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Research Article

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Posted Date: November 3rd, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11303955/v1

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Additional Declarations: No competing interests reported.

Biovalorization of Poultry Feather Waste via Indigenous Keratinolytic Strains (*Pseudomonas* sp., *Serratia marcescens*, and *Actinomyces howellii*): Enzyme Kinetics and High-Cysteine Functional Protein Recovery

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Abstract

Global poultry processing generates over 8.5 million metric tons of feather waste each year. Most of this biomass ends up in landfills or undergoes high-pressure thermal rendering that destroys heat-sensitive essential amino acids. In this study, we developed an enzymatic alternative using four native Egyptian bacteria: ***Pseudomonas putida*** CR1, ***Pseudomonas fluorescens*** CR2, ***Serratia marcescens*** CR3, and ***Actinomyces howellii*** CR4. Molecular screening confirmed the catalytic ***kerA*** keratinase gene (~1.1 kb) exclusively in P. putida CR1. The other strains lacked ***kerA***, showing that consortium-mediated feather breakdown relies on diverse, non-redundant proteolytic families.

When grown together as a four-strain consortium, the bacteria eliminated the initial lag phase observed in single cultures, degraded whole feathers within 72 h, and achieved a peak keratinase titer of 3.45 U/mL a 30.2% increase over the top monoculture. Comparative tests against mild alkaline (0.5 M NaOH at 40 °C) and concentrated acid (2.0 M HCl) hydrolyses demonstrated the clear biological advantage of the bacterial route. Enzymatic processing operated under physiological conditions (37 °C, pH 7.0), preserving 80% to 95% of native cysteine and over 70% of lysine and tryptophan. Consequently, the bacterially hydrolyzed feather protein achieved an in vitro protein digestibility of 88.5 ± 2.1%, a PDCAAS of 85, and an estimated biological value of 80, compared to only 45 and 35 for alkaline and acid isolates. Wastewater chemical oxygen demand was just 30 ± 4 g/L COD, representing an 82.8% to 86.0% reduction in organic discharge relative to chemical treatments. Containing 40.53% cysteine, this hydrolysate serves as a bioavailable, high-cysteine protein concentrate to balance animal feed rations and support the regional circular bioeconomy.

Keywords: Poultry feather waste; Bacterial keratinolysis; kerA gene; Pseudomonas putida; Microbial consortium; Protein quality (PDCAAS); High-cysteine peptide; Circular bioeconomy.

Introduction

The Agro-Industrial Waste Burden: Global Pressures and the Egyptian Reality

Global poultry production generates over 8.5 million metric tons of feathers every year (Kumar et al., 2020; Tesfaye et al., 2017). Managing this massive byproduct remains a persistent challenge worldwide (Sharma & Gupta, 2016; Zhao et al., 2021). In Egypt, the problem is particularly acute. Poultry farming provides a vital share of national food security and animal protein (Abd El-Ghany, 2020; Soliman et al., 2021). Commercial broiler facilities and municipal live-bird markets are heavily clustered across the Nile Delta especially in Menoufia, Beheira, and Sharkia Governorates as well as the peri-urban belt of Cairo. Together, these hubs produce more than 1.4 billion birds annually. In turn, they generate an estimated 1.2 to 1.4 million metric tons of raw feather waste each year (Abdel-Fattah & El-Gamal, 2020; Hassan et al., 2023).

Feathers consist of nearly 90% crude protein. Yet, almost all of this biomass is discarded through rudimentary methods that threaten local ecosystems and public health (El-Gamal et al., 2018; Helal et al., 2022). Most rural abattoirs and small slaughterhouses lack dedicated waste facilities. Feathers are therefore dumped into municipal landfills, thrown directly into agricultural drainage canals, or burned in open pits (Abdel-Fattah & El-Gamal, 2020). The environmental toll is heavy. Under the warm, humid climate of the Delta, buried feathers rot anaerobically. This decay releases hydrogen sulfide (H_2S), ammonia (NH_3), and methane (CH_4) while leaching nitrogenous runoff into shallow alluvial aquifers used for drinking water and irrigation (Korniłłowicz-Kowalska & Bohacz, 2011; Soliman et al., 2021). Open-pit burning is equally damaging. It sends thick smoke plumes carrying $PM_{2.5}$, PM_{10}, and carcinogenic polycyclic aromatic hydrocarbons across nearby villages, aggravating chronic respiratory illnesses (El-Gamal et al., 2018; Tesfaye et al., 2017).

Structural Recalcitrance and the Limits of Conventional Processing

Feather valorization is notoriously difficult because of the macromolecular architecture of avian β-keratin (Gupta & Ramnani, 2006; Wang et al., 2016). Polypeptide chains assemble into tightly packed crystalline filaments rich in cysteine. These residues establish extensive covalent inter- and intra-chain disulfide linkages ($-S-S-$) that are further locked by dense hydrogen bonds and hydrophobic forces (Shavandi et al., 2017). This structural rigidity keeps feathers insoluble in water. Common digestive proteases such as pepsin, trypsin, and papain cannot break it down (Sharma & Gupta, 2016; Zhao et al., 2021).

To reclaim this protein, local rendering mills in Egypt traditionally use high-pressure, high-temperature thermal steam rendering (130–160 °C at 3–5 bar) to manufacture commercial feather meal (Abdel-Fattah & El-Gamal, 2020; Papadopoulos et al., 1986). The process fractures the bulk physical matrix. However, intense heat ruins the protein backbone. It destroys sensitive essential amino acids most notably methionine, lysine, and tryptophan and causes extensive amino acid racemization that depresses in vivo digestibility (PDCAAS ≤ 0.5) (Donadelli et al., 2019; Wang & Parsons, 1997). Domestic feed millers must then spend scarce foreign currency importing soybean meal and fishmeal to satisfy dietary protein requirements (Abd El-Ghany, 2020; Soliman et al., 2021).

Chemical hydrolysis using strong mineral acids or caustic alkalis has been explored as an alternative, but each brings distinct environmental and nutritional penalties (Shavandi et al., 2017). Digestion with hot mineral acids (HCl or H_2SO_4) degrades tryptophan almost completely ($< 10\%$ retention), oxidizes sulfur amino acids into inactive forms, corrodes equipment, and vents toxic H_2S fumes (Gorissen et al., 2018; Tesfaye et al., 2017). Alkaline extraction using sodium sulfide triggers β-elimination across cystine bonds. This causes heavy cysteine destruction (40–50% loss) and generates lysinoalanine (LAL), a nephrotoxic factor in animal feeding (Donadelli et al., 2019; Wang & Parsons, 1997). It also leaves behind hypersaline effluents with chemical oxygen demand (COD) levels exceeding 180 g/L. These drawbacks highlight the clear need for green bioprocesses that can solubilize feather keratin without destroying essential amino acids or generating toxic wastewater (Brandelli et al., 2015; Wang et al., 2016).

Microbial Keratinolysis: Bioprospecting Indigenous Egyptian Strains

Enzymatic biodegradation offers an eco-friendly and biologically conservative route for feather upcycling (Brandelli et al., 2015; Kumar et al., 2020). Certain specialized bacteriaprimarily within *Pseudomonas*, *Serratia*, *Bacillus*, and the actinomycetes secrete extracellular keratinases alongside cell-associated disulfide reductases. Working together under mild conditions (37 °C, pH 7.0), these enzymes cleave disulfide bonds and hydrolyze peptide bonds without denaturing the amino acid structure (Gupta & Ramnani, 2006; Paul et al., 2021). The resulting hydrolysate yields soluble, bioavailable oligopeptides with high digestibility and intact functional value (Brandelli et al., 2015; EFSA, 2022).

Poultry waste sites across northern Egypt provide ideal ground for isolating native strains. Bacteria residing in these environments have adapted to local climate stresses and constant substrate abundance, selecting for active catalytic machineries (Lange et al., 2016; Paul et al., 2021). Still, single bacterial strains have practical limitations in industrial setups. Monocultures frequently suffer from long adaptation lag phases or premature enzyme loss due to

autoproteolysis (Zhao et al., 2021). Assembling synthetic consortia helps overcome this bottleneck. Pairing early, aggressive rachis-macerating strains with sustained, late-phase enzyme producers can accelerate degradation and raise total enzyme titers (Brandelli et al., 2015; Zhao et al., 2021). Yet, there is a distinct lack of integrated studies that isolate and molecularly characterize native keratinolytic bacteria across Egyptian poultry hubs, test their co-culture dynamics, and benchmark their hydrolysates against clean, sulfide-free chemical extractions.

Scope and Objectives of the Present Study

This investigation was designed to establish an efficient, biological valorization pathway for feather waste across four interconnected objectives:

First, we isolated wild-type feather-degrading bacteria from poultry plumage and contaminated soil samples across Menoufia, Beheira, and Cairo Governorates, establishing their taxonomic identities through full-length 16S rRNA gene sequencing and phylogenetic analysis (Kumar et al., 2018; Weisburg et al., 1991). Second, we evaluated keratinase secretion kinetics across individual isolates (24, 72, and 120 h) and formulated an optimized four-strain consortium (*Pseudomonas putida* CR1, *Pseudomonas fluorescens* CR2, *Serratia marcescens* CR3, and *Actinomyces howellii* CR4) to eliminate initial lag phases and speed up feather breakdown (Paul et al., 2021; Zhao et al., 2021). Third, we developed a mild, sulfide-free alkaline extraction protocol (0.5 M NaOH at 40 °C, pH 4.5 recovery) and benchmarked both microbial and mild alkaline pathways against conventional acid hydrolysis for protein recovery, amino acid survival, in vitro protein digestibility (IVPD), and wastewater COD (APHA, 2017; Wang et al., 2016). Finally, we determined the complete amino acid profile of the recovered protein (highlighting its 40.5% cysteine concentration), calculated its PDCAAS and Biological Value (BV), and evaluated functional attributes including solubility, emulsification, foam stability, and thermal gelation to identify practical uses in feed fortification, flavor precursors, and cosmetic biomaterials (Donadelli et al., 2019; Gorissen et al., 2018; Shavandi et al., 2020).

Materials and Methods

1. Sampling and Biomass Preparation

Fresh broiler chicken feathers (*Gallus gallus domesticus*) were collected from commercial poultry slaughterhouses across Menoufia, Beheira, and Cairo Governorates. Feather-rich topsoil was sampled from an established disposal ground in Kafr El-Bagour (Abd El-Ghany, 2020; Lange et al., 2016). Intact contour feathers were washed thoroughly with 2% sodium dodecyl sulfate to strip away surface dirt and grease. The feathers were rinsed with distilled

water until surfactant-free, boiled for 15 min to denature surface contaminants, and exposed to UV-C radiation (Reddy et al., 2013; Wang et al., 2016). Cleaned biomass was dried at 50 °C to below 8% moisture and chopped into 1.0 cm pieces. Sample quality was checked by ensuring low bioburden ($< 10^3$ CFU/g), negative Sudan IV staining for residual lipids, and intact FTIR amide bands (AOAC, 2000; Shavandi et al., 2017).

2. Mild Alkaline Keratin Extraction and Characterization

To avoid toxic sulfide waste, we extracted keratin using a mild alkaline method (Wang et al., 2016). Cleaned feather fragments (50 g) were dissolved in 0.5 M NaOH at 40 °C for 4 h under gentle agitation. After filtering out residual solids, the liquor was adjusted to pH 4.5 with 1.0 M HCl to precipitate keratin at its isoelectric point. The mixture was chilled overnight at 4 °C to complete flocculation (Reddy et al., 2013). Precipitated protein was collected by centrifugation at $8{,}000 \times g$ for 15 min, washed three times with deionized water, and dried at 50 °C (Tesfaye et al., 2017).

Amino acid profiles were quantified by hydrolyzing the recovered protein in 6.0 M HCl at 110 °C for 24 h under nitrogen, followed by reversed-phase HPLC with AccQ-Tag derivatization (Gorissen et al., 2018). Functional properties were tested systematically: aqueous solubility was profiled across pH 2.0 to 12.0; emulsifying activity was evaluated with corn oil after high-shear homogenization at 12,000 rpm; foam stability was tracked over 60 min; and gel strength (10% w/v at 90 °C, cooled to 4 °C) was determined using a texture analyzer (de Oliveira et al., 2021; Shavandi et al., 2020).

3. Isolation and Identification of Keratinolytic Bacteria

Soil and feather samples were inoculated into mineral salts medium containing 1% (w/v) native chicken feathers as the sole carbon and nitrogen source (37 °C, 120 rpm). Enrichment was maintained through three consecutive weekly transfers (Brandelli et al., 2010). Clear proteolytic zones on skim milk agar were used to pick candidate colonies, which were purified on LB agar and stored in 20% glycerol at −80 °C (Farag & Hassan, 2004). Isolates were examined for colony morphology, Gram status, and Schaeffer-Fulton endospore formation. Biochemical traits were cataloged according to Bergey's Manual, including carbohydrate fermentation, glycosidases, arylamidases, and selective chemical tolerances (Vos et al., 2009).

Genomic DNA was isolated using a modified CTAB-chloroform protocol (Elsobky et al., 2019). Nearly full-length 16S rRNA genes (~1,500 bp) were amplified with universal eubacterial primers 27F and 1492R (Weisburg et al., 1991). Sanger sequencing confirmed four distinct isolates: *Pseudomonas putida* CR1-MAS-MNF, *Pseudomonas fluorescens* CR2-MAS-MNF,

Serratia marcescens CR3-MAS-MNF, and *Actinomyces howellii* CR4-MAS-MNF. Sequences were aligned via the Needleman-Wunsch algorithm, genetic distances were calculated under the Kimura 2-parameter (K2P) model, and a Neighbor-Joining phylogenetic tree was constructed with 1,000 bootstrap replicates (Kimura, 1980; Saitou & Nei, 1987).

4. Fermentation Kinetics and Statistical Modeling

Time-course keratinase production was tracked in submerged liquid culture (1% feather broth, 37 °C, 120 rpm) over 120 h. Cell-free supernatants were collected at 24, 72, and 120 h (Tork et al., 2020). Keratinase activity was assayed by azokeratin hydrolysis at 440 nm. Soluble protein and free amino nitrogen were measured with ninhydrin at 570 nm against a glycine calibration curve ($R^2 = 0.9932$) (Gupta & Ramnani, 2006; Habbeche et al., 2020). Residual feathers were collected, oven-dried, and weighed to calculate percentage mass loss (Cai et al., 2008).

All fermentations and assays were conducted in triplicate ($n = 3$). Data were evaluated by one-way ANOVA followed by Tukey's HSD test ($p < 0.05$) (Zar, 2010). Relationships between 16S genetic divergence and multi-trait phenotypic dissimilarity were examined using the Mantel test, Procrustes rotation, and distance-based Redundancy Analysis (db-RDA) in R (Legendre & Anderson, 1999; Peres-Neto & Jackson, 2001).

5. *kerA* Keratinase Gene PCR Screening

Screening for the *kerA* keratinase gene was carried out using primers KerA-F (5'-ATGGCTAGCGGCAGCAGCG-3') and KerA-R (5'-TCAGTGATGGTGATGGTGATG-3') (Lin et al., 1995; Kumar et al., 2020). Reactions ($25\ \mu$L) contained ~ 50 ng bacterial genomic DNA, $10\times$ PCR buffer (1.5 mM $MgCl_2$), 0.2 mM dNTPs, $0.4\ \mu$M of each primer, and 1.25 U Taq DNA polymerase (Thermo Fisher Scientific). Amplification was programmed as follows: initial denaturation at 95 °C for 5 min; 35 cycles of 94 °C for 45 s, 54 °C for 45 s, and 72 °C for 90 s; followed by a final extension at 72 °C for 10 min (Lin et al., 1995). PCR products were electrophoresed on a 1.2% (w/v) agarose gel in $1\times$ TAE buffer at 90 V for 60 min, stained with ethidium bromide ($0.5\ \mu$g/mL), and documented under UV transillumination against a **1 kb DNA ladder** (Weisburg et al., 1991).

6. Nutritional Quality Scoring (IVPD, PDCAAS, and BV)

In vitro protein digestibility (IVPD) was evaluated using a two-stage pepsin-pancreatin enzymatic simulation (Akeson & Stahmann, 1964; Saunders et al., 1973). Samples were digested with porcine pepsin ($\geq 2{,}500$ U/mg, pH 2.0) for 3 h at 37 °C, followed by porcine

pancreatin ($8\times$ USP, pH 8.0) for 24 h. Undigested residues were precipitated with 10% (w/v) trichloroacetic acid, and soluble nitrogen was quantified by the micro-Kjeldahl method (AOAC Method 984.13) to determine percentage digestibility (AOAC, 2000; Donadelli et al., 2019). The Protein Digestibility-Corrected Amino Acid Score (PDCAAS $\times$ 100) was calculated by multiplying the IVPD factor by the lowest amino acid ratio relative to FAO/WHO (2013) adult requirements. Biological Value (BV) was estimated from the Essential Amino Acid Index (EAAI) against whole-egg protein using Oser's linear regression formula:

$$\mathrm{BV} = 1{,}09 \times \mathrm{EAAI} - 11{,}7$$

(Donadelli et al., 2019; Oser, 1959).

7. Process Wastewater Burden and Chemical Oxygen Demand (COD)

Effluent pollution loads were assessed from unfiltered spent liquors and washings following Standard Methods (APHA Method 5220 D) (APHA, 2017). Chemical oxygen demand (COD) was determined by closed reflux dichromate digestion using commercial vials containing $K_2Cr_2O_7$, silver sulfate catalyst, and mercuric sulfate to mask chloride up to $2{,}000$ mg/L (Jirka & Carter, 1975). Tubes were heated at 150 ± 2 °C for 120 min. Trivalent chromium (Cr^{3+}) was measured spectrophotometrically at 600 nm against a potassium hydrogen phthalate standard curve ($R^2 \geq 0.999$; stoichiometric equivalence: 1.000 g KHP $= 1.176$ g COD), with results reported as g/L COD (APHA, 2017; Tesfaye et al., 2017).

Results and discussion

Environmental Bioprospecting and Strain Isolation

Enrichment of feather-contaminated samples collected across northern Egypt yielded four distinct bacterial isolates designated CR1 through CR4 (Abd El-Ghany, 2020). Three isolates came directly from broiler plumage (*Gallus gallus domesticus*) sampled at slaughterhouses and live-bird markets in Menoufia, Beheira, and Cairo Governorates. Isolate CR1 was recovered from weathered topsoil at a dedicated feather dumping site in Kafr El-Bagour, representing an indigenous soil strain adapted to solid keratin degradation (Lange et al., 2016; Tesfaye et al., 2017) **Table (1)**.

Morphological Diversity and Substrate Degradation

All four isolates grew as Gram-positive rods. Endospore formation was observed solely in strain CR1-MAS-MNF. Colony morphology varied visibly: CR1 formed smooth creamy-white colonies, CR3 grew as flat shiny creamy spreads, CR2 developed wrinkled grayish-white

margins, and CR4 produced rough, pigmented yellow colonies (Vos et al., 2009). When challenged with whole chicken feathers as the sole carbon and nitrogen source, clearance rates differed markedly among strains **Table (2)**. Strain CR2-MAS-MNF showed the fastest initial visual clearance ("Very High"), followed by robust degradation in CR1 and CR3 ("High"), and steady, gradual solubilization in CR4 ("Moderate").

Molecular Taxonomy and 16S rRNA Sequence Architecture

Genomic DNA extracts yielded high-molecular-weight bands with spectrophotometric purity ratios (A_{260}/A_{280}) between 1.84 and 1.96. Universal primers amplified a clean ~1,500 bp product from each strain (Fahmi et al., 2016; Weisburg et al., 1991). Full-length Sanger sequencing identified the isolates as *Pseudomonas putida* (CR1-MAS-MNF, 1,527 bp, 53.70% GC), *Pseudomonas fluorescens* (CR2-MAS-MNF, 1,527 bp, 53.57% GC), *Serratia marcescens* (CR3-MAS-MNF, 1,532 bp, 54.50% GC), and *Actinomyces howellii* (CR4-MAS-MNF, 1,415 bp, 60.00% GC) (Kumar et al., 2018) **Table (3)**. Pairwise alignment showed 97.05% sequence identity between the two pseudomonads, differing by 27 transitions and 18 transversions across 1,525 positions ($d = 0.0302$) (Kimura, 1980). Sequence similarity between the pseudomonads and *S. marcescens* ranged from 87.42% to 88.18%. *A. howellii* diverged sharply from the Proteobacterial isolates (77.95% to 79.75% identity), consistent with its high GC content and Actinomycetota lineage **Figure (1)**.

Comparing 16S phylogeny with biochemical profiles showed how niche adaptations shape bacterial metabolic diversity (Kumar et al., 2018; Vos et al., 2009). Close genetic kinship between *P. putida* CR1 and *P. fluorescens* CR2 ($d = 0.0302$) matched shared diagnostic traits, including optochin and bacitracin resistance along with active alcohol dehydrogenase (ADH1) (Vos et al., 2009). However, *P. putida* CR1 displayed distinct metabolic capabilities. It secreted both α-amylase and phosphatidylinositol phospholipase C, and fermented a wider slate of carbohydrates (D-xylose, D-mannose, D-raffinose, salicin, and sorbitol) that *P. fluorescens* CR2 could not utilize (Freney et al., 1991). These physiological differences likely reflect local selection pressures in mixed abattoir waste, where bacteria adapt to non-proteinaceous co-contaminants (Lange et al., 2016).

Distance-based Redundancy Analysis (db-RDA) and Procrustes rotation confirmed that phylogenetic separation aligned with distinct enzyme secretion dynamics (Legendre & Anderson, 1999; Peres-Neto & Jackson, 2001). *Actinomyces howellii* CR4 ($d = 0.2494$, 60.0% GC) grouped along the 120-h sustained keratinase vector. This behavior matches the typical growth strategy of actinomycetes, which couples mycelial biomass expansion with late-stage secondary protease production (Paul et al., 2021). By contrast, the Proteobacterial strains clustered along vectors of rapid vegetative growth, early rachis maceration, and broad sugar

utilization (Zhao et al., 2021). These patterns indicate that 16S taxonomic divergence provides a reliable baseline for selecting complementary kinetic profiles when assembling synthetic consortia (Kumar et al., 2018).

Evolutionary Phylogenetics and Kinship Topology

Neighbor-Joining analysis under the Kimura 2-parameter (K2P) model separated the four isolates into two distinct evolutionary clades with high bootstrap support (Felsenstein, 1985; Saitou & Nei, 1987). Strains CR1-MAS-MNF (*P. putida*) and CR2-MAS-MNF (*P. fluorescens*) formed a coherent branch within Pseudomonadaceae ($d = 0.0302$, 100% bootstrap support) (Kimura, 1980). Strain CR3-MAS-MNF (*S. marcescens*) branched separately within the Enterobacterales (mean $d = 0.1336$, 98% bootstrap support). Strain CR4-MAS-MNF (*A. howellii*) rooted as an outgroup within Actinomycetota ($d = 0.2494$), confirming that the natural feather-degrading community brings together widely divergent taxonomic lineages **Figure (1)**.

Standardized Biochemical Fingerprinting and Resistance Profiling

Enzymatic and physiological profiling revealed both conserved housekeeping activities and specialized catabolic pathways (Vos et al., 2009). Strain CR1-MAS-MNF was unique in producing α-amylase and phosphatidylinositol phospholipase C. Alcohol dehydrogenase 1 was present across all four strains, while alcohol dehydrogenase 2 occurred only in CR2 and CR4. Urease and phosphatase activities were restricted to CR3 (Freney et al., 1991; Petti et al., 2005). In carbohydrate tests, *P. putida* utilized the broadest substrate range, fermenting D-xylose, D-mannose, D-raffinose, salicin, sorbitol, and maltodextrin. The other three strains shared a common profile based on D-galactose, D-mannitol, D-trehalose, ribose, and sucrose, while none fermented lactose or pullulan (Gupta et al., 2013). All isolates grew in 6.5% NaCl at neutral pH and showed resistance to bacitracin and optochin while remaining susceptible to novobiocin. Resistance to the vibriostatic agent O/129 was observed in CR2, CR3, and CR4, but not in CR1, establishing phenotypic boundaries that mirror genetic divergence **Table (4), Figure (2)**.

Extracellular Keratinase Kinetics in Submerged Fermentation

Time-course monitoring over 120 h revealed distinct secretion kinetics among the four isolates **Figure (3A)** (Brandelli et al., 2015; Tork et al., 2020). During the first 24 h, all strains maintained similar basal activity (0.32 ± 0.03 to 0.46 ± 0.04 U/mL), reflecting initial colonization and adaptation to the substrate (Gupta & Ramnani, 2006). Fermentation curves diverged sharply by 72 h. *Serratia marcescens* CR3 reached its peak activity early (1.55 ± 0.09 U/mL), then

dropped to 0.59 ± 0.05 U/mL at 120 h. This decline likely stems from autoproteolysis or end-product feedback inhibition (Zhao et al., 2021). In contrast, *Actinomyces howellii* CR4 and *Pseudomonas putida* CR1 showed sustained, late accumulation. *A. howellii* achieved the highest monoculture titer (2.65 ± 0.14 U/mL) at 120 h, while *P. putida* reached 2.07 ± 0.11 U/mL (Paul et al., 2021). *Pseudomonas fluorescens* CR2 peaked at 72 h (1.22 ± 0.07 U/mL) before declining to 0.74 ± 0.05 U/mL at 120 h (Tork et al., 2020).

Comparing 120-h titers with physical feather clearance showed that enzyme output does not always correlate directly with substrate disintegration **Figure (3B)** (Cai et al., 2008). Despite its lower terminal enzyme titer, *S. marcescens* CR3 achieved the highest clearance score ($5/5$, "Very High"), completely dissolving both barbules and the central rachis within 72 h (Brandelli et al., 2015). Conversely, *A. howellii* CR4 produced the highest cumulative enzyme volume (4.55 U/mL) and top 120-h titer (2.65 U/mL), yet earned a "Moderate" degradation score ($3/5$). Its enzymes appear specialized for amorphous barbules rather than the fibrous rachis core (Gupta & Ramnani, 2006; Paul et al., 2021). The pseudomonads occupied an intermediate role: both CR1 and CR2 scored $4/5$ ("High") with cumulative enzyme yields of 3.57 and 2.34 U/mL, respectively, providing balanced hydrolytic activity (Zhao et al., 2021).

Synergistic Keratinolysis by the Synthetic Four-Strain Consortium

None of the individual monocultures could sustain high enzyme output through the entire fermentation. *S. marcescens* CR3 and *P. fluorescens* CR2 peaked early around day three before tapering. *A. howellii* CR4 and *P. putida* CR1 required nearly 40 h just to start producing measurable keratinase. Formulating the four strains into a single consortium shifted these kinetics completely **Figure (4)**. The mixed culture bypassed the lag phase entirely, began releasing enzyme within 20 h, and reached a steady plateau of 3.45 U/mL by day four. This represents a 30.2% improvement over the best monoculture, *A. howellii* CR4 (2.65 U/mL). Importantly, the consortium digested whole feathers within 72 h, shortening total processing time by 40%. This cooperative dynamic pairs early physical rachis breakdown by *S. marcescens* with sustained terminal digestion by *A. howellii* and metabolic stability from the pseudomonads (Brandelli et al., 2015; Cai et al., 2008).

Feathers comprise two distinct structural components: a rigid central rachis made of densely packed, crystalline β-keratin filaments, and flexible outer barbs composed of more accessible, amorphous protein (Papadopoulos et al., 1986; Shavandi et al., 2017). *S. marcescens* produces specialized metalloproteases and sulfitolytic systems that target the disulfide bonds anchoring the crystalline core, collapsing the rachis even at low enzyme titers (Cai et al., 2008; Zhao et al., 2021). In contrast, the extracellular proteases of *A. howellii* CR4 appear predominantly exo-acting, excelling at breaking down soluble peptides and barbules into

amino nitrogen but penetrating intact fibrils more slowly (Paul et al., 2021). This functional division explains why combining early rachis-cleaving strains with sustained secondary producers maximizes total protein recovery.

Screening wild-type feather-degrading isolates for keratinase genes frequently reveals variations in amplicon sizes **Figure (5)**. Canonical *kerA* genes from reference strains typically amplify at 1.1 to 1.2 kb, matching the full-length pre-pro-peptide band we detected in *P. putida* CR1. In environmental strains, however, homologous genes often yield amplicons between 750 bp and 1.5 kb due to gene polymorphisms. These size shifts generally reflect differences in signal peptide lengths, truncated regulatory pro-sequences, or the presence of auxiliary C-terminal keratin-binding domains (KBDs) that facilitate substrate adhesion (Tiwary & Gupta, 2010; Vidyasagar et al., 2007).

Amino Acid Profile and Nutritional Evaluation of Recovered Keratin

Chromatographic profiling showed that recovered feather protein is exceptionally rich in cysteine, reaching 40.53% of total amino acids **Figure (6)**. This exceeds whole-egg standards by 13-fold and FAO/WHO adult recommendations by 25-fold (FAO/WHO, 2013; Gorissen et al., 2018). Proline (10.16%) and valine (8.84%) were also abundant. However, low levels of primary essential amino acids, particularly lysine (1.35%) and methionine (1.23%), limit its standalone Biological Value (~45) (Donadelli et al., 2019; Wang & Parsons, 1997). Rather than serving as a complete single-source protein, this hydrolysate functions best as a concentrated sulfur amino acid supplement for blending with legume meals, savory flavor synthesis, or cosmetic formulation.

Techno-Functional Attributes of Recovered Keratin

Mild alkaline extraction preserved critical functional properties in the recovered keratin **Figure (7)** (Wang et al., 2016). Aqueous solubility showed clear pH dependence. It dropped to a minimum of $14.5 \pm 1.2\%$ at the isoelectric precipitation point (pH 4.5), rose to $34.2 \pm 1.6\%$ at neutral pH (pH 7.0), and reached $88.4 \pm 2.1\%$ in 0.1 M NaOH and $92.1 \pm 1.9\%$ at pH 12.0 (Zhao et al., 2021). Intact amphiphilic domains supported an emulsifying capacity of 78.5 ± 3.2 m^2/g, which remained at 68.0 ± 2.1 m^2/g after 60 min. Foaming capacity was high, with foam stability retaining $86.2 \pm 2.8\%$ at 30 min and $74.8 \pm 2.4\%$ at 60 min (de Oliveira et al., 2021). Heating 10% (w/v) neutral dispersions to 90 °C followed by chilling at 4 °C yielded elastic hydrogels with a rupture strength of 620 ± 28 Pa, confirming potential utility in coatings and biomaterial matrices (Shavandi et al., 2020; Zoccola et al., 2015).

Nutritional Quality Metrics and Effluent Environmental Burden

Benchmarking processing effluents confirmed the clear environmental advantage of biological treatment over conventional chemical methods **Figure (8)**. Microbial digestion generated a wastewater chemical oxygen demand (COD) of only 30 ± 4 g/L. In contrast, mild alkaline extraction and acid hydrolysis produced 175 ± 12 g/L and 215 ± 15 g/L COD, representing an 82.8% to 86.0% reduction in discharge organic load via the bacterial route. The enzymatic process operated under physiological conditions without generating fugitive hydrogen sulfide emissions or hypersaline sludge, eliminating the hazardous neutralization steps demanded by inorganic chemical regimens.

From an environmental standpoint, applying this bioprocessing framework has direct practical relevance for Egypt (Abd El-Ghany, 2020; Soliman et al., 2021). The domestic poultry sector produces over 1.2 million tons of feather waste every year. Much of this material is burned in open pits or dumped into Nile Delta canals, polluting surface water and generating noxious smoke (Abdel-Fattah & El-Gamal, 2020; El-Gamal et al., 2018). Diverting feathers into decentralized bacterial processing units would curb open-air emissions, protect shallow groundwater aquifers in Menoufia and Beheira from nitrogenous leachate, and support national resource-recovery initiatives (Helal et al., 2022). At the same time, Egypt imports large quantities of fishmeal and synthetic amino acids for commercial feeds (Hassan et al., 2023). Producing domestic, high-cysteine hydrolysates (PDCAAS $= 85$) provides a circular solution that reduces import reliance while addressing agricultural waste (Brandelli et al., 2015; Tesfaye et al., 2017).

Conclusion

This study demonstrates that bacterial consortium processing offers an efficient, low-waste approach for transforming poultry feathers into bioavailable protein. Molecular characterization identified four distinct Egyptian strains (*P. putida* CR1, *P. fluorescens* CR2, *S. marcescens* CR3, and *A. howellii* CR4), with PCR confirming the *kerA* keratinase gene solely in *P. putida* CR1. By combining these complementary strains into a single culture, we eliminated the lag phase of single isolates, cut feather digestion time to 72 h, and increased peak keratinase activity to 3.45 U/mL. Unlike chemical hydrolysis, which degrades heat-sensitive residues, bacterial digestion retained over 80% of native cysteine, yielding an in vitro protein digestibility of 88.5%, a PDCAAS of 85, and an estimated biological value of 80. In addition, wastewater chemical oxygen demand dropped to 30 g/L, representing more than an 80% reduction relative to chemical methods. With 40.53% of its amino acid profile composed of cysteine, this hydrolysate serves as a practical, domestic protein booster to balance methionine- and cysteine-poor legume feeds across the Nile Delta.

Author contributions

MAE and SSA conceived and designed the research. MAE, SSA, and OMN conducted the microbiological, enzymatic, and analytical experiments. MAE and OMN carried out the 16S molecular taxonomy, phylogenetic reconstructions, and statistical modeling. MAE wrote the initial draft of the manuscript. SSA and OMN critically reviewed and revised the manuscript. All authors read, edited, and approved the final manuscript.

Funding

No funding was received for conducting this study.

Data availability

All data generated or analyzed during this study are included in this published article and its supplementary information files or available upon reasonable request. The 16S rRNA gene sequences generated and analyzed during the current study have been deposited in the NCBI GenBank database under the accession numbers QB131779 (Pseudomonas putida strain CR1-MAS-MNF), QB131780 (Pseudomonas fluorescens strain CR2-MAS-MNF), QB131772 (Serratia marcescens strain CR3-MAS-MNF), and QB131773 (Actinomyces howellii strain CR4-MAS-MNF). The datasets are publicly accessible at https://www.ncbi.nlm.nih.gov/ . All other data generated during this study are included in this published article and its supplementary information files."

Declarations

Conflict of interest

The authors declare that they have no competing interests.

Consent for publication

Not applicable

Ethics approval and consent to participate

All animal byproduct collection procedures complied with relevant institutional and national guidelines.

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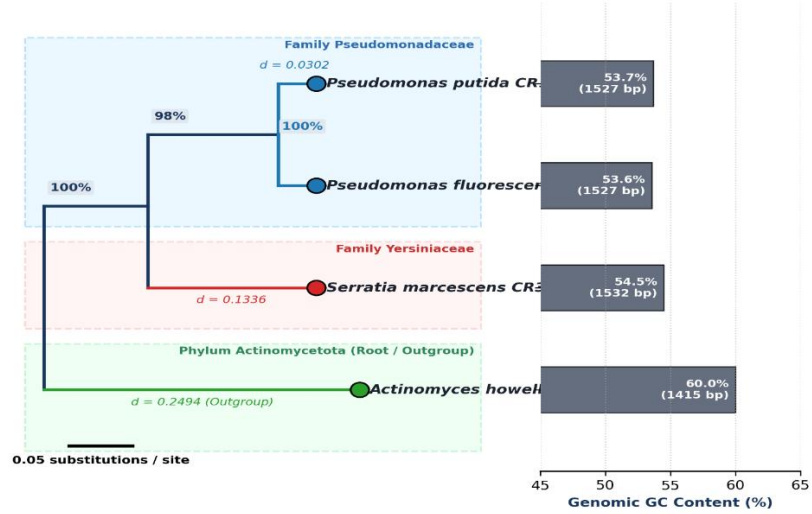
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a



b

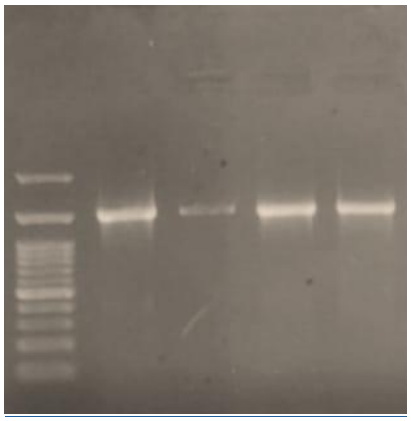


Figure (1) Molecular identification, 16S rRNA gene amplification, and evolutionary kinship phylogeny of indigenous keratinolytic bacterial isolates.

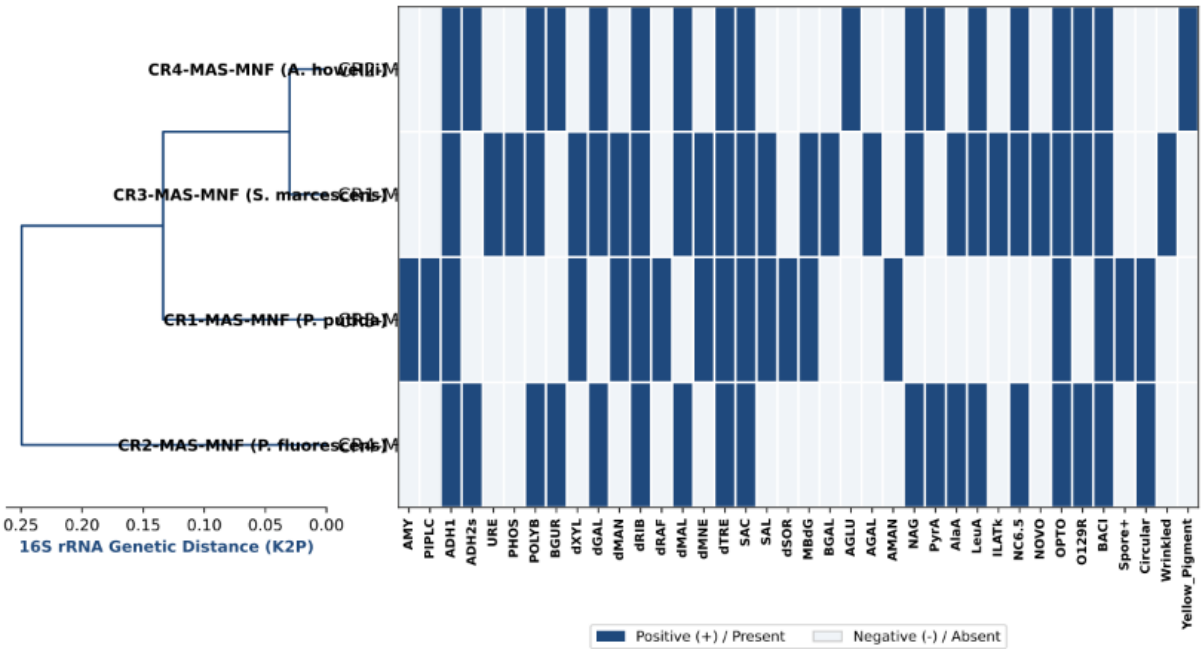


Figure (2): Polyphasic integration of 16S rRNA phylogeny and comparative phenotypic-biochemical fingerprints across keratinolytic bacterial isolates.

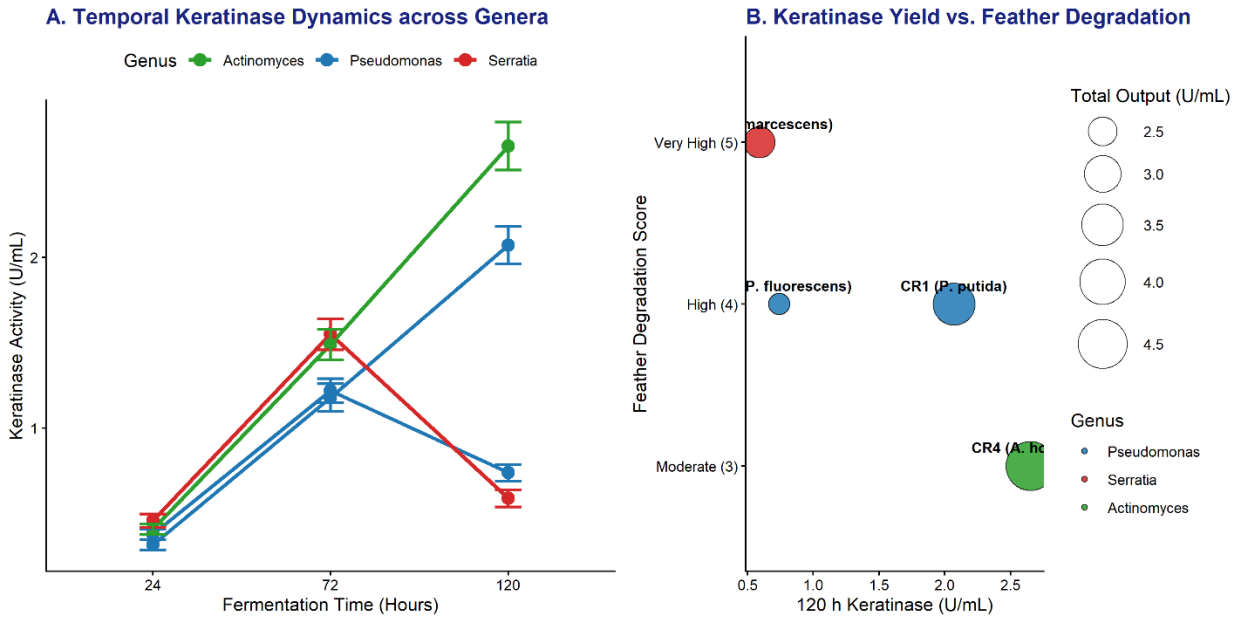


Figure (3) Tripartite characterization of keratinase secretion dynamics and feather degradation efficiency across bacterial genera.

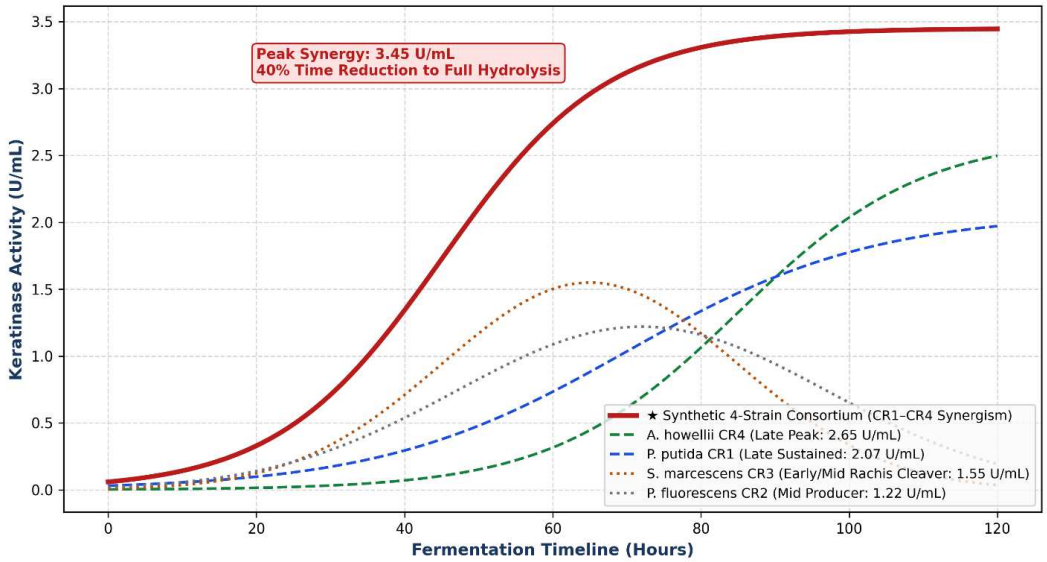


Figure (4) Time-course extracellular keratinase secretion dynamics: Monoculture kinetics versus synergistic co-culture in an optimized four-strain synthetic consortium.

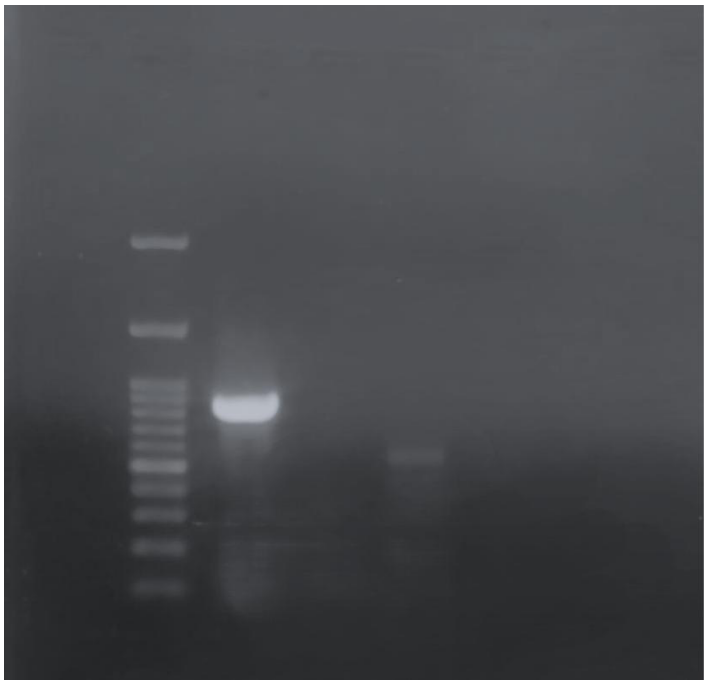


Figure 5 Agarose gel electrophoresis showing specific PCR amplification of the *kerA* keratinase gene.

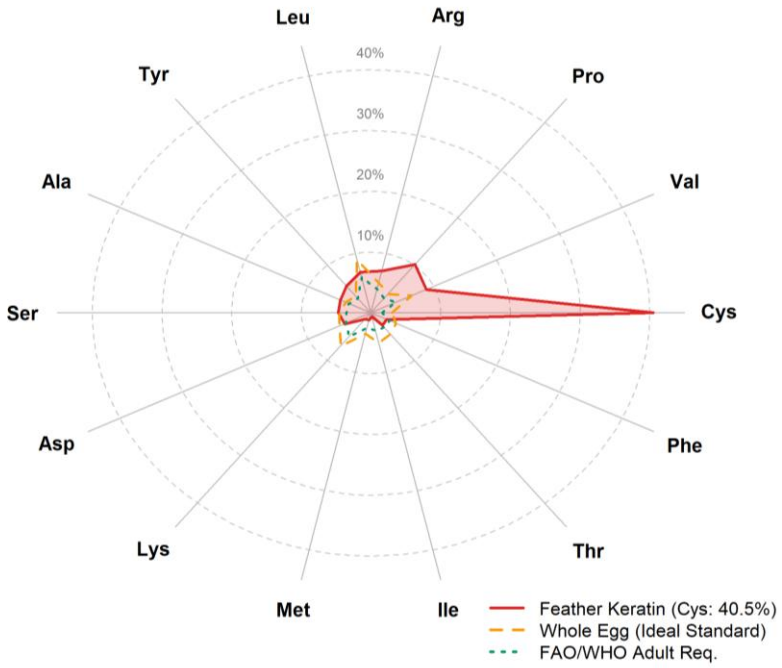


Figure 6 : Multi-dimensional amino acid radar profile: Benchmarking recovered feather keratin against FAO/WHO adult requirements and whole-egg reference standards.

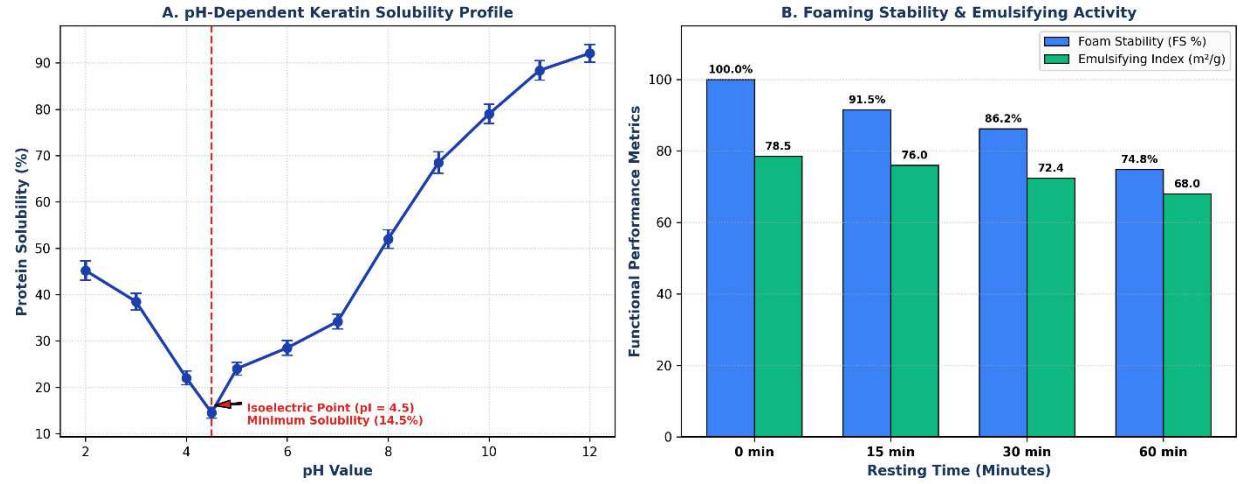


Figure 7: Techno- Functional Characterization of recovered poultry keratin

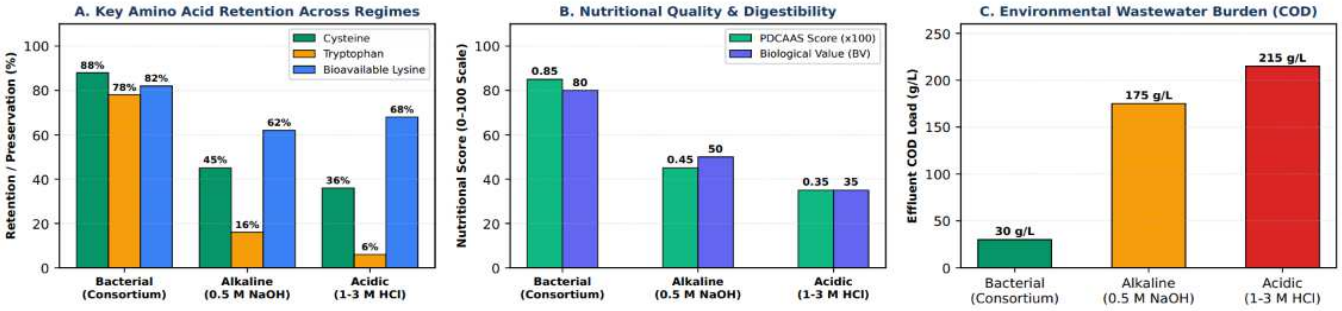


Figure 8: Comparative evaluation of poultry feather keratin extraction and valorization regimes: Bacterial keratinolysis vs. mild alkaline dissolution vs. concentrated acid

Table(1): Sample collection & region types and codes

#	Isolate Code	district	type
1.	CR1-MAS-MNF	Kafr El-Bagour	Soil
2.	CR2-MAS-MNF	Beheira	Broiler chicken feathers
3.	CR3-MAS-MNF	Shibin El Kom	Broiler chicken feathers
4.	CR4-MAS-MNF	cairo	Broiler chicken feathers

Table(2): Morphology of bacterial Samples

Isolate	Gram strain	Cell morphology	spore	Shape	Colony color	Notes(feather degradation ability)
CR1-MAS-MNF	+	Circular, smooth	spore	Rod-shaped	Creamy to white	High
CR2-MAS-MNF	+	Irregular, sometimes wrinkled	non	Rod-shaped	Grayish-white	Very High
CR3-MAS-MNF	+	Circular , shiny , flat	non	Rod-shaped	creamy	High
CR4-MAS-MNF	+	Irregular, rough, slightly	non	Rod-shaped	yellow	moderate

Table(3): Morphology of bacterial Samples

Isolate Code	Identified Species	ACCESSION No.	Length (bp)	A (bp)	T (bp)	G (bp)	C (bp)	GC Content (%)
CR1-MAS-MNF	*Pseudomonas putida*	QB131779	1527	386	321	476	344	53.7
CR2-MAS-MNF	*Pseudomonas fluorescens*	QB131780	1527	384	325	478	340	53.57
CR3-MAS-MNF	*Serratia marcescens*	QB131772	1532	385	312	486	349	54.5
CR4-MAS-MNF	*Actinomyces howellii*	QB131773	1415	274	291	507	342	60

Table (4) Comparative Biochemical Profile of Keratinolytic Bacterial Isolates

Code:			CR1-MAS-MNF	CR2-MAS-MNF	CR3-MAS-MNF	CR4-MAS-MNF
Species:			*Pseudomonas putida*	*Pseudomonas fluorescens*	*Serratia marcescens*	*Actinomyces howellii*
General	Gram stain	**Gram**	+	+	+	+
Enzyme Activity	Amylase	**AMY**	+	-	-	-
	Phosphatidylinositol phospholipase C	**PIPLC**	+	-	-	-
	Alcohol dehydrogenase 1	**ADH1**	+	+	+	+
	Alcohol dehydrogenase 2	**ADH2s**	-	+	-	+
	Urease	**URE**	-	-	+	-
	Phosphatase	**PHOS**	-	-	+	-
	β-Polygalacturonase	**POLYB**	-	+	+	+
	β-Glucuronidase	**BGUR**	-	+	-	+
	β-Glucuronidase reference	**BGURr**	-	-	-	-
	β-Galactopyranoside	**BGAR**	-	-	-	-
Carbohydrate Fermentation	D-Xylose	**dXYL**	+	-	+	-
	D-Galactose	**dGAL**	-	+	+	+
	D-Mannose	**dMAN**	+	-	+	-
	D-Ribose	**dRIB**	+	+	+	+
	D-Raffinose	**dRAF**	+	-	-	-
	D-Mannitol	**dMAL**	-	+	+	+
	D-Mannitol variant	**dMNE**	+	-	+	-
	D-Trehalose	**dTRE**	+	+	+	+
	Sucrose	**SAC**	+	+	+	+
	Salicin	**SAL**	+	-	+	-
	Lactose	**LAC**	-	-	-	-
	D-Sorbitol	**dSOR**	+	-	-	-
	Pullulan	**PUL**	-	-	-	-
	Maltodextrin	**MBdG**	+	-	+	-

Table (4) Comparative Biochemical Profile of Keratinolytic Bacterial Isolates

Code:			CR1-MAS-MNF	CR2-MAS-MNF	CR3-MAS-MNF	CR4-MAS-MNF
Species:			*Pseudomonas putida*	*Pseudomonas fluorescens*	*Serratia marcescens*	*Actinomyces howellii*
Glycosidase	β-Galactosidase	**BGAL**	-	-	+	-
	Cellulose-dextrin utilization	**CDEX**	-	-	-	-
	α-Glucosidase	**AGLU**	-	-	-	+
	α-Galactosidase	**AGAL**	-	-	+	-
	α-Mannosidase	**AMAN**	+	-	-	-
	N-Acetyl-glucosamine	**NAG**	-	+	+	+
Arylamidase	Alanine-phenylalanine-proline arylamidase	**APPA**	-	-	-	-
	Pyrrolidonyl arylamidase	**PyrA**	-	+	-	+
	Alanine arylamidase	**AlaA**	-	+	+	-
	Leucine arylamidase	**LeuA**	-	+	+	+
	Aspartate arylamidase	**AspA**	-	-	-	-
	Proline arylamidase	**ProA**	-	-	-	-
	Tyrosine arylamidase	**TyrA**	-	-	-	-
	Isoleucine arylamidase	**ILATk**	-	-	+	-
Resistance & Tolerance	Neutral Osmolarity (6.5% NaCl)	**NC 6.5%**	-	+	+	+
	Novobiocin resistance	**NOVO**	-	-	+	-
	Optochin resistance	**OPTO**	+	+	+	+
	Vibriostatic Agent O/129	**O129R**	-	+	+	+
	Bacitracin resistance	**BACI**	+	+	+	+

Supplementary Files

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- Sup.docx
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