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Enhancing spinach growth with a biofertilizer derived from chicken feathers using a keratinolytic bacterial consortium

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Abstract

This study was conducted to develop a cost-effective and environmentally friendly biofertilizer by utilizing chicken feather waste. Two bacterial strains were employed to biotransform the abundant keratin protein in feathers. The keratinolytic bacterial strains used in this study were identified as *Bacillus licheniformis* MW45 and *Bacillus paralicheniformis* MW48. The feather hydrolysate was assessed for its effectiveness as a nitrogen fertilizer with slow-release properties. The study employed a completely randomized design (CRD) with three replicates, and statistical analysis, including ANOVA followed by Tukey's test, was used to validate the differences between treatments. The test plant was spinach, and various growth parameters were observed. The growth promotion activity of the produced biofertilizer was compared with a commercially available NPK fertilizer. The results showed that the growth promotion effect of chicken feather hydrolysate was significantly higher than the control and commercially available NPK fertilizer. The feather hydrolysate displayed the highest germination percentage (48%), vigor index (1081.44), number of leaves (17), height (22.53 cm), and weight (3.493 g), compared to the chemical fertilizer's germination percentage (31%), vigor index (714.4), number of leaves (13), height (18.5 cm), and weight (1.904 g). Statistical analysis showed that the fermented chicken feather hydrolysate can be effectively applied as a slow-releasing nitrogen fertilizer in agricultural fields. The novelty of this study lies in the use of a bacterial consortium to transform chicken feathers into high efficiency biofertilizer. This production may not only supports the national economy by increasing crop yield but also contributes to a cleaner and greener Pakistan by recycling feather waste.

Keywords Keratinase, Keratin hydrolysate, Feather waste, Immobilization, Corncob, Biofertilizer, Spinach plant

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Introduction

One of Pakistan's major industries, the poultry industry adds 1.3% to the country's GDP, which is a substantial amount. Pakistan is the eleventh-largest producer of chicken worldwide. According to data from the Pakistan Poultry Association, 1.4 million tons of chicken meat were produced in Pakistan in 2018–2019 [1]. The use of chicken meat rendered chicken feathers as obstinate garbage as the feathers of a bird can weigh approximately 125 g (5–7%). These feathers are made up of 90% keratin protein which gives them strength and resistance to deterioration. Keratin is an insoluble protein constituent of skin and its appendages, including hair, nails, feathers, and wool [2]. To create 3-dimensional structural proteins, the keratin peptides are firmly bonded together in disulfide bridges by cysteine residues in either α-helix (α-keratin) or β-sheet (β-keratin) [3]. Based on sulfur content keratin can be grouped into soft keratins (e.g. skin) and hard keratins (e.g. feathers, hairs, etc.) [4]. Ecological challenges are being developed by this obstinate garbage pile for the land and water resources [5]. Keratin degradation can be obtained through various methods such as chemical, thermal, and enzymatic. Chemical methods include disruption of keratin structure by use of chemical reagents or strong alkali [6]. Thermal hydrolysis involves the use of high temperature and pressure to disrupt the structure of keratin, whereas enzymatic hydrolysis involves the degradation of keratin by proteolytic enzymes. Enzymatic hydrolysis is the most efficient method of obtaining keratin hydrolysate as it is eco-friendly and provides high-quality products [7].

To handle this obstinate garbage, recycling of chicken feathers is an absolute management which can be achieved cheaply through biodegradation by keratinolytic microorganisms such as *Microsporum*, *Doratomyces microsporum*, *Trychophyton*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Bacillus and Streptomyces.* These microbial species have potential to degrade keratin protein by producing extracellular enzyme known as keratinase [8]. To enhance keratinase production, immobilization of cells is employed which is an impressive strategy to enhance enzyme production by restricting cells either completely or partly through its attachment to a solid surface [9]. Cells are attached through various techniques i.e. adsorption, entrapment, encapsulation, cross-linking, and covalent bonding [10]. The matrix that is usually used in immobilization must have a greater surface area for the attachment of cells. Corn cob is an integral part and the waste of corn is spongy in the structure that provides greater surface area for absorption of cells [11].

The cost-effective keratin biodegradation imposes its various significant industrial applications such as in leather, cosmetics, detergent, as a feed supplement and nitrogen fertilizer, etc [5]. Pakistan as a developing and agricultural country needs a cheap and effective strategy to enhance its economy through the agricultural sector.

Several strategies have been used to improve crop growth, including chemical fertilizers, organic amendments such as compost and manure, and biological methods such as biofertilizers and mycorrhizal inoculants. While chemical fertilizers are widely used, their rapid nutrient release frequently causes environmental problems, such as nitrogen leaching and soil degradation. Organic amendments, while sustainable, are resource-intensive and slow-acting. Single-species biofertilizers have shown promise, but their efficacy is often limited due to restricted microbial activity. In contrast, using keratin-based biofertilizers derived from chicken feather waste is a sustainable and cost-effective solution. These biofertilizers not only provide slow-release nitrogen but also improve soil composition, making them ideal for achieving the dual goals of environmental conservation and agricultural productivity. Keratin is rich in nitrogen content of about 15% and releases nitrogen gradually upon degradation as compared to the commercially available chemical NPK fertilizer that releases nitrogen readily which flushes out with water and is inaccessible to plants [12]. Keratin hydrolysate also adjusts the composition and quality of soil besides supplying nitrogen to plants [13]. In Asian countries especially in Pakistan, spinach is used widely as a vegetable raising its demand as Pakistan ranked 8th among spinach-producing countries [14]. Nitrogen fertilizer helps obtain darker and more vigorous leaves of spinach plants with higher production rates [15]. The prior studies documenting the effect of microbially synthesized biofertilizer from chicken feather waste on spinach plants are very limited and primarily focused on single species [16, 17]. The objective of this study was to formulate a cell-free biofertilizer using a bacterial consortium with chicken feather waste. Additionally, this study explored the effectiveness of feather hydrolysate on seed germination and other plant growth parameters of spinach plants.

Materials and methods

Culture and seed collection

Spinach seeds were collected from a local nursery and processed in the laboratory at the Department of Microbiology, according to the guidelines and regulations of the University of Karachi. After processing, the seeds were planted, and all the pot plants were kept in the greenhouse of the Department of Botany, University of Karachi. Bacterial strains *Bacillus licheniformis* MW45 and *Bacillus paralicheniformis* MW48 were previously isolated from poultry farm soil samples and maintained on Luria-Bertani (LB) agar at 4 °C, sub-cultured after every 15-day interval [18].

Inoculum Preparation and culture conditions for keratinase production

To obtain the seed cultures, a loop full of the bacterial strains *Bacillus licheniformis* MW45 and *Bacillus paralicheniformis* MW48 were inoculated in nutrient broth for overnight cultures at 37 °C. Then their turbidity was matched with 0.5 Mc Farland standard solution (1.5×10^6 – 1.5×10^8 CFU/mL). An inoculum size of 8% and 2% was used for *B. licheniformis* MW45 and *B. paralicheniformis* MW48 respectively. For keratinase production, two distinct previously optimized LB media compositions (LB45 and LB48) were employed to cultivate *B.licheniformis* MW45 and *B.paralicheniformis* MW48 strains respectively (Table 1). These compositions were examined to enhance keratinase synthesis from individual keratinolytic strains as well as co-culture [19].

Immobilization of cultures on corn cobs

For enhanced production of keratinase enzyme, immobilization of the producer cultures on corncob was attempted. Corn cobs were primarily cut into 0.1- 0.5cm^3 size pieces, washed, and then boiled for 5–10 min followed by drying and autoclaving [20]. Six pieces of corn cobs and 5% overnight inoculum were then transferred into the 50 ml of nutrient broth and incubated at 37 °C for 24 h to let cells be immobilized on the cobs. After an incubation period, corncobs immobilized with keratinolytic strains were added to their respective medium.

Keratinase production

After adding both free cell inoculum and immobilized inoculum, culture media broths were incubated for 72 h at 45 °C with constant shaking (150 rpm). Later, the contents of each medium were centrifuged to get a cell-free supernatant containing enzyme i.e. keratinase. The keratinase production was then determined by the enzyme assay. This cell free supernatant consisting of the keratinase enzyme is termed feather hydrolysate and will be assessed as a biofertilizer in pot plant assay.

Table 1 Medium composition of LB45 and LB48

Components	Medium LB45	Medium LB48
Glucose	1%	1%
Peptone	5%	0.287%
Yeast	0.1%	0.2%
NaCl	0.2%	0.05%
Feathers	0.1%	0.5%
Inoculum size	8%	2%
pH	7	7
Temperature	45 °C	44 °C
Incubation time	72 h	72 h

Enzyme assay

For the detection of quantitative analysis of keratinase enzyme, the protocol for enzyme assay begins with the addition of 130µL of casein solution (0.66% w/v) and 25µL of crude keratinase enzyme extract (feather hydrolysate) in a microcentrifuge tube followed by incubation at 37 °C for 20 min [21]. Then addition of 130µL of 110mM TCA and incubation at 37 °C for 20 min followed by centrifugation at 2500 rpm for 10 min. After centrifugation, 250µL of the supernatant was transferred to the separate microcentrifuge tube. The addition of 625µL of 500mM sodium carbonate solution and 125µL of Folin and Ciocalteu's Phenol Reagent was followed by incubation at 37 °C for 30 min. Optical density was recorded at 660 nm by using a spectrophotometer.

One unit will hydrolyze casein to produce a color equivalent to 1.0µmole (181 µg) of tyrosine per minute at pH 7.5 at 37 °C (color by Folin and Ciocalteu's Reagent) [21].

$$Units/mL = \frac{\mu\text{mole of tyrosine x reaction volume}}{\text{sample volume x reaction time x volume assayed}}$$

Feather degradation percentage

Degraded pieces of feathers were picked out from the medium after incubation using sterilized forceps. After removal, feathers were washed thoroughly with ethanol followed by drying, and then weighed to observe the final weight after degradation [22]. The feather degrading percentage was calculated according to the following formula,

$$\text{Percentage of weight loss} = \frac{\text{Initial weight - Final weight}}{\text{Initial weight}} x100$$

Nitrogen Estimation

The nitrogen content of the media was estimated by the Kjeldahl method. In a Kjeldahl flask, 0.5 g of keratin hydrolysate sample and the mixture of Na_2SO_4 and $CuSO_4$ in a ratio of 5:1 were taken. Then, the mixture was digested with 15 ml of conc. H_2SO_4. 40% NaOH was used to neutralize the digested solution and then distilled into a 4% boric acid solution. The borate anions formed were then titrated with 0.5M H_2SO_4, which was converted into the nitrogen in the sample and recorded in mg/L [23].

Application of keratin hydrolysate

The efficacy of the feather hydrolysate (being rich in keratin) as a biofertilizer was assessed on the *Spinacia oleracea* (Spinach) plant [24]. Pots (20 cm deep and 25 cm in diameter) containing 1000 g of garden soil were divided into three groups. The control group did not receive any fertilizer treatment. The two test groups include the pots amended with NPK chemical fertilizer and those that

received freshly prepared feather hydrolysate as biofertilizer. Thirty (30) seeds of *Spinacia oleracea* (Spinach) plant per pot were sowed and watered regularly. The pot soil was amended with feather hydrolysate as a 20% root dose after sowing seeds and then at an interval of 15 days [25] while 0.1 g of commercially available NPK chemical fertilizer was added to the soil at the same intervals mentioned for feather hydrolysate. The experiment was run in triplicates.

Germination percentage

After 15 days, the number of seedlings was counted, and the germination percentage was calculated by the following formula [26].

$$\text{Germination } \% = \frac{\text{Number of germinated seeds}}{\text{Numberoftotalseeds}}x100$$

Agronomic parameters

After 50 days of sowing seeds, a plant was harvested from each pot for data collection in terms of the following parameters [27].

Seedling Vigor index

The root length and stem length of the plant were measured in centimeters and the seedling vigor index was calculated by the following formula:

Vigor index = [Mean root length (cm) + mean shoot length (cm)] × germination percentage.

Plant height

The height of the plant was measured in centimeters from the ground level to the tip of the stem by using a measuring scale and the average height of the plant per pot was recorded.

The number of leaves

The total number of leaves from each pot were counted and the data was recorded as an average number of leaves per pot.

Weight of plant

The dry weight of the plant including stem and leaves was measured and recorded. The average weight per pot was presented in grams per plant.

Statistical analysis

All the experiments were run in triplicates and data values were expressed as mean ± S.D. One way ANOVA was applied using Minitab (release 19) on data to find efficacy of feather hydrolysate as biofertilizer at 5% level of significance. Tukey's Post Hoc test was applied where $\alpha < 0.05$.

Results and discussions

Being a recalcitrant inedible protein, keratin renders chicken feathers with low solubility in water and other organic solvents, leading to extensive ecological difficulties [28]. The profound disposal of a chicken feather by biodegradation is the only way to manage this surplus waste [29]. Thus, the present study focused on the recycling of feather waste and its application. When grown individually, the *B. licheniformis* MW45 strain was found to be more potent (20.889 keratinase units/mL) than the *B. paralicheniformis* MW48 strain (10.390 keratinase units/mL) for the production of the keratinase enzyme. In addition, it was noticed that immobilization enhanced the production of the keratinase enzyme by about 12% (32.347 keratinase units/mL). To further enhance the keratinase enzyme production and rate of feather degradation, both keratinolytic strains were grown together as free cells and in immobilized form for 72 h. Interestingly, an immobilized consortium of *B. licheniformis* MW45 and *B. paralicheniformis* MW48 strains on corn cob in medium LB45 was found to be most effective in degrading feathers by actively producing keratinase enzyme, as shown in Fig. 1. The co-immobilized strains showed a 17% increase in keratinase production compared to the free cells (45.790 keratinase units/mL), with the feather degradation percentage reaching up to 95–100% within 72 h, as shown in Figs. 1 and 2. These results are also supported by a study where a bacterial consortium, KMCG6, featuring chiefly members of *Bacteroidetes* and *Proteobacteria*, exhibited a high keratin degradation level as compared to a single microbial species [30]. It has been claimed that immobilization results in improved biocatalyst steadiness and better efficiency over free systems [31]. Corncob is a cheap and easily available substrate that does not require any harsh treatment before use, making it an ideal matrix for the adsorption technique of immobilization [32].

The recycling of keratinous material has attracted great attention in recent times and raised its various applications in different industries such as agriculture, leather, cosmetology, food, and pharmaceutical industries [33]. In Pakistan, chicken meat is widely consumed; hence, recycling feathers is one of the major problems faced by Pakistan. Our study revealed the promising difference in nitrogen content of feather hydrolysate obtained through a monoculture of *B. licheniformis* and a consortium of *B. licheniformis* & *B. paralicheniformis*. Strains in consortium with a nitrogen content of 1.8 mg/L were found to be more competent than monoculture with a nitrogen content of 1.2 mg/L, as shown in Fig. 3. A study revealed that nitrogen content between 3.06 and 10.94% in hair wastewater significantly improves the germination percentage of the Abelmoschus esculenthus (Okra) plant when compared to plants without fertilizer. Nitrogen has

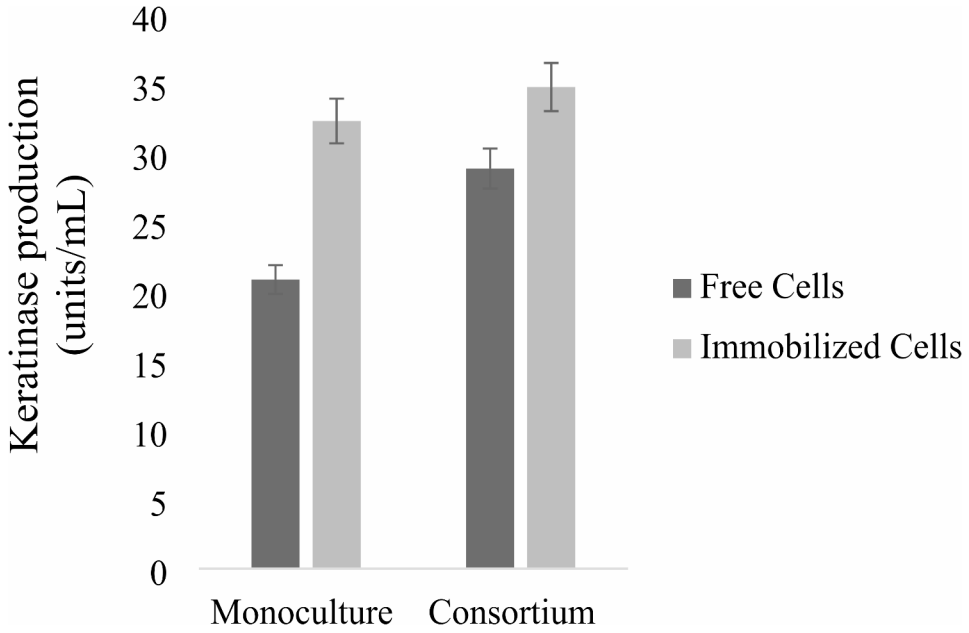

Fig. 1 Keratinase production by *B. licheniformis* MW45 monoculture and a consortium of *B. licheniformis* MW45 & *B. paralicheniformis* MW48 strains using chicken feathers

been observed to significantly influence both the growth and irritability of plants [34].

Several studies noted that strains in consortia were found to be more significant as compared to single strains. One of the study findings showed that strains in consortium can improve productivity and cope with undesirable environmental conditions better than single strains. when different strains of *Penicillium*, *Bacillus*, and *Pseudomonas* were inoculated as a single and in consortium with various environmental challenges such as high alkaline pH (7.9), low fertility, less organic carbon availability (0.08%), and sandy soil (96% sand) with low precipitation for the cultivation of *Lycopersicum esculentum L.* (tomato) plants. It was noted that weight of the fruit has increased by 20–30% and the tomato yield increased by 10% when these consortium strains were used [35]. Feathers rich in nitrogen content (> 15%) open a door to the agricultural industry as a slow-releasing nitrogen fertilizer. In developing countries with limited resources, the usage of feather waste as a promising, inexpensive, and eco-friendly nitrogen fertilizer will surely contribute to managing this garbage.

To meet the rising demand for food as a result of the world population's continual growth, it is crucial to raise the productivity of food crops [36]. Around the world, synthetic fertilizers are utilized to boost crop output [37]. Despite producing good results, these fertilizers have many negative side effects [38]. Overuse of fertilizers can cause eutrophication, severe soil acidity or alkalinity, and water and soil contamination. As a result, the soil's quality and microflora have drastically declined [39]. About 15% of the nitrogen in chicken feathers, which are formed of keratin, can be utilized as fertilizer for plants [40]. It has been established that feather hydrolysate, which is produced when fungi and bacteria break down feathers, could be used as a slow-release nitrogen fertilizer for plants. Plants can use the combination of amino acids and peptides found in feather hydrolysate [41]. Tryptophan, another amino acid present in feather hydrolysate, is used by microbes to synthesize indole acetic acid (IAA), a phytohormone [42].

As per the current study, the growth promotion effect of chicken feather hydrolysate was found to be significantly higher than that of the control and commercially available NPK fertilizer (Fig. 4). When compared, feather hydrolysate displayed the highest germination percentage (48%), vigor index (1081.44), average number of leaves/pot (17), height (22.53 cm), and average weight/plant (3.493 g) whereas in the case of chemical fertilizer, these parameters were recorded as germination percentage (31%), vigor index (714.4), the average number of leaves/pot (13), average height/plant (18.5 cm) and average weight/plant (1.904 g) (Table 2). The addition of feather hydrolysate improves soil fertility by altering the N, P, K, and C/N ratios. It not only stimulates seed germination and seedling growth, but it also improves nodule development. Chicken feather hydrolysate showed promising results on the growth enhancement of Bengal gram (*Cicer arietinum*) with a germination rate of 87.5% [43]. According to one of Nayaka and Vidyasagar's studies, the feather compost outperformed all other composts (such as mulberry, cow dung, and urea) tested for their

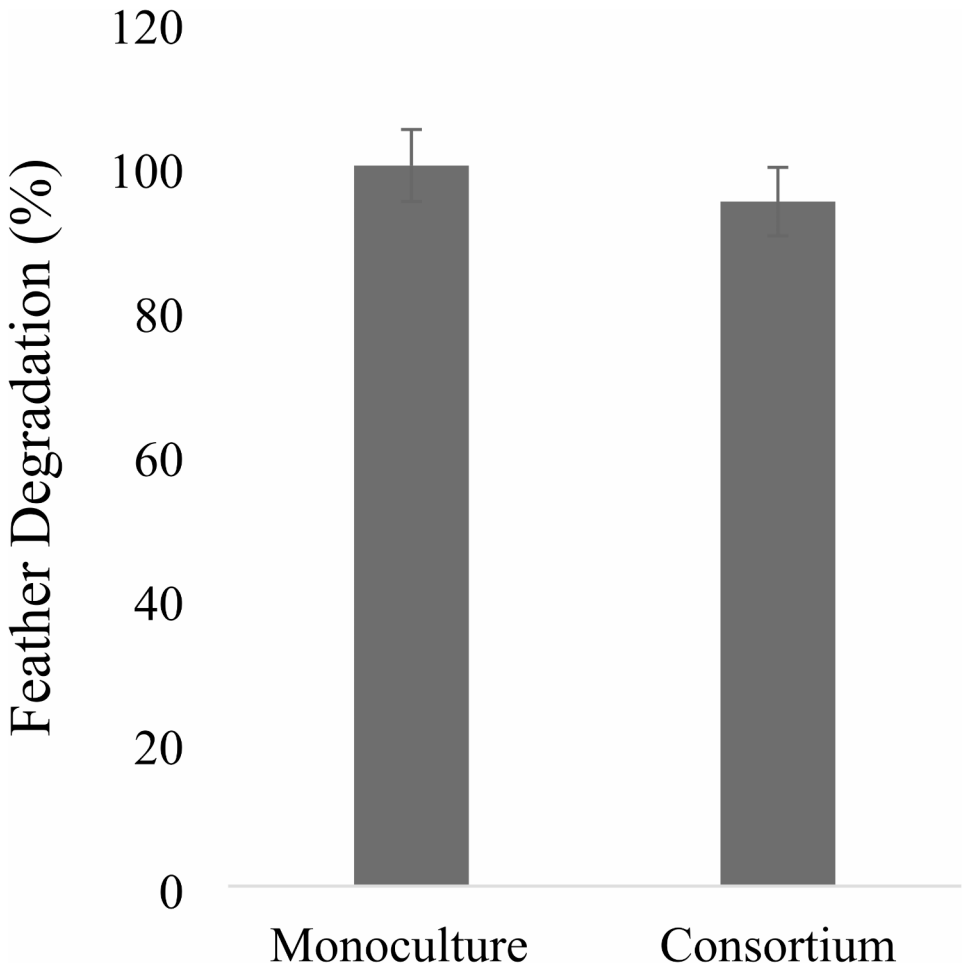

Fig. 2 Feather Degradation (%) by *B. licheniformis* MW45 monoculture and a consortium of *B. licheniformis* MW45 & *B. paralicheniformis* MW48 strains using chicken feathers

significant effect on the growth of *C. Roseus* (Sada bahar) plant [44]. Likewise, biofertilizer derived from feather waste was found to be an effective substitute for expensive and environmentally harmful chemical fertilizers by increasing soil carbon content and improving tomato plant enzymatic activity [45]. In another study, keratinolytic actinomycete *Streptomyces* CN1 was also found to be a potent degrader to develop slow-releasing nitrogen fertilizer for a longer duration [46].

It has been discovered that microbes that break down feathers also generate several elements that aid the growth of plants, including siderophores, acids that break down insoluble phosphate so that plants can utilize it. Phosphate solubilizers and free-living nitrogen fixers are also found to be more abundant in soil treated with feather hydrolysate [43]. In a study by Jeong et al. (2010b) *Stenotrophomonas maltophilia* R13, a bacterium was discovered capable of digesting chicken feathers in a minimal medium by producing disulfide reductase, which generates large quantities of free amino acids. This strain also demonstrated positive traits for plant growth such as the high production of indole-3-acetic acid and the ability to inhibit phytopathogenic fungi [12]. Kucinska et al. (2014) investigated the effects of compost and feather hydrolysate on plant growth and found that the characteristics of soil were found to be significantly improved by these supplements, and the growth of white cabbage, tomatoes, and maize was promoted [13]. These treatments were found to dramatically enhance chlorophyll content, reduce phenylalanine ammonia-lyase activity, and boost guaiacol peroxidase activity. In a study by Bose et al. (2014), feather degradation by *Bacillus amyloliquefaciens* 6B was explored. The growth of *Vigna radiata* var. meha (mung plant) was positively impacted by the feather hydrolysate produced after the complete chicken feathers degradation [47]. Bhange et al. (2016) studied the effects on the stimulation of Mung plant (*V. radiata*) by a keratinolytic *Bacillus subtilis* PF1 strain which was recovered in their study owing to the ability to break down large quantities of chicken feathers. In Tamreihao et al.'s study, the strain of *Amycolatopsis sp.* MBRL 40 was found to be capable of digesting chicken feathers. Rice plants treated

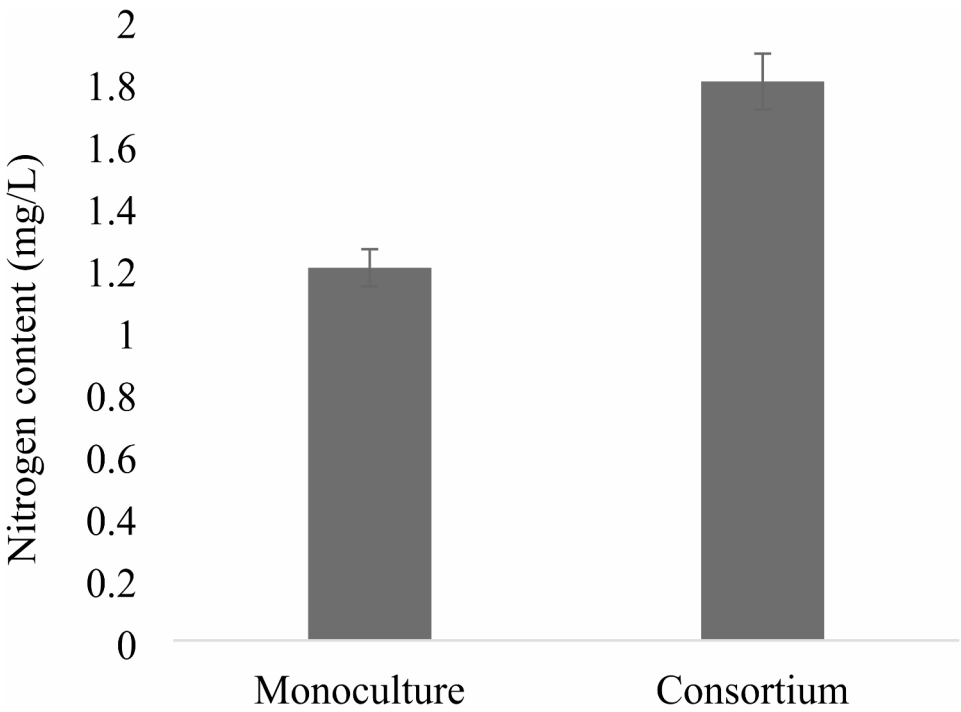

Fig. 3 Nitrogen content (mg/L) of chicken feather hydrolysate by *B. licheniformis* MW45 monoculture and a consortium of *B. licheniformis* MW45 & *B. paralicheniformis* MW48 strains

Fig. 4 Spinach plant with different amendments: (**a**) control (**b**) chemical fertilizer (**c**) keratin hydrolysate

Table 2 Agronomic parameters of spinach plant grown with different amendments

Parameters	Control	Chemical Fertilizer	Keratin hydrolysate	*P* value α= < 0.05
Germination %	38 ± 0.051	31 ± 0.017	48 ± 0.040	0.006*
Vigor Index	714.40 ± 111.8	573.50 ± 40.7	1081.44 ± 20.3	0.000*
No. of leaves	13 ± 2	12 ± 1.528	17 ± 2.31	0.065
Height (cm)	18.80 ± 1.323	18.50 ± 1.266	22.53 ± 2.06	0.015*
Weight (g)	1.826 ± 0.863	1.904 ± 0.425	3.493 ± 0.778	0.049*

*α= < 0.05 is statistically significant

with feather hydrolysate showed a significant increase in fresh weight, dry weight, and the number of tillers when compared to control plants grown without these supplements [48]. A study by Nafady et al. (2018) discovered a strain of *Bacillus licheniformis* ASU that could break down chicken feathers [49]. Moreover, this strain was competent to dissolve insoluble phosphate and generated large levels of IAA. Together with feather hydrolysate and the arbuscular mycorrhizal fungi *Acaulospora bireticulata* and *Glomus caesaris*, this strain was inoculated to promote the growth of the *Vicia faba L plant*. Following inoculation, the plants showed improved photosynthesis rate, nodulation, and nitrogen fixation. In another study, it was demonstrated that chicken feathers when inoculated in tomato plants, significantly decreased the bulk density of the soil while increasing its porosity, organic matter, nutrient content, and tomato yield [50].

For both plants and animals, antioxidants are essential because they scavenge free radicals and shield cells from damage [51]. The presence of free amino acids & soluble peptides in the feather hydrolysate has been shown in numerous studies to possess significant antioxidant characteristics [52, 53]. The feather hydrolysate prepared after the degradation of 50 g/L of chicken feather using *Bacillus pumilus* A1 displayed a very high antioxidant activity [48]. Likewise, when the biodegradation of chicken feathers by a consortium of *S. maltophilia* BBE11-1 and *B. licheniformis* BBE11-1 was studied by Peng et al. (2019), the hydrolysate displayed strong antioxidant capabilities, according to the ferric-reducing antioxidant power (FRAP) assay [54]. The current study revealed that our potent bacterial degraders can effectively synthesize the keratinase enzyme to cleave the defiant keratin protein, a main component of the feather. Keratin hydrolysate obtained through the biodegradation of chicken feathers has been found to have the potential to be applied as a biofertilizer for the cultivation of the spinach plant. It was noted that keratin hydrolysate significantly enhances the growth of spinach plants when compared to the effect of chemical fertilizer on the growth of plants, making it a more cost-effective approach than the use of expensive chemical NPK fertilizer.

Conclusions

The present research explored the potential of indigenous, co-immobilized *B. licheniformis* MW45 and *B. paralicheniformis* MW48 strains for keratinase production, with the chicken feather degrading percentage reaching up to 95–100% within 72 h. The feather hydrolysate obtained through this bioconversion has great potential to serve as an eco-friendly, slow-release nitrogen fertilizer for plants such as spinach. This approach can be promising for the goal of a cleaner and greener environment as it also involves the recycling of otherwise recalcitrant feather waste.

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Author contributions
IS, WA, TK, SBA did conceptualization, formal analysis and original draft writing, WA, AAMAH and TAS did reviewing, and editing: IS, and SK did investigations, and editing, LOM, and WA provided resources and financial assistance.

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Data availability
Data will be available upon request from the corresponding author.

Declarations

Ethical approval
The study used spinach seeds collected from a local nursery and processed in the laboratory at the Department of Microbiology, according to the guidelines and regulations of the University of Karachi and no additional ethical approval was required for this study.

Consent for publication
Not applicable.

Informed consent
Not applicable.

Competing interests
The authors declare no competing interests.

Statement of human and animal rights
Not applicable.

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