volume in the reaction mixture, as reported by Nnolim *et al.*⁹.

Thiol concentration determination: The thiol in the filtrate was determined using 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB)¹⁴. The reaction mixture was prepared by dissolving the crude enzyme in DTNB, 100 mM phosphate buffer (pH 8.0), and distilled water. The mixture was allowed to stand at room temperature for 5 minutes. The control was prepared using the same procedure but without the crude enzyme. Absorbance was measured at 412 nm to determine the amount of free sulfhydryl produced. The concentration of the thiol group liberated in the medium was extrapolated from the cysteine standard curve prepared using 0- 550 µM of cysteine.

Whole genome sequencing and annotation: Genomic DNA was extracted from *C. proteolyticum* using the "salting out method" as described by Sunnucks and Hales¹⁵, and purified using magnetic beads as reported by Liu *et al.*¹⁶. The extracted gDNA was quantified on a Qubit™ 4 Fluorometer using the Qubit™ 1X dsDNA High Sensitivity (HS) Assay Kit (ThermoFisher Scientific), following the manufacturer's protocol. The quantified DNA was then screened using a NanoDrop® ND-1000 (ThermoFisher Scientific) to assess purity, and its genomic quality score (GQS) was determined using the LabChip GX Touch 24 Nucleic Acid Analyser (PerkinElmer, Waltham, MA, USA), following the manufacturer's protocol. The Ion Plus Fragment Library Kit (ThermoFisher Scientific) was used to prepare the quantified gDNA into a library, and the

library was quantified using the Ion Library TaqMan™ Quantitation Kit. The library was then amplified on the StepOnePlus™ Real-Time PCR System (ThermoFisher Scientific) using quantitative PCR to determine the library molarity and the respective barcode. Finally, the library was diluted to 40 pM and combined in equimolar amounts for template preparation using the Ion 510™, Ion 520™, and Ion 530™ Chef Kit (ThermoFisher Scientific) and loaded onto the Ion GeneStudio™ S5 Prime System for sequencing. The generated sequence reads were assembled with SPAdes 3.13.0 (Stellenbosch University HPC, Western Cape, South Africa)¹⁷, evaluated with QUAST V4.1¹⁸, and annotated with Prokka 1.14.6¹⁹.

Multiple amino acid sequence comparison: The Prokka gene annotation was used to identify the amino acid sequence of the target *trxA*. The National Centre for Biotechnology Information (NCBI) (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) was used to identify amino acid sequences similar to those of available *Chryseobacterium* reference strains on NCBI. The obtained similar amino acid sequences were aligned with those of *trxA* using Clustal Omega (CLUSTAL O (1.2.4) multiple sequence alignment to pinpoint the differences²⁰.

Establishment of the physicochemical properties of the *trxA*: The ExPASy-ProtParam tool was used to calculate the physicochemical properties of the amino acid sequences retrieved from Prokka, including the instability index, molecular weight (MW), aliphatic index, and theoretical isoelectric point (pI), as previously reported by Gasteiger *et al.*²¹.

Amplification of the *trxA* gene and visualization: The forward and reverse primers were designed and verified for target specificity using the Primer BLAST tool from NCBI (<http://www.ncbi.nlm.nih.gov/tools/primer-blast>). The forward primer sequence was identified as 5'-GGA TAG CTC ATT TCA G-3', while the reverse primer was 5'-CAC TTC TTT TGG TGA TAC TC-3'. A 25 µL PCR mixture was prepared, containing 12.5 µL of Master mix, 5 µL of DNA template, 1 µL of each primer, and 5.5 µL of nuclease-free water. The PCR was performed in a Thermocycler (Bio-Rad Laboratories (Pty) Ltd, South Africa) with the following profile: initial denaturation at 94°C for 5 min (one cycle), then 30 cycles of 94°C for 30 sec, 50°C for 1 min, 72°C for 1 min, followed by a final extension at 72°C for 10 min. The resulting amplicons were run on a 1.5% agarose gel for 45 min at 100 V, and visualised using a UV transilluminator (United Scientific (Pty) Ltd), revealing a 271 bp band consistent with the expected size.

Cloning of the *trxA* from the *C. proteolyticum*: The Wizzard® SV Gel and PCR Clean-up System was used to purify the PCR amplicons, and the purified amplicons were used as DNA templates for cloning. The purified PCR products and the p-GEM®-T Easy vector were digested with *EcoRI* (Promega, Madison, Wisconsin, United States), loaded onto a 1% agarose gel, run at 100 V for 45 min, and visualised on a UV transilluminator to confirm digestion. The *trxA* digest was cloned into the p-GEM®-T Easy vector using T4 DNA ligase. The recombinant plasmid p-GEM-*trxA* was transformed into *E. coli* JM109 cells following Promega's protocol. Clones were cultured on LB agar containing ampicillin (50 µg) and incubated at 37°C to select successful clones, and colony PCR was used to identify p-GEM-*trxA*-positive clones. The negative control for colony PCR was prepared by amplifying *E. coli* JM109 cells without the insert. To further confirm successful cloning, the recombinant pGEM-*trxA* was subjected to a restriction digest with the *EcoRI* endonuclease, which had been used previously for cloning. In brief, plasmid DNA was extracted from *E. coli* cells containing the pGEM-*trxA* recombinant DNA using the PureYield™ Plasmid Miniprep System (Promega, Madison, Wisconsin, United States). The extracted plasmid DNA was treated with *EcoRI* endonuclease following the user's protocol. The digested DNA was loaded onto a 2% agarose gel and run for 45 min at 100 V, then visualised on the UV transilluminator.

Expression of the recombinant gene (p-GEM-*trxA*) and

SDS-PAGE: The selected p-GEM-*trxA*-positive clone was cultured in 100 mL of LB ampicillin broth (50 µg) and incubated at 37°C until an OD₆₀₀ of 0.6 was reached. 0.1 mM IPTG was added to induce protein expression once the OD₆₀₀ of 0.6 was reached. The incubation temperature was then adjusted to 25°C to promote soluble protein expression, and the culture was incubated for a further 18 hrs. Cells were harvested by centrifugation at 13,000 rpm for 15 min and resuspended in cell lysis buffer containing 50 mM NaH₂PO₄·H₂O, 300 mM NaCl, and 10 mM imidazole, with the pH adjusted to 8.0 using NaOH²². Cells were disrupted by ultrasonication, and the recombinant protein was harvested by centrifugation at 13,000 rpm for 10 min at 4°C. The successfully extracted recombinant protein was stored at -20°C until further analysis. Subsequently, the extracted recombinant protein was subjected to a keratinase assay and thiol determination.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

(SDS-PAGE): The SDS-PAGE was used to estimate the molecular weight of the recombinant p-GEM-*trxA* clone on a 12% separating polyacrylamide gel, as described by Laemmli²³. A duplicate experimental run was carried out, and in each case, a total of 50 µL of sample buffer (0.5 M Tris-HCl, pH 6.8; 10% glycerol, v/v; 0.5% bromophenol blue, w/v; 10% SDS, w/v; and 50 µL of β-mercaptoethanol) was mixed with 25 µL of the extracted recombinant protein. The negative control was prepared by adding only the sample buffer without the protein. The reaction mixtures (sample and negative control) were heated for 5 min at 100°C before electrophoresis. The gel was stained with Coomassie Brilliant Blue R-250 and destained with acetate-methanol solution.

Evaluation of the keratinase production and disulfide reduction by the recombinant strain:

The recombinant p-GEM-*trxA* construct (2 mL) was inoculated into 98 mL of basal salt media supplemented with chicken feathers as the sole carbon and nitrogen source in 250 mL Erlenmeyer flasks. The flasks were incubated for 96 hrs at 37°C and 130 rpm to allow fermentation. Following incubation, the crude enzyme was extracted and subjected to the standard keratin azure method to determine the keratinolytic activity of the recombinant strain. The SYNERGYMx 96-well microplate reader (Biotech Instrument Inc., Winooski, VT, USA) was used to measure the released free azo dye from the reaction mixture, along with a control, at 595 nm. Consequently, the crude enzyme was also subjected to the DTNB method to determine the production of sulfhydryl groups. The thiol concentration was determined by measuring the free sulfhydryl produced at 412 nm using the SYNERGYMx 96-well microplate reader (Biotech Instrument Inc., Winooski, VT, USA).

Biochemical characterisation of the recombinant enzyme

(p-GEM-*trxA*): The activity and stability of the p-GEM-*trxA* recombinant enzyme were evaluated using two physicochemical parameters, namely pH and temperature.

Effect of pH on the recombinant keratinolytic enzyme activity and stability:

The effect of pH on the recombinant enzyme's activity was evaluated using the following buffer solutions: 0.1 M sodium citrate buffer (pH 4.0-5.0), phosphate buffer (pH 6.0-7.0), Tris-HCl (pH 8.0-9.0), and glycine-sodium hydroxide (pH 10.0-11.0). The recombinant enzyme's pH stability was evaluated by incubating the crude enzyme with buffer solutions at pH 6.0-9.0 at 30°C for 4 hrs, and the keratinolytic activity assay was measured at 1 hr intervals.

Effect of temperature on the recombinant keratinolytic enzyme activity and stability:

The effect of temperature on the recombinant enzyme was investigated by performing the standard enzyme assay over a wide temperature range, from 30 to 80°C, in 10°C increments. The enzyme thermostability profile was determined by incubating the enzyme at varying temperatures (50, 55, 60, 65, and 70°C) for 150 min, and aliquots were withdrawn at 30-minute intervals to assess residual keratinase activity.

Effect of laundry detergents on p-GEM-trxA stability:

The effect of laundry detergents on the stability of pGEM-trxA was investigated using selected commercially available solid laundry detergents, including OMO, Sunlight, and Surf (Unilever, La Lucia, Durban, South Africa), Maq (Bliss Brands (Pty) Ltd., Johannesburg, Gauteng, South Africa), and Ariel (Procter and Gamble, Sandton, Johannesburg, Gauteng, South Africa)²⁴. Briefly, 7 mg of each detergent was dissolved in 1 mL of tap water, heated at 100°C for 30 min to deactivate any endogenous enzymes, and then cooled to 50°C. The crude enzyme was added to the cooled detergent solution at a 4:1 ratio and incubated at 40°C for 60 min. The control was prepared by incubating the crude enzyme in tap water, then incubating it in the detergent solution described above.

Construction of optimal process variables for enhanced enzyme production by the pGEM-trxA:

The optimal process conditions for enhanced enzyme production by the recombinant strain were determined using the one-variable-at-a-time (OVAT) method, which evaluated the effects of pH and temperature on enzyme production.

Effect of temperature on the keratinase and thiol production by the recombinant strain:

The effect of temperature on keratinase and sulfhydryl group production was assessed by inoculating the pGEM-trxA strain into basal salt medium and fermenting at different incubation temperatures (25 and 50°C, at 5°C intervals) over 96 hrs. After 96 hrs of incubation, the cell-free extract was collected by centrifugation and used to determine keratinase activity using the standard keratinase assay protocol discussed above. Additionally, the cell-free extract was used to determine thiol concentrations in the fermentation media using the standard DTNB protocol.

Effect of pH on the keratinase and thiol production by the recombinant strain:

The p-GEM-trxA clone was inoculated into a basal salt medium adjusted to pH values ranging from

4.0 to 10.0 in 1-unit increments and incubated for 4 days at 30°C. Subsequently, the cells were used to recover crude enzyme by centrifugation at 000 rpm for 15 min. The recovered crude enzyme was used to determine keratinase activity using the standard keratinase assay and thiol content using the DTNB method.

Time course profile of keratinase activity by the pGEM-trxA:

To determine the time-course profile of keratinase activity produced by pGEM-trxA, a basal medium consisting of 0.03% K₂HPO₄, 0.04% KH₂PO₄, 0.02% MgCl₂, 0.022% CaCl₂, and 1% PCF was prepared and sterilised at 121°C for 15 min. The pH of the sterilised medium was aseptically adjusted to 7.0, and 3% (v/v) of the bacterial suspension was inoculated. The flasks were incubated at 30°C and 150 rpm for 96 hrs. During the 96 hrs fermentation, aliquots were aseptically withdrawn at 24 hrs intervals to determine keratinase activity, keratinase production, and thiol concentrations.

Statistical analysis: All the experiments were performed in triplicate unless otherwise stated. The data accrued were subjected to analysis of variance using the IBM Statistical Package for Social Science version 23, and the results were presented as mean with standard deviation. The mean significant difference was determined at p<0.05, using Duncan's Multiple Range Test.

RESULTS

Validation of the enzyme activity from the previously isolated *C. proteolyticum*:

The strain demonstrated significant potential to produce keratinase-associated enzymes, as it exhibited a remarkable keratinase activity of 706.36 U/mL and a thiol concentration of 123.68 µM.

Analysis of whole genome sequencing and gene annotation:

The whole-genome analysis showed that the *C. proteolyticum* genome contains 2,484,843 nucleotide bases and spans 804,134,986 bp. These sequences were then uploaded to NCBI GenBank, which assigned the accession number JBJLWK000000000. Prokka genome annotation identified 38 genes encoding keratinolytic enzymes, including the thioredoxin (*trxA*) gene. Further details indicated that the *trxA* gene is 271 bp long, has a locus tag MLPAAHBK_03368, and encodes 105 amino acids, with the amino acid compositions listed in Table 1.

```

WP_034741495.1  MALETIDSSFQETVLKSDKPVLVDFWAVWCGPCRTLGPPIIEEVASDFEGKAVVGKVDVDN  60
MLPAAHBK_03368  MALETIDSSFQETVLKSDKPVLVDFWAVWCGPCRTLGPPIIEEVATDFEGKAVVGKVDVDN  60
WP_034682284.1  MALETIDSSFQETVLKSDKPVLVDFWAVWCGPCRTLGPPIIEEVATDFEGKAVVGKVDVDN  60
WP_076561138.1  MALETIDSSFQETVLKSDKPVLVDFWAVWCGPCRTLGPPIIEEVATDFEGKALVGKVDVDN  60
*****

WP_034741495.1  NQEISMQYGIRNIPTVLIFKNGEVVDKLVGVTPKEVIAEKLSAHL  105
MLPAAHBK_03368  NQEISMQYGIRNIPTVLIFKNGEVVDKLVGVTPKEVIAEKLSAHL  105
WP_034682284.1  NQEISMQYGIRNIPTVLIFKNGEVVDKLVGVTPKEVIAEKLSAHL  105
WP_076561138.1  NQEISMQYGIRNIPTVLIFKNGEVVDKLVGVTPKEVIAEKLSAHL  105
*****

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Fig. 1: Multiple alignments of the recently established *Chryseobacterium* trxA (MLPAAHBK_03368) amino acid sequence to other thioredoxin *Chryseobacterium* genes from NCBI with accession numbers WP_034682284.1, WP_076561138.1 and WP_034741495.1

Table 1: Amino acid composition of the keratinolytic trxA

Amino acid	Frequency	Composition (%)
Alanine (A)	6	5.7
Arginine (R)	2	1.9
Asparagine (N)	4	3.8
Aspartic acid (D)	7	6.7
Cysteine (C)	2	1.9
Glutamine (Q)	3	2.9
Glutamic acid (E)	9	8.6
Glycine (G)	7	6.7
Histidine (H)	1	1.0
Isoleucine (I)	8	7.6
Leucine (L)	8	7.6
Lysine (K)	8	7.6
Methionine (M)	2	1.9
Phenylalanine (F)	4	3.8
Proline (P)	5	4.8
Serine (S)	5	4.8
Threonine (T)	6	5.7
Tryptophan (W)	2	1.9
Tyrosine (Y)	1	1.0
Valine (V)	15	14.3

Multiple amino acid sequence comparison: The comparison of thioredoxin amino acid sequences on the NCBI portal showed that our sequences were 100% identical to the thioredoxin sequence of *Chryseobacterium* species with accession number WP_034682284.1. Additionally, the trxA sequences showed 99% similarity to those of *Chryseobacterium* species with accession numbers WP_076561138.1 and WP_034741495.1. Multiple alignment revealed a dissimilarity of Leu52 between our trxA and the *Chryseobacterium* strain with accession number WP_076561138.1. In contrast, the variation between our trxA and the *Chryseobacterium* strain with accession number WP_034741495.1 was at Ser45. These amino acid differences and similarities are clearly displayed in the multiple alignment shown in Fig. 1.

Establishment of the physicochemical properties of the trxA: The ExPASy-ProtParam indicated that the established trxA exhibited an aliphatic index, molecular weight, theoretical

PI, and instability index of 106.57, 11.5 kDa, 4.64, and 9.21, respectively. The summary of the trxA physicochemical parameters is further graphically presented in Fig. 2.

Amplification of the trxA gene and visualisation: The polymerase chain reaction revealed that the primer sequences identified by the NCBI were specific for amplifying the trxA. The visualisation of the gel electrophoresis using a UV transilluminator revealed a 271 bp band, which is equivalent to the expected gene product, as shown in Fig. 3.

Cloning of the trxA from the *C. proteolyticum*: The digestion of the cleaned trxA amplicon was successful, as the gel electrophoresis after digestion by the *EcoRI* exhibited the product size of the trxA (271 bp), illustrated in Fig. 4a. Furthermore, the digestion of the pGEM easy vector was consequently successful, as the gel electrophoresis of the *EcoRI*-digested pGEM easy vector displayed the 3015 bp, which is the product size of the pGEM easy vector (Fig. 4b).

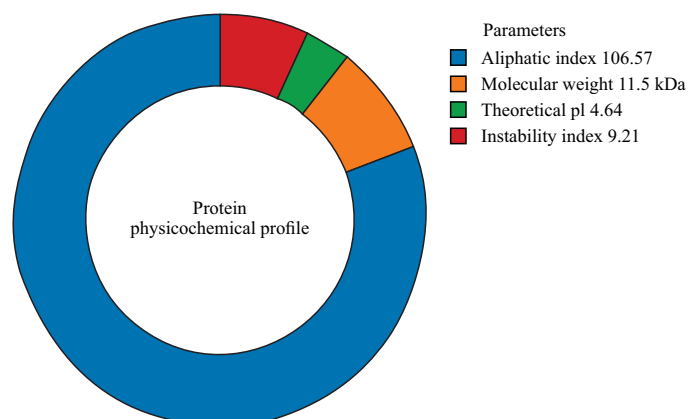


Fig. 2: Physicochemical properties of the thioredoxin, illustrating the overall biochemical profile of the protein

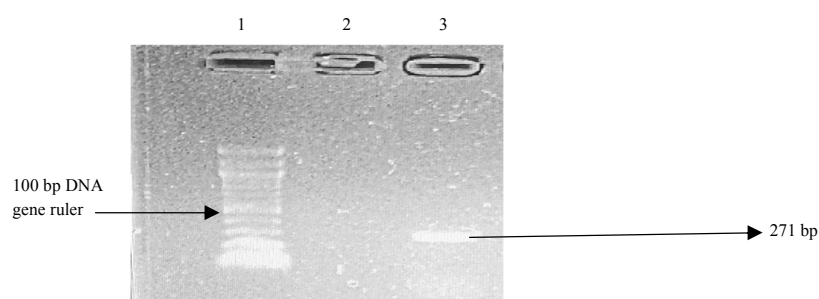


Fig. 3: Gel electrophoresis representing the gene product of trxA following amplification by the PCR using a specific primer sequence

Lane 1 consists of a 100 bp DNA marker, Lane 2 consists of a negative control, and Lane 3 consists of the band presenting positive amplification of the trxA (271 bp)

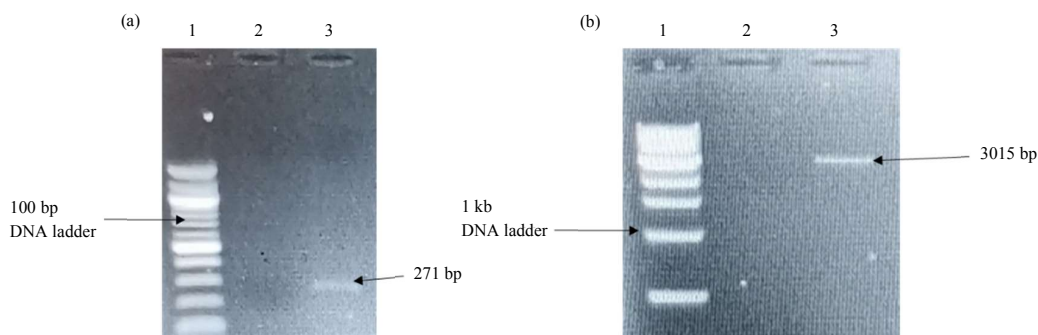


Fig. 4(a-b): Gel electrophoresis representing the restriction digest of the trxA amplicon and p-GEM easy vector with the *EcoRI* endonuclease (a) Display the restriction digest of the trxA amplicon, exhibiting the 271 bp gene product size; Lane 1: A 100 bp gene ruler, Lane 2: Negative control, Lane 3: Digested trxA DNA and (b) Gel electrophoresis showing the restriction digest of the p-GEM easy vector

Lane 1: A 1 kb gene ruler, Lane 2: Negative control, Lane 3: Digested p-GEM easy vector

After 24 hrs of incubation of the transformed *E. coli* JM109 cells in LB-ampicillin agar, the ampicillin-LB agar revealed white colonies representing presumptive colonies of the *E. coli* JM109 cells with the recombinant DNA (pGEM-trxA). Furthermore, colony PCR revealed a 271 bp band, confirming the presence of the insert DNA

in the *E. coli* JM109 colonies that grew on the LB-ampicillin agar plates (Fig. 5). Furthermore, the agarose gel of the restricted plasmid DNA of the pGEM-trxA cloned construct exhibited both the plasmid expected band size (3015 bp) and insert band size (271 bp), Fig. 6.

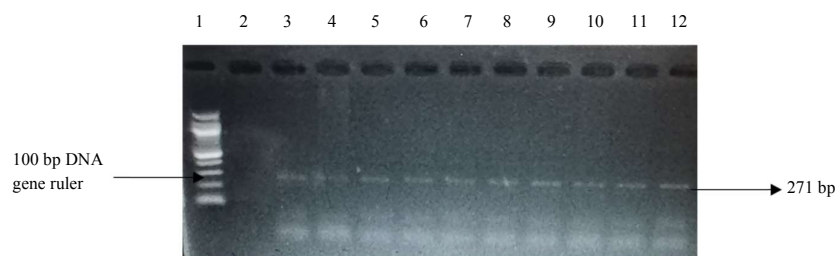


Fig. 5: Gel electrophoresis of the colony PCR confirming positive cloning of the pGEM-trxA

Lane 1 consists of a 100 bp DNA gene ruler, Lane 2 consists of the negative control (*E. coli* JM109 cells with no insert), and Lanes 3 to 12 consist of the positive pGEM-trxA constructs (271 bp)

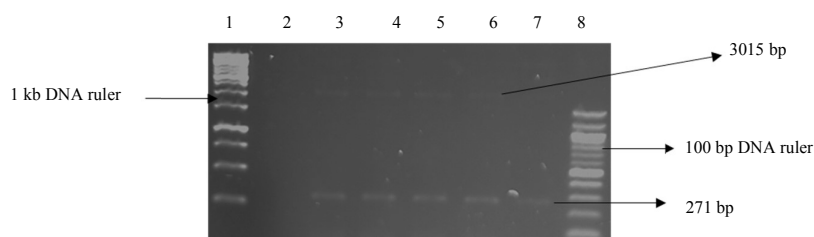


Fig. 6: Gel electrophoresis of the restriction digest confirming positive cloning of the pGEM-trxA

Lane 1 consists of a 1 kb DNA gene ruler, Lane 2 consists of the negative control, and Lanes 3 to 7 consist of the positive pGEM-trxA constructs with the band size of the insert (271 bp) and of the vector (3015 bp)

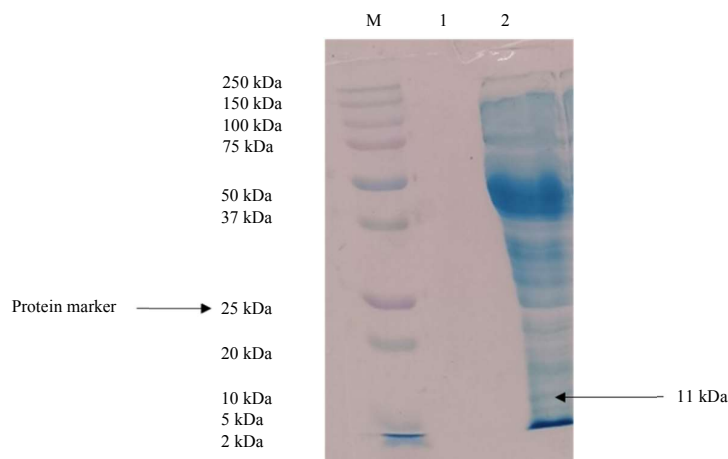


Fig. 7: SDS-PAGE analysis of the expressed recombinant enzyme in *E. coli* JM109

Lane M: Molecular weight marker, Lane 1: Negative control, Lane 2: p-GEM-trxA (11.5 kDa)

Expression of the recombinant gene (p-GEM-trxA) and SDS-PAGE

Keratinase assay and thiol concentration determination of the recombinant enzyme: The recombinant enzyme exhibited a tripled keratinase activity of 2166.37 U/mL, as compared to the wild strain, which possessed a keratinolytic activity of 706.36 U/mL. Equally, the recombinant enzyme demonstrated a high thiol concentration of 742.76 μ M, which is way higher than that of the wild strain (123.68 μ M).

SDS-PAGE: The SDS-PAGE confirmed successful expression of the recombinant p-GEM-trxA with IPTG, as the gel exhibited an 11.5 kDa protein band size (Fig. 7).

Evaluation of the keratinase production and disulfide reduction by the recombinant strain: Following the incubation of the recombinant strain on basal media with only chicken feathers as a sole carbon and nitrogen source, the strain demonstrated complete hydrolysis of the chicken

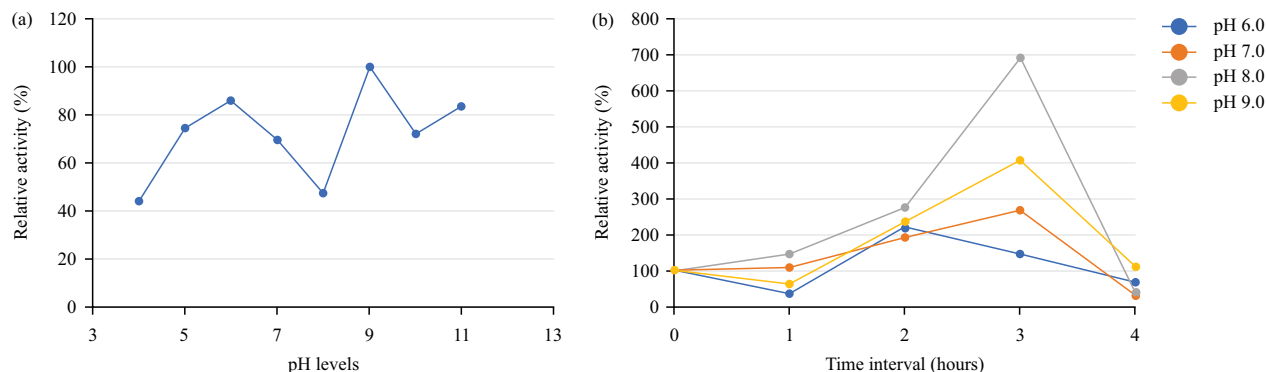


Fig. 8(a-b): Effect of pH (a) and stability (b) on p-GEM-trxA
Each point represents the mean value of duplicate assays

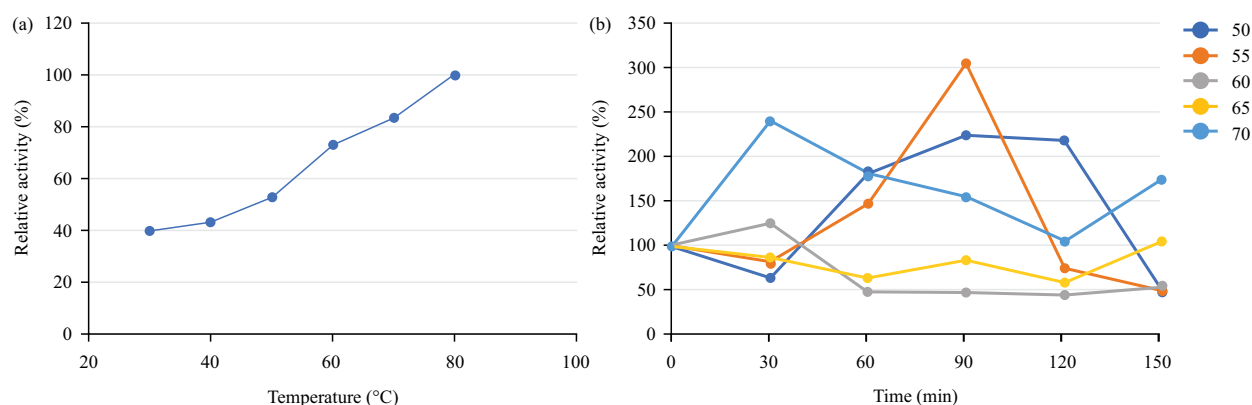


Fig. 9(a-b): Effect of temperature (a) and thermostability (b) of p-GEM-trxA

feathers. Additionally, the keratinase assay revealed that the recombinant strain exhibited an activity of 672.73 U/mL. Furthermore, a high thiol concentration level of 237.45 μ M was detected in the fermentation broth.

Biochemical characterisation of the recombinant enzyme (p-GEM-trxA)

Effect of pH on the recombinant keratinolytic enzyme activity and stability: The assessment of pH influence on pGEM-trxA activity revealed that the recombinant enzyme remained active across a broad pH spectrum, with peak catalytic efficiency observed at pH 9.0, as shown in Fig. 8a. The enzyme exhibited low activity at pH 4.0 (44.06%), which modestly increased to 74.32% at pH 5.0 and 85.77% at pH 6.0. Notably, enzyme activity sharply declined at pH 8.0 to 47.28%. The stability analysis (pH 6.0 to 9.0) indicated that pGEM-trxA was highly stable in different buffer solutions, with residual activities of 220.44, 266.02, 684.86, and 403.75% at pH 6.0, 7.0, 8.0, and 9.0%, respectively (Fig. 8b), suggesting exceptional stability at pH 8.0. Additionally, results showed that enzyme activity remains more stable between 2 and 3 hrs of incubation, compared to the first and fourth hours.

Effect of temperature on the recombinant keratinolytic enzyme activity and stability:

The investigation into how temperature affects pGEM-trxA showed that temperature significantly influences enzyme activity. The recombinant enzyme reached its peak activity at 80°C, with 100% relative activity. The lowest activity occurred at 30°C, with just 39.82%. As incubation temperature increased, enzyme activity also rose (Fig. 9a). The thermostability analysis indicated the enzyme was quite stable, retaining 118.21%, 161.02%, and 154.61% of its initial activity after 60, 90, and 120 min of preincubation at 50°C (Fig. 9b). Interestingly, the enzyme was even more stable at 70°C, with residual activities of 241.71, 180.82, 155.68, 105.35, and 175.02% after 30, 60, 90, 120, and 150 min of heating, respectively.

Effect of laundry detergents on p-GEM-trxA stability:

p-GEM-trxA showed high stability across the test laundry detergents, though to varying degrees. The enzyme showed optimum activity of 768.04% against MAQ washing powder. The enzyme showed activities of 707.15, 636.4, 636.4, 367.35, and 377.56% against Sunlight, OMO, ARIAL, and Surf washing powders, respectively (Fig. 10).

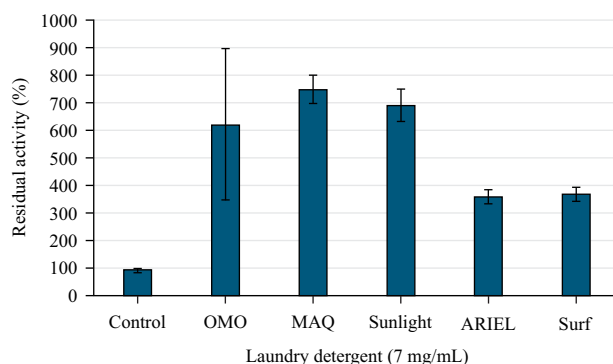


Fig. 10: Residual activity of the pGEM-trxA against the test commercial laundry detergents

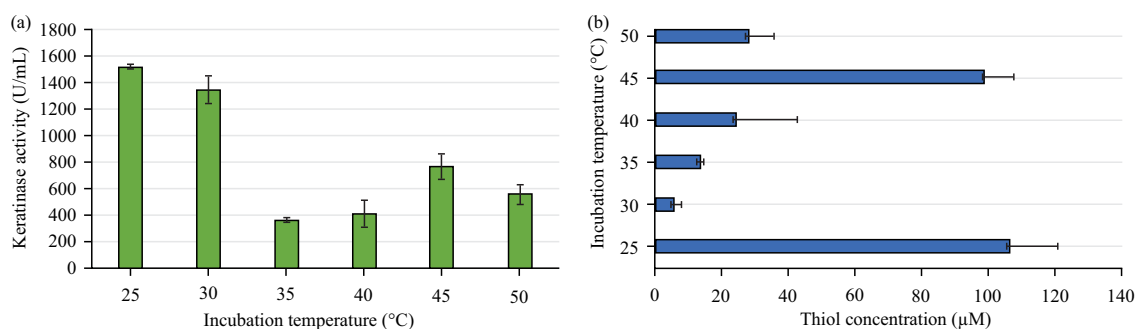


Fig. 11(a-b): (a) Effect of temperature on keratinase production and (b) sulfhydryl groups

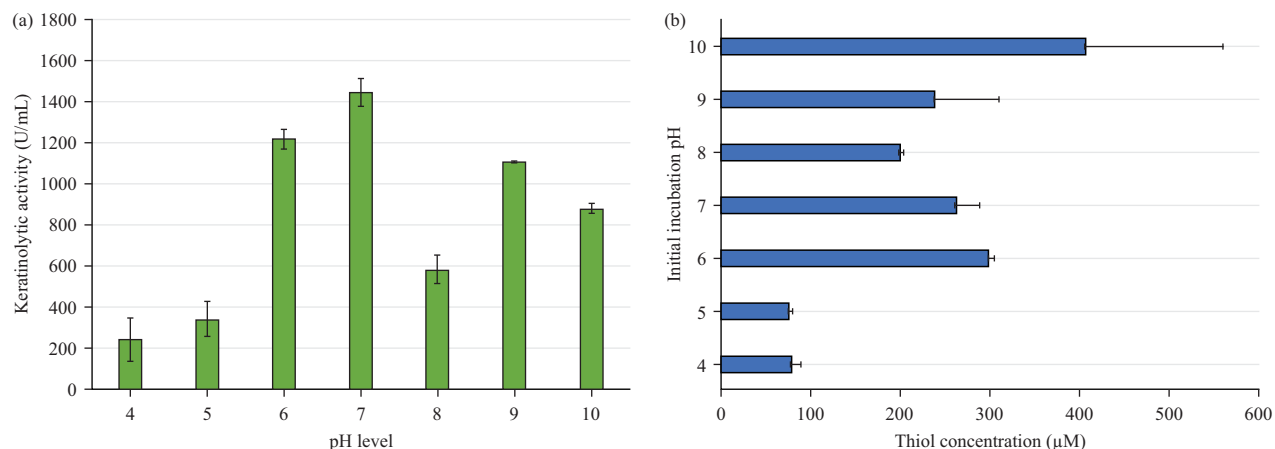


Fig. 12(a-b): Effect of pH on enzyme production by the pGEM-trxA strain over 96 hours of incubation (a) Represents the effect of pH on keratinase production by the pGEM-trxA strain and (b) Represents the effect of pH on thiol production by the pGEM-trxA

Evaluation of optimal process variables for enhanced enzyme production by the pGEM-trxA

Effect of temperature on the keratinase and thiol production by the recombinant strain: A study examining the impact of temperature on enzyme production found that the recombinant strain produced keratinases, along with sulfhydryl groups in the fermentation medium. The highest keratinase activity

(1511.82 U/mL) and thiol levels (104.595 μM) were recorded at 25°C. Keratinase production decreased at 30 to 1340.91 U/mL and dropped significantly to 358.185 and 403.645 U/mL at 35 and 40°C (Fig. 11a). Similarly, thiol concentration sharply declined at 30 and 35°C, reaching 5.655 and 13.425 μM, respectively (Fig. 11b). Interestingly, both keratinase and thiol production showed a slight increase at 45°C.

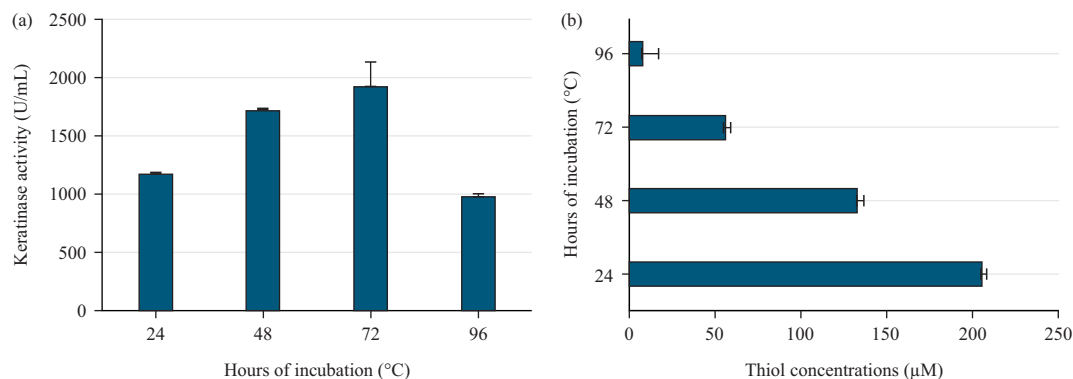


Fig. 13(a-b): Time course profile for the recombinant strain over 96 hours of incubation (a) Presenting keratinase production over the 96 hrs of incubation and (b) Representing the production of sulfhydryl groups

Effect of pH on the keratinase and thiol production by the recombinant strain:

The strain demonstrated keratinase production across all tested pH values; however, pH 4.0 and 5.0 appear to disfavour keratinase production, yielding 246.365 U/mL and 347.27 U/mL, respectively. Similarly, thiol yields were low at these pH levels, with 72.08 and 74.17 μM at pH 4.0 and 5.0, respectively. Keratinase production increased at pH 6.0 (1240.91 U/mL) and 7.0 (1471.82 U/mL), as illustrated in Fig. 12a. Subsequently, a drastic increase in thiol production was observed from pH 6.0 to pH 10.0 (Fig. 12b).

Time course profile of keratinase activity by the pGEM-trxA:

During the first 24 hrs of incubation of the recombinant strain, keratinase production was already high at 1192.73 U/mL, surpassing that of the wild strain, which produced 706.36 U/mL after 96 hrs. The strain continued to show increased keratinase output at 48 hrs (1750.91 U/mL) and 72 hrs (1963.635 U/mL). However, keratinase levels decreased to 999.09 U/mL after 72 hrs (Fig. 13a). Conversely, thiol concentrations were high between 24 hrs (206.36 μM) and 48 hrs (133.57 μM), as shown in Fig. 13b. A significant decline in thiol levels occurred between 72 hrs and 96 hours of incubation.

DISCUSSION

An organism's remarkable ability to degrade keratinous biomass stems from a group of enzymes that break the cross-linked disulfide bonds in keratin, which make this protein resistant to standard proteolytic enzymes. Several species naturally produce these enzymes, including members of the *Bacillus* genus, as well as *Chryseobacterium* spp., *Xanthomonas* spp., *Burkholderia* spp., *Pseudomonas* spp., *Thermoanaerobacter* spp., and *Fervidobacterium* spp.^{3,6,25}

Although many bacterial and fungal strains can degrade keratinous waste like chicken feathers, these strains are often fastidious, toxic (especially fungi), and require longer incubation times. This limits their effectiveness despite their significant enzymatic potential in biotechnology and industry. Moreover, the keratinase production from these strains typically does not meet industrial demands. Therefore, there is a need to develop cost-effective, environmentally friendly methods to overcome these limitations, with genetic engineering being a promising approach. Consequently, this study focused on cloning the *trxA* gene to improve keratinase yields for converting feather waste into useful peptides.

The validation of the wild strain's ability to produce keratinases confirmed that it could still secrete these enzymes. Giwu *et al.*²⁴ previously reported a keratinolytic titre of 693.63 U/mL for this isolate, which is comparable to the 706.36 U/mL observed in this study. Furthermore, the high thiol concentrations measured indicate effective disulfide bond breakdown in the chicken feathers added to the media.

Analysis of the entire genome of the previously isolated *C. proteolyticum* showed it contained approximately thirty-eight genes related to keratin breakdown. These genes play various roles, including sulfitolysis and proteolysis. The presence of both sulfitolytic and proteolytic genes indicates that keratin degradation involves multiple enzymatic steps, as noted by Yamamura *et al.*²⁶. Among the identified keratinolytic genes was thioredoxin, known for its ability to reduce disulfide bonds, which is a key initial step in keratinolysis.

The target *trxA* consisted of a chain of 105 amino acids, with the proportions of each amino acid specified. Among these identified amino acids, cysteine and serine residues were present. This confirms that *trxA* was responsible for keratin degradation in chicken feathers, as cysteine is well known for its ability to reduce cystine disulfide bonds to two cysteine thiols. Additionally, the presence of serine in the *trxA*

amino acid composition indicates that the enzymes responsible for keratin degradation possess serine catalytic activity²⁷. These amino acids were also identified in the hydrolysate generated from the degradation of chicken feathers by keratinolytic *Chryseobacterium proteolyticum* FGNn, as reported by Giwu *et al.*²⁴.

The amino acid sequence of the target trxA showed 100 and 99% similarity to three trxA sequences identified in previously isolated keratinolytic *Chryseobacterium* species, suggesting that this enzyme is a novel keratinase. The 1% divergence in amino acid sequences between the current trxA sequence of *C. proteolyticum* and the *Chryseobacterium* species with accession numbers WP_076561138.1 and WP_034741495.1 might be due to strain variation.

The aliphatic index is an essential physicochemical property that contributes to protein thermal stability. The target trxA displayed a high aliphatic index, indicating that this enzyme is thermostable, as proteins with a high aliphatic index are more thermostable than those with a low aliphatic index²⁸. Furthermore, this aliphatic index indicates that the enzyme contains high concentrations of hydrophobic amino acids, such as alanine, isoleucine, leucine, aspartic acid, and valine. The instability index of the trxA protein was 9.21, indicating that it is stable, as amino acid sequences with an instability index below 40 are considered stable. The theoretical PI of this enzyme suggested it was basic, as proteins with a PI <7 are considered basic²⁹. Unlike the widely reported keratinolytic enzymes, the current trxA enzyme had a molecular weight of 11.5 kDa, whereas reported keratinases range from 17 to 240 kDa^{5,25}.

The amplification of trxA was successful, as amplicon visualisation showed a prominent 271 bp band corresponding to the expected gene product size. Additionally, the presence of the 271 bp band indicates that the NCBI system is an excellent tool for primer design. Digestion of the amplicon and the vector with *EcoRI* was successful, indicating that *EcoRI* was a suitable restriction enzyme for both the vector and the target gene.

Subsequently, ligation and transformation of the resulting recombinant DNA into competent *E. coli* JM109 cells were successful. This was evidenced by the growth of *E. coli* on LB-Ampicillin agar, facilitated by the presence of the ampicillin resistance gene in the p-GEM® Easy vector system. All plasmids carry a genetic marker, such as a resistance gene, that facilitates the selection of positive transformants³⁰. In this study, the p-GEM® Easy vector system comprises a lac operator and a β -lactamase coding region, which confers ampicillin resistance; therefore, transformants that grew on LB ampicillin plates were considered presumptive positive clones, as they

are predicted to have acquired the target recombinant DNA. Furthermore, growth of the transformants on LB ampicillin agar indicates excellent transformation efficiency, meaning the cells could take up the foreign DNA from the external environment and express the genes encoded by it³⁰.

Observation of *E. coli* JM109 cells on LB-ampicillin agar plates does not conclusively confirm successful cloning and transformation; therefore, colony PCR was used to provide further confirmation. The colony PCR confirmed successful cloning, as the insert size was detected. The detection of the insert or plasmid size can confirm successful cloning. In this study, the colony PCR was used to target the insert size. However, colony PCR is sometimes associated with false positives, underscoring the need to further confirm successful cloning. The restriction digest of the cloned construct showed both the insert and vector sizes, indicating that the clones were successfully generated.

The SDS-PAGE results revealed a prominent band of about 11.5 kDa, confirming expression of trxA in response to IPTG. In addition, the prominent bands on the SDS-PAGE gel indicate that the expressed proteins were completely removed from inclusion bodies by sonication. However, this molecular weight is unusual compared with other characterised keratinolytic genes. Most reported keratinolytic-associated enzymes range from 17 to 240 kDa^{5,25}. Keratinolytic enzymes have a wide range of molecular weights, and the low amino acid sequence of trxA may have contributed to its low molecular weight.

When the recombinant strain was incubated in basal salt media with chicken feathers as the sole carbon and nitrogen source, it achieved complete feather hydrolysis. The ability of the recombinant strain to completely valorise chicken feathers indicated that it possessed biochemical characteristics like those of native keratinases^{1,9,24,27}. Furthermore, the recombinant strain exhibited high keratinolytic activity and elevated free-thiol concentrations in the fermentation broth. This indicates that the *E. coli* JM109 cells possessed trxA, which enabled them to break down cross-linked disulfide bonds; hence, the fermentation broth had high thiol concentrations (237.45 μ M).

In this study, the pGEM-trxA recombinant enzyme showed peak activity at pH 9.0. Moreover, pGEM-trxA exhibited remarkable stability across different buffer solutions. This was consistent with other previously keratinolytic proteases, which demonstrated highest catalytic activity under neutral to alkaline conditions, with an optimal pH between 7.0 and 10.0, regardless of microbial origin^{4,5,9}. Consequently, pGEM-trxA holds significant promise for various industrial applications, such as leather processing and animal feed production^{1,24}.

The recombinant enzyme exhibited stability across various buffer solutions and showed remarkable thermostability over a broad pH range, with maximum activity at 80°C. Notably, the optimal temperature for enzyme stability in this study exceeded the typical range reported for many bacterial keratinases, which generally have optimal activity between 30 and 75°C^{9,27}. The pGEM-trxA enzyme can be classified as hyperthermophilic. Such enzymes are known to have optimal activity above 75°C, including *Fervidobacterium islandicum* AW-1 with an optimum at 100°C²⁴, *Bacillus circulans* DZ100 with an optimum at 85°C, and *Sphingobacterium multivorum* HNFx with an optimum at 90°C³¹. The temperature tolerance observed in this keratinolytic clone highlights its potential for biotechnological and industrial applications.

The world faces high carbon dioxide emissions, and traditionally, laundry soaps contain high levels of phosphates and phosphonates, which contribute to 60% of carbon dioxide emissions. This has emphasised the need to investigate enzymes to replace phosphates and phosphonates in detergents. The current recombinant keratinase enzyme showed a high degree of tolerance to the test commercial laundry detergents. This activity was relatively high compared with that of previously characterised keratinases from wild strains in a similar detergent panel^{24,31,32}. Therefore, the current recombinant retains efficacy as an additive in detergent formulations, replacing phosphates and phosphonates in the laundry industry.

Temperature and pH are key physicochemical factors influencing enzyme production, especially keratinase. In this study, these parameters were examined. The pGEM-trxA strain showed high keratinase activity at mesophilic temperatures of 25 and 30°C, indicating it is a mesophilic bacterium with significant potential to promote the green economy and bioprocessing. Most keratinase-producing organisms, including *Chryseobacterium* species where the cloned gene was originally isolated^{24,27} and *Bacillus* species^{11,33}, also exhibit keratinase activity at mesophilic temperatures (20-50°C). These variations in keratinase output across different temperatures highlight the crucial role of temperature in extracellular enzyme synthesis.

The study revealed that pH plays a vital role in the production of extracellular enzymes, as the recombinant strain showed varying keratinase production across different incubation pH ranges. The pH of the culture medium is an essential factor for microbial growth, plasma membrane integrity, and transport protein activity⁹. High yields of keratinase production by the recombinant strain were observed at slightly acidic to neutral pH. These findings align with keratinase outputs reported for previously described keratinolytic species^{12,24,27,33}.

The time-course study revealed that the recombinant strain produced significantly higher enzyme levels as early as 24 hrs of incubation, whereas most keratinolytic wild strains only show high yields after 48 hrs^{24,31,32}. Most wild strains require optimised conditions to achieve the enzyme activity demonstrated by the pGEM-trxA within the first 24 hrs. This highlights the immense potential of gene cloning to enhance keratinase outputs and advance waste management, particularly for chicken feather waste. The decrease in enzyme production observed after 72 hrs of incubation may be attributed to rapid cell proliferation, which may have led to the depletion of nutrients in the fermentation media and the accumulation of secondary metabolites. Consequently, the high concentrations of free thiols observed in the first 48 hrs of incubation signify the cleavage of disulfide bonds from the chicken feathers, which were supplemented as the sole carbon and nitrogen source. The cleavage of these disulfide bonds enables the proteolytic hydrolysis of chicken feathers into amino acids with vast application potential^{24,33}.

CONCLUSION

The pGEM-trxA strain has shown significant keratinase production, demonstrating that disulfide reductase plays a key role in keratin degradation by loosening the tightly coiled keratin and exposing it to proteolytic enzymes. Furthermore, the high keratinase yields from the recombinant strain highlight the potential of genetic engineering as a rapid, cost-effective, and powerful approach for valorising keratin-rich biomass. The strain not only produced high keratinase levels but also exhibited excellent catalytic properties, including thermostability and stability in laundry detergents. With these attributes, this recombinant strain offers promising applications in various biotechnological fields such as laundry, waste management, and agriculture. Overall, this study emphasises the importance of genetic engineering to enhance keratinase production for industrial-scale applications.

SIGNIFICANCE STATEMENT

Keratinous waste generated from agricultural and poultry industries, including chicken feathers, hair, nails, claws, and animal hides, poses significant environmental and public health concerns due to its resistant structural composition and slow degradation. Despite its potential for conversion into valuable bio-products such as peptides, animal feed, and biofertilizers, efficient recycling remains limited because of low keratinase production by microorganisms. This study

highlights the significance of genetic engineering to enhance keratinase yield through molecular modification, thereby improving the biodegradation and bioconversion efficiency of keratin-rich waste into value-added products. The findings provide a sustainable strategy for managing increasing keratinous waste and promoting environmental bioremediation.

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