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Decomposed chicken feather: A biostimulant to lettuce (*Lactuca sativa*) growth

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ABSTRACT

It is crucial to produce plant growth biostimulants from waste and renewable resources. A study was carried during 2020 at the Soil and Water Research Institute, Karaj, Iran to evaluate the effect of decomposed chicken feather as a biostimulant on lettuce (*Lactuca sativa* L.) growth. A total of 29 soil samples from 15 Iranian regions were used to isolate and identify the bacteria and 31 strains that were able to produce keratinase and capable of feather degrading were isolated. Then, 8 isolates that were able to degrade the feather during 7 days were selected as the superior strains. Based on the results *Bacillus siamensis* c11, *Bacillus methylotrophicus* gh1 and *Bacillus methylotrophicus* a2 were identified as the new strains that can produce keratinase enzyme. Then, the effect of foliar spraying of the solutions produced from feather degradation by the new strains (c11, gh1, and a2) on the growth of lettuce was investigated. The results showed that the solution produced by *methylotrophicus* gh1 strain significantly increased the fresh and dry weight of the shoot and root (respectively, 25.9, 36.9, 34.1 and 51.9% increase compared to the control). It is concluded that the microbial solution of these three microbes as a consortium could be quite a new addition on one hand, could also reduce the human allergy as a via- medium of waste disposal.

Keywords: Biodegradation, Growth regulators biostimulants, Soil bacteria, Waste

To date, numerous advances have been made toward the use of new technologies to improve the level of agriculture outcomes and prevent damage to the environment (Mousavi *et al.* 2022). Some of these causing factors includes, use of soil bacterial degrading wastes and contaminants (Mousavi *et al.* 2018a, Moshiri *et al.* 2019) and use of their potential to promote plant growth and yield (Sheikhy *et al.* 2018, Srivastava *et al.* 2021, Cheraghi *et al.* 2022, Basirat *et al.* 2023). Amino acids are organic compounds and the most important parts of protein with various functions. They facilitate nutrient transfer, reduce heavy metal toxicity, regulate stoma opening, increase environmental stress resistance, improve nutrient absorption, and regulate ionic transfer (Radkowski 2018). The production of amino acids can be significantly reduced by utilizing biotechnology processes and reusing waste from various industries, which contain various proteins (Kshetri *et al.* 2019). Disposing of beneficial sources like feathers, which contain keratin, which is a non-soluble protein, through burning or burying in the ground leads to environmental contamination (Callegaro *et al.* 2019). In order to break these bonds, using of keratinase enzyme is considered an efficient method. Kshetri *et al.* (2019) used the *Chryseobacterium sediminis* RCM-SSR-7 strain in order to feather degradation. Feather protein hydrolysate prepared with this organism is rich in essential amino acids and nutrients and can be uses as plant growth biostimulant. Liaqat *et al.* (2022), reported that the enzyme produced by *B. licheniformis* dcs1 strain completely degraded feathers in 14 days. The effect of degraded feathers by *Bacillus pumilus* JYL on the growth of wheat was studied by Sun *et al.* (2021). They reported that fresh and dry biomass yield was significantly increased compared to the control. The objectives of this study were, to isolate and identify bacteria capable of producing keratinase enzymes from a culture that is a mix of soil and chicken feather; to determine the amount of amino acids resulting from feather degradation by the strains and; and to investigate the effect of the products resulting from feather degradation as a plant growth stimulant on lettuce (*Lactuca sativa* L.).

MATERIALS AND METHODS

Present study was carried out during 2020 at Soil and Water Research Institute, Karaj, Iran. A total of 29 soil samples from 29 regions in 15 provinces of Iran (Mazandaran, East Azerbaijan, West Azerbaijan, Semnan, North Khorasan, Bushehr, Chaharmahal and Bakhtiari, Fars, Isfahan, Khuzestan, Hormozgan, Kerman, Kermanshah, Golestan and Lorestan) were used to isolate and identify bacteria producing Keratinas enzyme. One hundred gram of each soil sample was mixed with chicken feathers and preserved

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in an incubator at 30°C for 3 weeks. In the second phase, feather meal agar (FMA) was prepared consisting of 10 g/litre of the ground feather, 0.5 g/litre of NaCl, 0.3 g/l of K_2HPO_4, 0.4 g/l KH_2PO_4, and 15 g/litre of agar. For initial isolation with the aid of the serial dilution method, serial dilution of samples was transferred to feather meal agar and single colonies were selected, isolated, and purified (Bach *et al.* 2011). In the third phase, a liquid culture media was prepared, and the *p*H was adjusted to 7.5. Next, it was autoclaved and the purified isolates were added to the culture media and placed in a shaker incubator (30°C and 150 rpm). After 7 days, the culture media was passed through a filter paper, and the culture feathers were separated. Then, they were placed inside the oven at 50°C, and the weight of the samples was determined after drying, and the difference between the remaining feather weights with the blank sample was considered as the degradation criteria. The isolates that were capable of completely degrading the feather were selected as the superior samples. Ultimately, the screened culture was preserved at 4°C (Srivastava *et al.* 2011).

Analytical procedures: The keratinase enzyme activity was measured by centrifuging the filtered media, using the supernatant as the crude enzyme source. The selected samples' filtered culture media were centrifuged, and the supernatant was analyzed using HPLC to identify free amino acids. The amount of ammonia nitrogen was measured using Kjeltec Auto (1030 Analyzer) (Emami 1996).

A pot experiment as a randomized arrangement with 3 replications was conducted in order to examine the effect of foliar spraying of 3 chicken feather degradation solutions (gh1, b1, c11) on lettuce growth. The soil samples were taken from an agricultural soil, in Karaj, Iran and its physical [soil texture (Gee and Bauder 1986)] and chemical properties [*p*H (Klute 1986), EC (Bremner 1982), OC % (Walkley and Black 1934), calcite % (Loeppert and Suarez 1996), N (Bremner 1996), and P (Olsen 1954)] were determined. The results are as follows, soil texture loamy; *p*H 8.2; EC 0.63 dS/m; OC 0.45%; calcite 8.7%; N 0.08%; P 14 mg/kg; K 210 mg/kg. The lettuce seedlings were transplanted into the pots.

Plastic pots 22.5 cm tall with a 15.5 cm mouth opening diameter were used in the experiment. The pots had drainage and were filled with 5 kg of air-dried soil passed through a 4 mm sieve. The lettuce seedlings were transplanted into the pots. The effect of the solution resulting from the complete degradation of 2% feather in the culture media by the superior strains (including *B. methylotrophicus* gh1, *B. siamensis* c11, and *B. velezensis b1)* on the growth of lettuce was studied. Before spraying, the population of all three strains in the culture media was equalized based on the concentration of half McFarland CFU/Ml) (1.5×10^8). Because the amino acid requirement per hectare is 2 to 3 kg, the required amount of amino acid for each plant was calculated and according to the total amount of free amino acids in the culture media of each isolate, the amounts of the supernatant of each media were obtained for spraying on the plant. From the supernatant of strains of c11, gh1, and b1 the amounts were respectively 34 cc, 56 cc, and 64 cc. After that they were brought to 100 cc, and this amount was the basis of the original amount of spraying on the plant. The spraying was done in two stages with an interval of 5 days. After 15 days, the plants were harvested and plant growth and yield were measured. The data were analyzed using SAS Ver.9.1 (SAS Institute 2008) and the means were compared with the Least Significant Difference (LSD) test at the 5% significance level.

RESULTS AND DISCUSSION

Keratinas enzyme activity: Intense growth and development of food processing industries have caused a considerable amount of waste as a by-product that is mostly discharged into the environment. Chicken feather remains one of the significant by-products from the poultry industry, mainly due to keratin protein that is hard to degrade. Our results showed that 31 isolates were able to grow on feather meal agar (FMA), of which 8 isolates were able to degrade the feather during 7 days. The results from molecular diagnostics of the 8 isolates showed the 7 strains of the *Bacillus* genus included: *Bacillus subtilis*, *Bacillus velezensis*, *Bacillus siamensis*, *Bacillus methylotrophicus*, and one species, *Stenotrophomonas rhizophil*. The ability to degrade feathers by keratinolytic bacteria is different. *Bacillus* species are one of the best producers of keratinase enzyme that can transform the feather into amino acids (Almahasheer *et al.* 2022). The maximum activity of keratinase enzyme was measured in the strain of *Bacillus methylotrophicus* gh1 (8.56 U/ml). The lowest activity of keratinase enzyme belonged to *Bacillus subtilis* dr2 (Table 1). Different *Bacillus* species include *B. licheniformis* dcs1, *B. cereus* wps1 (Liaqat *et al.* 2022), *Bacillus subtilis* PF1 (Bumbra *et al.* 2022), *Bacillus pumilis* JYL (Sun *et al.* 2021), and *Bacillus tropicus* strain Gxun-17 (Shen *et al.* 2022) were reported as the bacteria that can degrade feather. According to literature reviews, there was no report on the activity of *Bacillus siamensis* and *Bacillus methylotrophicus* for feather degradation. Hence, they are introduced as new strains with keratinolytic activity.

Abdel-fattah *et al.* (2015) reported that the enzyme activity in the culture media of *Cyberlindnera fabianii* NRC3 strain appeared on the 1st day, reached the maximum level on the 3rd day, and the complete decomposition was observed on the 4th day. Hence, it can be concluded that the higher the bacteria's capability to produce the keratinase enzyme, the more amino acids will produce due to complete feather decomposition.

The concentration of ammonia nitrogen: The bacteria significantly affected the Keratinas enzyme activity and the percent of ammonia nitrogen on the 7th day. The concentration of ammonia nitrogen resulting from feather degradation in the culture media was obtained between 3.95 to 5.68%. The maximum content of ammonia nitrogen was produced by *B. methylotrophicus* gh1 and *B. methylotrophicus* a2 (Table 1). The minimum activity of Keratinas enzyme was measured in *B. subtilis* dr2. *S. rhizophila* dr4 caused the minimum

Table 1 Keratinas enzyme activity and the per cent of ammonia nitrogen on 7th day

Bacteria	Keratinase enzyme activity (U/ml)	Ammonia nitrogen (%)
B. methylotrophicus gh1	8.56[a]	5.68[a]
B. methylotrophicus a2	8.46[ab]	5.59[ab]
B. siamensis c11	8.28[ab]	5.12[bc]
B. velezensis b1	7.77[abc]	5.09[c]
B. velezensis gh2	7.61[bc]	5.07[c]
B. velezensis d2	7.03[cd]	5.04[c]
S. rhizophila dr4	6.53[d]	3.95[e]
B. subtilis dr2	5.41[e]	4.47[d]

concentration of ammonia nitrogen. Accumulated studies have reported that feathers can be efficiently degraded by various microorganisms and different priceless products like Keratinas enzyme and ammonia nitrogen can be produced in result of this degradation (Li *et al.* 2019). Therefore, microbial conversion of feathers into value-added products such as biofertilizer should be considered in poultry industries.

The concentration of free amino acids: A total of 17 free amino acids (asparagine, glutamine, serine, glycine, histidine, arginine, threonine, alanine, proline, tyrosine, valine, methionine, cysteine, isoleucine, leucine, phenylalanine and lysine) were separately identified. Eight of them were essential amino acids, namely leucine, lysine, isoleucine, methionine, threonine, phenylalanine, valine and tryptophan. These findings showed that some amino acids were more abundant than other amino acids; hence, they will be utilized based on the type of amino acids required in different industries. For example, in plants, osmotic regulation happens via the aggregation of amino acids such as glycine, alanine, and valine. Also, glutamic acid plays a role in pollination (Radkowski 2018). Therefore, amino acids produced from feathers can be used as growth stimulants for plants to increase the quantitative and qualitative yield of plants (Bhari *et al.* 2021).

The maximum content of amino acids was observed in the strains of *Bacillus siamensis* c11 (1065 µg/ml), *B. methylotrophicus* gh1 (1032 µg/ml), *B. methylotrophicus* a2 (1027 µg/ml) and *Bacillus velezensis* b1 (954 µg/ml). In the supernatant, all of the 4 bacteria had the maximum content of phenylalanine amino acid (Table 2). Prajapati *et al.* (2021) reported the considerable potential of *B. amyloliquefaciens* KB1 in production of glutamic acid, leucine, proline, valine, and aspartic acid from degradation of feather waste.

The influence of the solution resulting from degradation of the feather on plant growth: Different potentials of soil bacteria related to plant growth were evaluated. The soil bacteria can directly and indirectly affect the bioavailability of both essential nutrients and none-essential elements like toxic metals (Mousavi *et al.* 2018b, Moshiri *et al.*

Table 2 The amount of free amino acids in the culture media containing 1% feather

Isolate	Asp	Glu	Ser	Gly	His	Arg	Thr	Ala	Pro	Tyr	Val	Met	Cys	Ile	Leu	Phe	Lys	Sum
	(µg/ml)																	
B. methylotrophicus a2	6.2	51.2	34.9	172.8	29.3	19.3	16.9	31.5	11.3	106.9	138.9	9	<0.1	41.8	81.3	216.2	59.3	1026.7
B. velezensis b1	2.5	24.6	35.1	130.1	13.4	20.4	17.3	12.5	1.9	113.6	149.6	0.8	12.1	22.3	65.2	280.9	51.1	953.5
B. siamensis c11	12.3	100.3	30.5	200.3	40.2	42.2	15.3	15.4	<0.1	132.7	41.2	31.6	<0.1	19.3	36	268.2	79.5	1064.9
B. velezensis d2	5.7	46.3	31.5	26.1	13.6	3.1	10.2	15.7	8.6	112.7	116.8	0.4	19.9	39.8	113.7	264.6	44.8	873.5
S. rhizophila dr4	7	15.5	10.8	11.9	1	56.7	10.9	0.8	2.1	70	14.8	7.6	6.7	3.1	10.9	116	3.1	348.8
B. subtilis dr2	<0.1	1.5	10.9	17.4	3.9	2.3	2.7	29.8	6.2	2.2	25	<0.1	3.8	8.9	28.6	8.1	3.3	154.6
B. methylotrophicus gh1	8.4	43.3	25	103.3	26.7	24	16.6	5.7	<0.1	111	160.8	1.9	10.2	21.3	139.8	276.9	57	1031.9
B. velezensis gh2	4.1	27.7	24.6	72.6	3.7	21.5	16.3	7.6	4.6	102.5	148.2	2.6	5.8	28.5	68.2	235.9	62.5	836.7

Asp, Asparagine; Glu, Glutamine; Ser, Serine; Gly, Glycine; His, Histidine; Arg, Arginine; Thr, Threonine; Ala, Alanine; Pro, Proline; Tyr, Tyrosine; Val, Valine; Met, Methionine; Cys, Cysteine; Ile, Isoleucine; Leu, Leucine; Phe, Phenylalanine; Lys, Lysine.

Table 3 Effect of foliar spraying of different treatments on the growth and yield of lettuce

The solution resulting from degradation of feather	Fresh weight of shoot	Dry weight of shoot	Fresh weight of root	Dry weight of root
	g/pot			
gh1	63.5 a	7.79 a	22.4 a	2.72 a
c11	61.0 a	7.90 a	19.1 b	2.31 ab
b1	57.9 ab	6.33 ab	18.7 b	1.89 bc
Control (distilled water)	50.4 b	5.69 b	16.7 b	1.79 c

Means in the same column followed by the same letter are not significantly different according to LSD at ($P \leq 0.05$).

2019). These bacteria also affecting the condition of the soil environment and therefore growth of the roots affect the uptake of water by the plant. The sums of these changes significantly affect the plant growth and yield. The measurements of present study showed that the highest fresh weight was recorded in gh1 strain, with no significant difference between c11 and b1. Root fresh and dry weights were significantly improved by gh1 treatment. The greatest dry weight was recorded in c11, but no significant difference was found with gh1 and b1 strains (Table 3). The solutions significantly improved lettuce growth compared to the control treatment. Similar results were reported on other bacterial strains, confirming the findings of the study (Sun *et al.* 2021). Although, there are different unknown aspects related to the biodegradation of feathers that need to be identified, however, microbial conversion of feathers can be considered as an efficient tool for improving plant growth and yield.

The studied soil bacteria showed different potentials in the degree of degradation of feathers. Furthermore, the differences in the amount and the compound of the products resulted from the degradation of feathers were considerable. The present study introduced 3 new strains including *Bacillus siamensis* strain c11 (MT229226), *Bacillus methylotrophicus* strain gh1 (MT229227) and *Bacillus methylotrophicus* strain a2 (MT229228) in terms of keratinase enzyme for feather degradation and amino acids. Based on the measurements, the solutions resulting from the degradation of chicken feathers by the mentioned strains had a significant effect on improving the growth and yield of lettuce, and can be used as plant growth stimulants. The findings of this study will be useful in the exploitation of these microorganisms and/or their enzymes in the reduction of feather waste and the production of amino acids as efficient plant growth stimulants, and the conversion of feather waste into rich amino acid hydrolysate could be an efficient way of feather waste management. In general, the application of bacterial keratinases in keratin waste hydrolysis is not only economical and eco-friendly but also allows for an effective management of post-slaughter waste.

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