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RESEARCH ARTICLE OPEN ACCESS

Genome Mining of *Chryseobacterium Proteolyticum* for Keratinolytic Properties and Prospective Applications

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Abstract

The manipulation of keratinolytic determinants provides an avenue for enhancing keratinase output, thereby facilitating the conversion of wasted chicken feathers into valuable products. Thus, this study investigated the keratinolytic determinants expressed by *Chryseobacterium proteolyticum* during the degradation of chicken feathers. The bacterial genome was sequenced using the Ion GeneStudio™ S5 Prime System, and SPAdes was used to assemble the raw sequences, while Prokka was used to annotate the obtained sequences. The keratinolytic genes were amplified by polymerase chain reaction (PCR). A total of 4.84 Mb bases were generated from the bacterial genome, which yielded thirty-seven contigs ranging between 165 bp and 1242714 bp when assembled by SPAdes. Prokka annotated 4798 genes, of which thirty-eight were identified as keratinolytic enzyme-coding genes. The *resA* coding for thiol-disulfide oxidoreductase was the most occurring keratinolytic determinant with a proportion of 26.3% (10/38), followed by *dap* coding for D-aminopeptidase (13.2%; 5/38). PCR revealed that *resA* exhibited a band size of 646 bp. The successful amplification of the keratinolytic determinants from this potent keratin degrader, *C. proteolyticum,* confirms its dexterity for keratinolysis and may pave the way for genetic engineering to enhance keratinase output.

Keywords: Keratinase, Chicken Feathers, Disulfide Reductase, Aminopeptidases, *Chryseobacterium proteolyticum*

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INTRODUCTION

The increased consumption of chickens has resulted in the generation of millions of tons of chicken feathers annually, a waste with no efficient recycling strategies to manage the waste biomass.[1] If handled poorly, poultry waste may pose a hazard to the environment and humans. Chicken feathers are insoluble and resistant to degradation due to the presence of a high amount of fibrous protein - keratin (90%). Keratin is structurally stabilized by the inherently interconnected disulfide, hydrogen, and hydrophobic bonds, and thus is resilient to decomposition under natural environmental conditions.[2] Numerous waste management methods have been devised to curb chicken feather's environmental accumulation. Typically, chicken feathers are disposed of through landfilling, and this disposal approach could promote the growth of pathogenic microbes, the release of offensive odours within the local vicinity and the eutrophication of waterbodies by the leachate.[3] Incineration increases air pollution and contributes to the rising carbon footprint.[3,4]

Considering the keratin content in chicken feathers, many approaches have been explored to transform this waste into useful products with application potential in many sectors of the economy, which include the detergent, agro-sector, and pharmaceutical industries.[5-7] The approaches that have been applied for the valorisation of the chicken feathers include high-pressure and temperature heating, mechanical crushing and chemical treatment of the biomass to generate protein hydrolysates for potential use in other sectors, such as animal feed and organic fertiliser. These processing technologies geared towards adding value to chicken feather biomass often damage the heat-sensitive amino acids, produce indigestible and low-quality products, consume energy, and introduce hazardous chemicals to the environment.[8] The challenges orchestrated by traditional techniques necessitate the exploration of different ecological niches for microbial sources with the potential to express robust metabolic enzyme cocktails for valorising poultry feathers into high-value products.

Microbial keratinolytic enzymes, including disulfide reductases, carboxypeptidases, and aminopeptidases, are enzyme cocktails that display dexterity in dismembering keratinous biomass into amino acids, functional peptides, and non-protein nitrogenous compounds.[9,10] The disulphide reductases are responsible for hydrolyzing densely populated cysteine disulfide bonds, and they include membrane-associated extra-cytoplasmic disulfide reductase (*ResA*)[11] and Cytoplasmic disulfide reductase/Thioredoxin reductases (*trxA* and *trxB*).[12] Similarly, aminopeptidases such as dipeptidyl aminopeptidase BIII (*dapb-3*) and aminopeptidase C (*pepC*) have all been associated with the bioconversion of chicken feathers to high-value products, post the breakage of disulfide bonds.[13,14]

Microbial keratinase-directed chicken feather degradation is a cost-effective and eco-friendly recycling method that is currently gaining traction in the bioindustry space. That, therefore, underscores the need to explore microbial keratinolytic enzyme-encoding genes to enhance keratinase yield for efficient bioconversion of chicken feathers into high-value products. The feather-degrading *C. proteolyticum* used for this study was previously isolated and characterized for its keratinolytic potential.[15] Therefore, this study was implemented to sequence the genome of *C. proteolyticum*, identify the secondary metabolite determinants encoding for keratinolysis of chicken feathers and amplify the identified genes for enhancing keratinolytic activity. The identified keratinolytic enzyme-encoded genes have the potential to be amplified and used in genetic engineering to improve keratinase production. The sequenced genome was annotated to explore the keratinolytic determinants responsible for the keratinolytic activity of *C. proteolyticum.* The putative keratinolytic enzyme-encoding genes identified were successfully amplified.

MATERIALS AND METHODS

Reagents

Media constituents were purchased from Merck Life Science Limited (Johannesburg, South Africa) and Lasec (Pty) Ltd. (Port Elizabeth, South Africa). Keratin Azure was obtained from Sigma-Aldrich (St. Louis, MO, USA). All the reagents used in the polymerase chain reaction (PCR), including primers, master mix, and nuclease-free water, were purchased from Inqaba Biotechnical

Industries (Pty) Ltd. (Pretoria, South Africa); meanwhile, reagents used in gel electrophoresis were purchased from Lasec (Pty) Ltd. (Port Elizabeth, South Africa).

Feather keratin substrate preparation

Chicken feathers were collected from a local poultry farm in Middledrift, Raymond Mhlaba Municipality, South Africa. The chicken feathers were exhaustively washed with water, air-dried, and oven-dried at 60 °C for 48 h. The dry biomass was milled into powdered chicken feathers (PCF) using a 2 mm mesh-fitted pulverizer. The chicken feathers were kept in an air-tight container for subsequent use as a keratinous substrate.

Keratinolytic strain and culture media

The previously established keratinolytic *C. proteolyticum* identified through the 16S ribosomal RNA gene sequencing[15] and maintained on basal salt feather-agar slants was utilized for this study. The bacterial strain was inoculated on basal salt medium (K_2HPO_4; 0.3%, KH_2PO_4; 0.4%, $MgCl_2$; 0.2%, $CaCl_2$; 0.22%) with chicken feather; 10%, and incubated at 30 °C for 48 h.[16] This passaging process was repeated 4 times to reacclimate the bacterial strain to utilise keratinous feathers as the only source of carbon and nitrogen.

Keratinase production and enzyme assay

Keratinase production was carried out following the method previously reported.[17] Concisely, a sterile basal salt media containing powdered chicken feather (PCF), 10 g/L as the only carbon and nitrogen source, was inoculated with a freshly prepared bacterial suspension (2%, v/v). The culture flasks were incubated for 96 h at 30 °C and 130 rpm (Labotec IncoShake (Pty) Ltd, Midrand, Gauteng, South Africa). Subsequently, the culture medium was filtered using a 0.45 µm pore filter and spun at 15,000x g in a centrifuge (Lasec SA (Pty) Ltd., Port Elizabeth, South Africa) for 10 min. The cell-free filtrate was used as a crude enzyme for keratinase assay, following the method described by Jaouadi et al.[18] with some modifications. The keratinase assay reaction mixture was prepared by coupling 500 µL of crude enzymes with 500 µL of keratin azure solution (pH 7.5). The reaction mixture was incubated at 40 °C for 1 h with continuous shaking at 200 rpm. After the incubation, the reaction was terminated by placing the assay tubes on ice for 10 min. The keratinolytic activity was determined by measuring the amount of free azo dye released in the solution at 595 nm using a SYNERGYMx 96-well microplate reader (BioTek Instrument Inc., Winooski, VT, USA).

Whole genome sequencing (WGS) of the bacterial isolate

The genome of the *C. proteolyticum* was sequenced using Ion GeneStudio™ S5 Prime System (Thermo Fisher Scientific, Waltham, MA, USA). Briefly, the genomic DNA (gDNA) of *C. proteolyticum* was isolated using the "salting out" method previously described by Sunnucks and Hales[19] and purified using the magnetic beads method. The gDNA was quantified by the Qubit™ 4 Fluorometer using the Qubit™ 1X dsDNA High Sensitivity (HS) Assay Kit (Thermo Fisher Scientific), following the manufacturer's protocol. The purity of the quantified gDNA was evaluated using NanoDrop® ND-1000 (Thermo Fisher Scientific). The genomic quality score (GQS) was determined on the LabChip GX Touch 24 Nucleic Acid Analyzer (PerkinElmer, Waltham, MA, USA) according to the manufacturer's protocol. The gDNA sample was subjected to library preparation using the Ion Plus Fragment Library Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. The Ion Library TaqMan™ Quantitation Kit quantified the prepared library by following the manufacturer's protocol. The StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific) was used to amplify the library using quantitative PCR to determine the molarity of the library and respective barcode. Subsequently, the amplified 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 (Thermo Fisher Scientific) and loaded on the Ion GeneStudio™ S5 Prime System for sequencing.

Sequence analysis and gene annotation

The obtained sequences from the Ion GeneStudio™ S5 Prime System were assembled using SPAdes assembler (Version 3.13.0)[20] on the Stellenbosch University HPC (Western Cape, South Africa) and evaluated using QUAST V4.1.[21] Genome annotation was performed using Prokka 1.14.6.[22]

The determination of the genetic bases for the expression of keratinolytic enzymes from *C. proteolyticum*

The primer basic local alignment search tool (BLAST) in the National Centre for Biotechnology Information (NCBI) (tool available publicly at http://www.ncbi.nlm.nih.gov/tools/primer-blast) was used to design primers for the target keratinolytic determinants from Prokka-annotated sequences. The PCR reaction consisted of 12.5 µL of Mastermix, 5 µL of DNA template, 1 µL of each primer, and 5.5 µL of nuclease-free water. PCR was executed in a Thermocycler (Bio-Rad Laboratories (Pty) Ltd, South Africa) programmed at 94 °C for 5 min (one cycle), followed by 30 cycles of 94 °C for 30 s, 50 °C for 1 min, 72 °C for 1 min, and one cycle of 72 °C for 10 min. The amplicons were loaded on 1.5% agarose gel and electrophoresed to confirm positive amplification of the test genes. The UV transilluminator (United Scientific (Pty) Ltd.) was used to view the DNA bands on the ethidium bromide-stained agarose gel.

Multiple-sequence alignment

The protein sequences similar to the successfully amplified target genes were searched on the protein database using the coding amino acid sequences retrieved from Prokka 1.14.6, and multiple sequence alignment was done to compare the sequences.

Determination of the physicochemical properties of the identified keratinolytic enzyme

The ExPASy-ProtParam tool was used to determine the molecular weight, amino acid composition, instability index, and theoretical isoelectric point (pI), as reported by Gasteiger and colleagues.[23]

RESULTS

Evaluation of keratinase production and activity

Revalidation of the keratinolytic potential of *C. proteolyticum* used for this study showed that the isolate secreted a significant amount of enzyme, which subsequently mediated the degradation of keratinous feather biomass in the fermentation medium, with keratinolytic activity and thiol concentration of 706.36 U/mL and 123.68 µM, respectively.

Whole genome sequencing, assembly and annotation

The Ion GeneStudio™ S5 Prime System generated 2,484,843 nucleotide base sequence reads from the *C. proteolyticum* genome length of 804,134,986, and SPAdes generated thirty-seven contigs. The maximum contig had a length of 1242714 bp, while the minimum contig consisted of 134 bp. The sequenced whole genome was uploaded on NCBI GenBank, and the accession number JBJLWK000000000 was generated. The Prokka annotation revealed that the bacterial genome consisted of 4733 coding sequences (cds), six rRNA, fifty-eight tRNA, and one tmRNA. Additionally, the annotation revealed that the keratinolytic *C. proteolyticum* under investigation possessed 4798 genes, where thirty-eight of these genes were coding for enzymes associated with keratinolysis, which included disulphide reductases, aminopeptidases, dipeptidyl peptidases, thioredoxin reductases, dipeptidases, and dipeptidyl carboxypeptidase. The various keratinolytic genes from *C. proteolyticum*, gene products, gene locus tag, and EC number are presented in Table.

The predominant keratinolytic enzyme-encoding gene that was observed from the *C. proteolyticum* genome was the thiol-disulfide oxidoreductase encoding gene (*resA*) with a proportion of 26.3% (10/38), followed by a D-aminopeptidase encoding gene (*dap*) with a proportion of 13.2% (5/38). While other keratinolytic-associated enzymes encoding genes such as dipeptidyl aminopeptidase encoding gene (*dapb*), dipeptidyl-peptidase encoding gene (*dpp*), peptidase T encoding gene (*pepT*), dipeptidyl carboxypeptidase encoding gene (*dcp*), and sulfite reductase [NADPH] flavoprotein alpha-component (*cysJ*) were identified in a proportion of 5.3% (2/38). While the genes coding for peptidase (*pepE*), aminopeptidase C (*pepC*), aminopeptidase S (*pepS*), Xaa-Pro aminopeptidase (*pepP*), Xaa-Pro dipeptidase (*pepQ*), protease 4 (spa), protease HtpX (*htpX*), protease 2 (*ptrB*), thioredoxin A (*trxA*), thioredoxin reductase (*trxB*), thioredoxin-dependent 5'-adenylylsulfate reductase (*cysH*),

sulfite reductase [NADPH] hemoprotein beta-component (*cysI*), and lon protease 2 (*lon2*) were identified in a proportion of 2.6% (1/38). The high prevalence of the thiol-disulfide oxidoreductase encoding gene highlights the robust sulfolytic system of *C. proteolyticum* biosynthetic machinery and its involvement in the reduction of keratin's disulfide bonds.

Table. Identified keratinolytic enzyme-encoding genes from the genome of *C. proteolyticum*

No.	Gene	Gene Locus Tag	EC Number	Clusters of Orthologous Genes (COG)	Gene product
1	*pepE*	MLPAAHBK_00960	3.4.13.21	COG3340	Peptidase E
2	*pepC*	MLPAAHBK_01375	3.4.22.40	COG3579	Aminopeptidase C
3	*resA_1*	MLPAAHBK_01466	-	-	Thiol-disulfide oxidoreductase
4	*sppA*	MLPAAHBK_01967	3.4.21.-	COG0616	Protease 4
5	*htpX*	MLPAAHBK_02208	3.4.24.-	-	Protease HtpX
6	*ptrB*	MLPAAHBK_02848	3.4.21.83	COG1770	Protease 2
7	*trxA*	MLPAAHBK_03368	-	-	Thioredoxin A
8	*trxB*	MLPAAHBK_04313	1.8.1.9	COG0492	Thioredoxin reductase
9	*dapb1*	MLPAAHBK_03502	3.4.14.-	-	Dipeptidyl aminopeptidase BI
10	*dapb3*	MLPAAHBK_01060	3.4.14.-	-	Dipeptidyl aminopeptidase BIII
11	*pepS*	MLPAAHBK_00223	3.4.11.24	COG2234	Aminopeptidase S
12	*pepP*	MLPAAHBK_00699	3.4.11.9	COG0006	Xaa-Pro aminopeptidase
13	*cysH*	MLPAAHBK_00709	1.8.4.10	-	Thioredoxin-dependent 5'-adenylylsulfate reductase
14	*cysJ_1*	MLPAAHBK_00715	1.8.1.2	COG0369	Sulfite reductase [NADPH] flavoprotein alpha-component
15	*cysJ_2*	MLPAAHBK_00716	1.8.1.2	COG0369	Sulfite reductase [NADPH] flavoprotein alpha-component
16	*cysI*	MLPAAHBK_00717	1.8.1.2	COG0155	Sulfite reductase [NADPH] hemoprotein beta-component
17	*dpp5*	MLPAAHBK_01391	3.4.14.-	COG1506	Dipeptidyl-peptidase 5
18	*dpp7*	MLPAAHBK_02697	3.4.14.-	-	Dipeptidyl-peptidase 7
19	*pepQ*	MLPAAHBK_01509	3.4.13.9	-	Xaa-Pro dipeptidase
20	*dap_1*	MLPAAHBK_01627	3.4.11.19	-	D-aminopeptidase
21	*dap_2*	MLPAAHBK_01852	3.4.11.19	-	D-aminopeptidase
22	*dap_3*	MLPAAHBK_01862	3.4.11.19	-	D-aminopeptidase
23	*dap_4*	MLPAAHBK_02277	3.4.11.19	-	D-aminopeptidase
24	*dap_5*	MLPAAHBK_04392	3.4.11.19	-	D-aminopeptidase
25	*resA_2*	MLPAAHBK_01665	-	-	Thiol-disulfide oxidoreductase
26	*resA_3*	MLPAAHBK_01869	-	-	Thiol-disulfide oxidoreductase
27	*resA_4*	MLPAAHBK_01991	-	-	Thiol-disulfide oxidoreductase
28	*resA_5*	MLPAAHBK_02098	-	COG0526	Thiol-disulfide oxidoreductase
29	*resA_6*	MLPAAHBK_02246	-	-	Thiol-disulfide oxidoreductase
30	*resA_7*	MLPAAHBK_02658	-	-	Thiol-disulfide oxidoreductase
31	*resA_8*	MLPAAHBK_02659	-	-	Thiol-disulfide oxidoreductase
32	*resA_9*	MLPAAHBK_03252	-	-	Thiol-disulfide oxidoreductase
33	*resA_10*	MLPAAHBK_04551	-	-	Thiol-disulfide oxidoreductase
34	*lon2*	MLPAAHBK_02324	3.4.21.53	COG0466	Lon protease 2
35	*pepT_1*	MLPAAHBK_02676	3.4.11.4	COG2195	Peptidase T
36	*pepT_2*	MLPAAHBK_02677	3.4.11.4	COG2195	Peptidase T
37	*dcp_1*	MLPAAHBK_03703	3.4.15.5	COG0339	Dipeptidyl carboxypeptidase
38	*dcp_2*	MLPAAHBK_03704	3.4.15.5	COG0339	Dipeptidyl carboxypeptidase

Multiple amino acid sequence alignment of the keratinolytic enzymes

Interestingly, about eight (80%) of the amplified keratinolytic enzymes (*pepE, pepC, resA, trxA, trxB, cysH, dapb3,* and *dcp_1*) displayed 99% similarity with the proteolytic enzymes from *Chryseobacterium daecheongense* strain PL22-22A (CP115858.1). While *dcp_2* and *cysJ_1* showed 98% and 97% sequence similarities with PL22-22A (CP115858.1), respectively. The multiple-sequence alignments of the keratinolytic enzymes showed variations in specific amino acid residues (Figure 1). Notably, the most variation in the sequence residues was observed from

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WP_261512302.1      MENNLKKEFEELLEKASDNTINTDFLQVVVEKFPGKVIFSTSFSYEDQIVTHLIKNLDVD      60
WBV57611.1          MENNLKKEFEKLLEEVSDHAVNTEFLQLLAERFPGEVIFSTSFSYEDQIITHLIKNLDID      60
MLPAAHBK_00709      MENNLKKEFEKLLEEVSDHAVNTEFLQLLAERFPGEVIFSTSFSYEDQIITHLIKNLDID      60
WP_172282549.1      MENNLKKEFEKLLEEVSDHAVNTEFLQLLAERFPGEVIFSTSFSYEDQIITHLIKNLDID      60
                    **********:***:.**:::**:***::.*:***:************:********:*

WP_261512302.1      IFTLDTGRLFEQTYETWTASRAFFKKEIKAYYPDTEEIREFVSQNGPDSFYQSVEQRKAC      120
WBV57611.1          IFTLDTGRLFEQTYETWTASRAFFKRNIKAYYPDTEEIREFVSQNGPDSFYQSVEQRKAC      120
MLPAAHBK_00709      IFTLDTGRLFEQTYETWTASRAFFKKNIKAYYPDTEEIREFVSQNGPDSFYQSVEQRKAC      120
WP_172282549.1      IFTLDTGRLFEQTYETWTASRAFFKKNIKAYYPDTEEIREFVSQNGPDSFYQSVEQRKAC      120
                    ************************::**********************************

WP_261512302.1      CTIRKVHPLKKALQGYKVWITGLRSEHSTNRQNMPQLEWDEDNQIIKFHPLLHWTSEQVL      180
WBV57611.1          CTIRKVHPLKKALQGYKVWITGLRSEHSANRQNMPQLEWDEDNRIIKFHPLLHWTSDQVL      180
MLPAAHBK_00709      CTIRKVHPLKKALQGYKVWITGLRSEHSVNRQNMPQLEWDEDNRIIKFHPLLHWTSDQVL      180
WP_172282549.1      CTIRKVHPLKKALQGYKVWITGLRSEHSANRQNMPQLEWDEDNRIIKFHPLLHWTSDQVL      180
                    **************************.**************:************:***

WP_261512302.1      EYVQMYHLPYNYLHKKGFVSIGCEPCTRAIKEGEDFRAGRWWWEDANKKECGLHIHK   237
WBV57611.1          EYVQTNHLPYNHLHKKGFVSIGCEPCTRAVKEGEDFRAGRWWWEDASKKECGLHIHK   237
MLPAAHBK_00709      EYVQTNHLPYNHLHKKGFVSIGCEPCTRAVKEGEDFRAGRWWWEDASKKECGLHIHK   237
WP_172282549.1      EYVQTNHLPYNHLHKKGFVSIGCEPCTRAVKEGEDFRAGRWWWEDASKKECGLHIHK   237
                    ****  *****:****************:****************.**********
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a

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WP_273648577.1      MKNLIQRTIISGTLGSLFLLIPSSFHAQKQQNHMEMTESEMQSSSYTIPQKPEDISPLLN      60
WP_317168537.1      MKNLIQRTIISGTLGSLFFLTPSSFHAQKQQNHMEMTESEMQSPAYTIPQKPEDISPLLN      60
MLPAAHBK_01466      MKNLIQRTIISGTLGSLFLLIPSSFHAQKQQNHMEMTESEMQSPAYTIPQKPEDISPLLN      60
WP_306614786.1      MKNLIQRTIISGTLGSLFFLIPSSFHAQKQQNHMEMTESEMQSPAYTIPQKPEDISPLLN      60
                    *****************:* ********************** :***************

WP_273648577.1      GEKIPVATLPDANGKRFDLNAAVSQKPTVLIFYRGGWCPYCSKQLSGLQEIAPDLEKTGY      120
WP_317168537.1      GEKIPVATLPDANGKRFDLNAAVSQKPTVLIFYRGGWCPYCSKQLSGLQEIAPDLEKTGY      120
MLPAAHBK_01466      GEKIPVATLPDANGKRFDLNAAVSQKPTVLIFYRGGWCPYCSKQLSGLQEIAPDLEKTGY      120
WP_306614786.1      GEKIPVATLPDANGKRFDLNAAVSQKPTVLIFYRGGWCPYCSKQLSGLQEIAPDLEKTGY      120
                    ************************************************************

WP_273648577.1      QIIAISTDQPEGLMQSATKEKLSYTLLSDADLKLSKQFGIAFKAPKGYWEMLPKTTGGKN      180
WP_317168537.1      QIIAISTDQPEGLMQSATKEKLSYTLLSDADLKLSKQFGIAFKAPKGYWEMLPKTTGGKN      180
MLPAAHBK_01466      QIIAISTDQPEGLMQSATKEKLSYTLLSDADLKLSKQFGIAFKAPKGYWEMLPKTTGGKN      180
WP_306614786.1      QIIAISTDQPEGLMQSATKEKLSYTLLSDADLKLSKQFGIAFKAPKGYWEMLPKTTGGKN      180
                    ************************************************************

WP_273648577.1      TDLLLPVPSVFILDRKGTIHFEYINPDFKQRLNPELLRSAAHHLINDL    228
WP_317168537.1      TELLLPVPSVFILDRKGTIHFEYINPDFKQRLNPELLRSAAHHLINDL    228
MLPAAHBK_01466      TELLLPVPSVFILDRKGTIHFEYINPDFKQRLNPELLRSAAHHLINDL    228
WP_306614786.1      TELLLPVPSVFILDRKGTIHFEYINPDFKQRLNPELLRSAAHHLINDL    228
                    *:*********************************************
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b

Figure 1. The multiple sequence alignments of the keratinolytic enzymes. (a). Sequence alignments of cysJ_1 (MLPAAHBK_00709) with other highly similar thioredoxin-dependent 5'-adenylylsulfate reductases of the different *Chryseobacterium* spp. (b). Sequence alignments of resA (MLPAABK_01466) with *Chryseobacterium* sp. B21-037 (WP_273648577.1), *Chryseobacterium* sp. LAM-KRS1 (WP_317168537.1), and *Chryseobacterium* sp. CKR4-1 (WP_273648577.1) enzyme sequences. The variance between sequences is denoted by the colon (:)

the thioredoxin-dependent 5'-adenylylsulfate reductase amino acid sequence (*cysJ_1*), where the *C. proteolyticum cysJ_1* (MLPAAHBK_00709) showed 90% sequence identity with a similar sequence from *Chryseobacterium paludism* (WP_261512302.1) and 99% with the unclassified *Chryseobacterium* (WP_172282549.1) and *Chryseobacterium daecheongense* (WBV57611.1) (Figure 1a). Variation in amino acid between the recent thioredoxin-dependent 5'-adenylylsulfate reductase amino acid sequence and *Chryseobacterium paludism* amino acid sequence was noticeable in position eleven, where the current sequence had lysine instead of glutamic acid. In position 15, the current sequence exhibited valine, while *Chryseobacterium paludism* exhibited lysine in this position. Another difference in amino codes in these sequences was observed in position 16, where alanine replaced valine, while in position 19, histidine replaced asparagine, and in position 20, alanine replaced threonine.

Similarly, the multiple sequence alignments of *resA* (MLPAABK_01466) with other related sequences from *Chryseobacterium* spp. showed that *resA* has 98% sequence homology with the amino acid sequences of enzymes from *Chryseobacterium* sp. B21-037, and 99% sequence similarity with the enzymes from *Chryseobacterium* sp. CKR4-1 and *Chryseobacterium* sp. LAM-KRS1 (Figure 1b). The dissimilarity of the amino acid

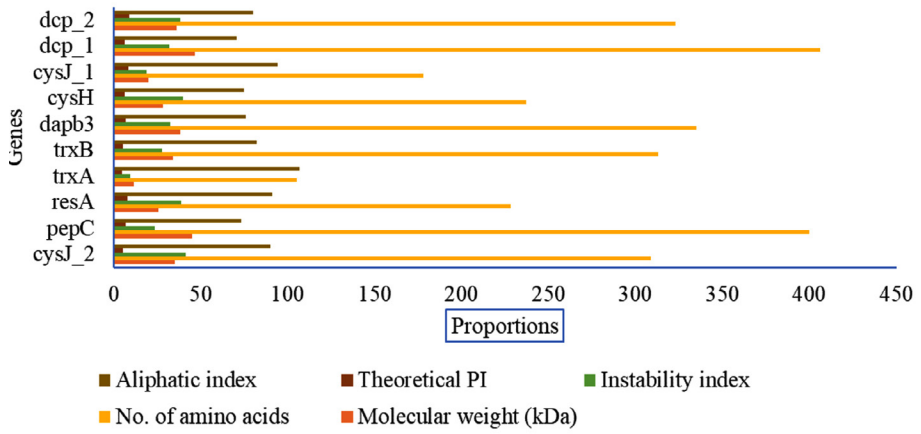

Figure 2. Determination of the physicochemical properties of the keratinolytic enzymes

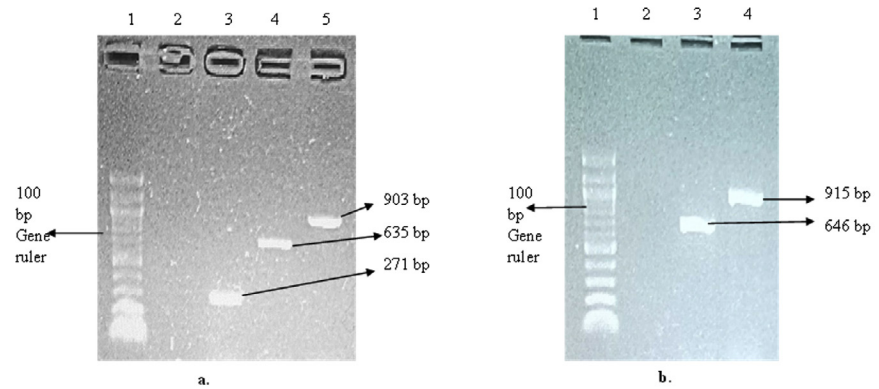

Figure 3. Gel electrophoresis of the amplified keratinolytic enzyme-encoding genes. (a). Gel picture showing *trxA*, *dapb-3,* and *pepC* genes from *C. proteolyticum;* Lane 1: 100 bp DNA gene ruler, Lane 2: Negative control, Lane 3: *trxA* gene (271 bp), Lane 4: *dapb-3* (635 bp) gene, and Lane 5: *pepC* gene (903 bp). (b). Gel picture showing *resA* and *trxB* genes; Lane 1: 100 bp DNA gene ruler, Lane 2: Negative control, Lane 3: *resA* gene (646 bp), and Lane 4: *trxB* gene (915 bp)

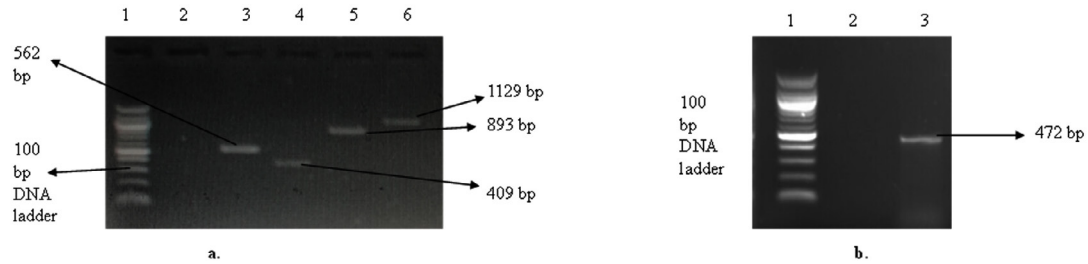

Figure 4. Gel electrophoresis of the amplified keratinolytic enzymes-encoding genes from *C. proteolyticum*. (a). Gel picture of *cysH*, *cysJ_1*, *dcp_1*, and *dcp_1*; Lane 1: 100 bp DNA ladder, Lane 2: negative control, Lane 3: *cysH* (562 bp), Lane 4: *cysJ-1* (409 bp), Lane 5: *dcp_2* (893 bp), Lane 6: *dcp_1* (1129 bp). (b). Gel picture of the *cysJ_2* gene; Lane 1: 100 bp DNA ladder, Lane 2: negative control, Lane 3: *cysJ_2* (472 bp)

sequences of the *resA* gene of this study with that of *Chryseobacterium* sp. B21-037 was observed in two amino acids, including serine in the 45th base, replaced in the query sequence by alanine, and aspartic acid in the 182nd base, replaced by glutamic acid. There was only one dissimilarity noted in the *Chryseobacterium* sp. LAM-KRS1 and the dissimilarity was at the 19th phenylalanine exchanged by Leucine.

Physiochemical properties of the identified keratinolytic genes

The determination of the physicochemical properties of the keratinolytic enzymes showed that sulfite reductase [NADPH] flavoprotein alpha-component demonstrated a molecular weight of 19.62 kDa, an instability index of 18.83, a theoretical pI of 8.49, aliphatic index of 94.27 (Figure 2). On the other hand, thioredoxin reductase properties were determined to be 33.8 kDa, 27.82, 5.24, and 82.3 for molecular weight, instability index, theoretical pI, and aliphatic index, respectively. Meanwhile, peptidyl carboxypeptidase's properties computation showed molecular weight, instability index, theoretical pI, and aliphatic index of 46.76 kDa, 31.73, 6.08, and 70.39, respectively (Figure 2). A total of 60% (6/10) of the analysed keratinolytic enzymes (*pepC, trxA, trxB, dapb_3, cysH,* and *dcp_1,*) showed to have theoretical pI of more than 7 (Figure 2). On the contrary, 40% (4/10) of the enzymes which include *resA, cysJ-1, cysJ_2,* and *dcp_2* had a theoretical pI of less than 7.

Amplification of selected keratinolytic enzyme-encoding genes from *C. proteolyticum* genome

Ten (10) selected genes coding for keratinolytic enzymes were amplified, and the results showed three genes coding for thioredoxin A (*trxA*), aminopeptidase C (*pepC*), and dipeptidyl aminopeptidase (*dapb_3*), with band sizes of 271 bp, 635 bp, and 903 bp, respectively (Figure 3a). Likewise, the amplification of reductases coding genes showed that *resA* was sitting at 646 bp and *trxB* was at 915 bp (Figure 3b). The two carboxypeptidases coding genes *dcp_1* and *dcp_2* were successfully amplified, with a respective band size of 1129 bp and 893 bp (Figure 4a). The genes coding for 5'-adenylylsulfate reductase (*cysH*) and sulfite reductase (*cysJ_1*) were also among the amplified genes with band sizes of 562 bp and 409 bp, respectively (Figure 4a). In addition, *cysJ*_2, which also codes for sulfite reductase, was sitting at 472 bp (Figure 4b).

DISCUSSION

Keratinous waste biomass accumulation is one of the public health challenges seeking urgent attention. The keratinases from different microbial species have proved that these enzyme batteries are effective towards keratinous waste valorisation. Identifying the metabolic pathways involved in the secretion of these enzyme batteries is essential in enhancing their production for large-scale biotechnological products. Hence, this study aimed at mining the *C. proteolyticum* genome to identify the production of secondary metabolites involved

in keratinolysis for prospective biotechnological applications. The revalidation of the keratinolytic potential of the *C. proteolyticum* strain proves that the complete chicken feather hydrolysis was due to the secretion of keratinolytic proteases. The expressed keratinase activity was similar to the previously reported keratinase activity of characterized *C. proteolyticum* by Giwu et al,[15] who reported 693.63 U/mL. The high keratinase titre demonstrated by *C. proteolyticum* underscores its robust metabolic diversity and keratinolytic potential.

The presence of free thiols in the media indicates the reduction of disulfide bonds to cysteine thiol and cysteine-S-sulphonate residue, which is facilitated by the series of reductases, including thioredoxin reductases, thioredoxin-dependent 5'-adenylylsulfate reductase, sulfite reductases, and thiol-disulfide oxidoreductases.[24-26] The high thiol groups detected in the fermentation broth prove that the chicken feather degradation by *C. proteolyticum* involved sulfitolysis.[17,27,28] In addition, the thiol concentration may indicate the biocatalytic efficiency of the keratinolytic and sulfitolytic systems of keratin-degrading bacteria.[17,27,29] The thiol concentration detected in this study was higher than that observed by Kshetri and Ningthoujam[29] and He et al.,[30] who recorded thiol concentrations of 82 µM and 14.1 µM, respectively, during chicken feather degradation, highlighting that this strain strongly degrades keratin as the carbon and nitrogen source.

The discovery of the keratinolytic enzymes-encoding genes in the genome of *C. proteolyticum* suggested that keratinolysis is a two-step enzymatic process, which is sulfitolysis and proteolysis, involving enzyme cocktails[31] that include reductases, carboxypeptidases, dipeptidases, and aminopeptidases in the bacterial genome.[31] During the chicken feather biodegradation, the tightly packed cysteine disulfide bonds are reduced by reductases, leaving the keratin vulnerable to proteolysis by the peptidases.[9,10,32,33] The genome profiling further demonstrated that the effective keratin-degrading ability of this bacterium might be associated with the expression of these batteries of genes and cooperative actions of the reductase and peptidase cocktails.[34,35] Bacteria genome mining has been identified as an important approach that has been effectively employed in discovering important secondary metabolites with keratinolytic dexterity.[5]

Multiple-sequence alignment helps to identify the evolutionary relationships among sequences with similar biological activity.[36] The disparity in amino acid residues at some positions on the aligned sequences might be attributed to the bacterial strain variations, and this residue substitution could suggest the uniqueness of the keratinolytic enzymes under investigation.[17] The observed sequence divergence could also influence the catalytic tendency of the biocatalysts towards substrate utilization. *In silico* determination of bacterial enzyme's physicochemical properties provides interesting data relevant to the enzyme application potential. The aliphatic index is one of the essential physicochemical properties that suggest a protein's thermal stability profile, and a protein with a high aliphatic index is more thermally stable than those with a low aliphatic index.[37] All the investigated keratinolytic enzymes displayed prospects of thermal stability, with an aliphatic index between 70 and 106. Additionally, the possessed aliphatic index proves that these enzymes consisted of hydrophobic amino acids such as alanine, isoleucine, leucine, aspartic acid, and valine.[38] The observed theoretical pI was between 4 units, suggesting that these enzymes would show activity optima from acidic to a basic region of the pH spectrum. According to Halligan,[39] a theoretical pI value greater than 7 suggests that the protein or enzyme is acidic.

The successful amplification of the selected keratinolytic enzymes-encoding genes suggests the feasibility of manipulating the isolate's keratinolytic determinants and their subsequent use in the transformation of industrially competent cells through genetic engineering to enhance keratinolytic activity. Scalable production of keratinolytic enzymes will not only promote sustainable valorization of the recalcitrant agroindustrial waste but also revolutionize the application of the enzymes in different sectors of the bioeconomy.[40]

CONCLUSION

The genome of a competent feather-degrading bacterium, *C. proteolyticum,* was sequenced and annotated to identify its keratinolytic determinants. Out of the 4798 genes obtained through Prokka annotation, thirty-eight proved to be putative keratinolytic enzyme-encoding genes. Multiple-sequence alignment demonstrated that the amino acid sequences of the putative keratinolytic enzymes displayed some variations with related sequences retrieved from the protein database. *In silico* analysis of the physicochemical characteristics showed that the enzymes displayed variable properties with a high thermostability profile. Successful amplification of the selected putative keratinolytic enzyme-encoding genes from the *C. proteolyticum* genome highlights its endowed keratinolytic tendencies for the sustainable valorization of keratinous biomass. The results of this study confirm the feasibility of heterologous expression of the identified genes in competent hosts to upscale the keratinolytic enzyme production and demand further investigation.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

LM, NEN and UUN conceptualized the study. UUN performed funding acquisition. UUN collected resources. LM applied methodology. LM and NEN performed formal analysis and investigation. LM wrote the original draft. NEN and UUN wrote, reviewed and edited the manuscript. NEN and UUN performed supervision. All authors read and approved the final version of the manuscript for publication.

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DATA AVAILABILITY

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

ETHICS STATEMENT

This article does not contain any studies on human participants or animals performed by any of the authors.

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