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Original Article

Valorization of chicken feather into organic liquid fertilizer through two species of Bacillus bacteria fermentation

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Abstract

The valorization of industrial poultry waste by microbes into value-added products has received significant attention for reducing pollutants and producing a healthy environment. The current study aims to explore the use of liquid fertilizer from fermented chicken feathers by *Bacillus cereus* and *Bacillus subtilis*. The study was carried out experimentally using a completely randomized trial design with 10 different treatments of liquid fertilizer, each of which was repeated 12 times. The current study showed that both bacteria can secrete keratinase enzymes, degrade chicken feathers, and increase the nitrogen content of liquid fertilizer. However, the keratinase activity, degradation, and nitrogen content of chicken feather liquid fertilizer produced by *B. subtilis* fermentation were significantly higher ($P<0.05$) than those of *B. cereus*. Likewise, the growth, anthocyanin content in the leaves, and yield of red spinach plants nurtured with chicken feather liquid fertilizer from *B. subtilis* fermentation were significantly ($P<0.05$) higher than those given liquid fertilizer from *B. cereus*. The current study concluded that chicken feathers could be valorized into liquid fertilizer for plants, especially red spinach, by both *B. cereus* and *B. subtilis* bacteria. The current study recommends liquid fertilizer from chicken feathers fermented by *B. subtilis* at 0.05 g/plant to replace chemical fertilizers in plants, especially red spinach.

Keywords: *Bacillus cereus*, *Bacillus subtilis*, Chicken feather, Keratinase, Red spinach

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Introduction

Waste from the poultry industry, especially chicken feathers, is a big concern for a clean environment because the amount continues to increase (Ali et al., 2021), and contributes about 5-7% of chicken body weight (Kani et al., 2012; Li, 2019), and produces more than 7.7 million tons of feathers per year, making it a severe environmental problem (Grazziotin et al., 2006). Increased consumption of poultry products is expected to increase the amount of chicken feather waste generated each year by the

global poultry industry (Verma et al., 2017; da Silva, 2018).

Burning is considered an effective method for dealing with feather waste (Sharma et al., 2016) because it can destroy infectious agents that may be present in feathers (Tesfaye et al., 2017). Various methods of processing chicken feathers into valuable product materials in various fields have been developed (Shanmugasundaram et al., 2018). However, large amounts of feathers are still released into the environment without proper handling (Li, 2019). Chicken feathers are a source of pollutants that are difficult to degrade naturally (Brandelli et al., 2015) because chicken feathers have a stable structure in the form of keratin (Wang et al., 2016). Untreated chicken feather waste can harbor many pathogenic microorganisms and emit pollutants, such as nitrous oxide, ammonia, and hydrogen sulfide, threatening the environment and human health (Tamreihao et al., 2019).

Several methods of feather degradation, such as pressurized hydrolysis, puffing, and the use of acids and bases (Lee et al., 2016; Liu et al., 2016), have limitations such as high energy consumption during the production process and considerable product damage (Holkar et al., 2016). Utilization of keratinolytic microorganisms such as bacteria and fungi (Mini et al., 2017; Zhang et al., 2016) attracts many researchers to degrade chicken feathers because it is not only environmentally friendly (Fang et al., 2017), but also maintains the original structure and activity of the product (Daroit and Brandelli, 2014). Several researchers have reported the biodegradation of keratin by bacteria of the genus *Bacillus*, wild-type, and recombinant *Bacillus sp.* C4 was reported to be able to degrade 75% of 5% chicken feather suspension in 8 days but was considered a long process with low efficiency (Patinvoh et al., 2016). *B. licheniformis* BBE11-1 was reported to be able to hydrolyze chicken feathers but was not suitable for degrading large amounts of feathers (Liu et al., 2016). *B. megaterium* KC405251 was reported to be able to hydrolyze chicken feathers with an efficiency of 80% within 3 days at an optimum temperature of 50ºC and pH 7 (Iqtedar et al., 2017). The recombinant *B. subtilis* DB 100 could completely degrade 2% of chicken feathers in the Bio Flo 110 14 L fermenter (Zaghloul et al., 2011). Recombinant *B. amyloliquefaciens* K11 was reported to degrade chicken feathers entirely within 12 hours but did not degrade the feather stem (Yang et al., 2016).

Chicken feather waste is a great potential source of protein and antioxidant peptides (Fontoura et al., 2014). The hydrolysis products of chicken feather keratinase are mainly soluble amino acids and peptides and exhibit antioxidant properties (Wan et al., 2015). The valorization of chicken feathers into economically value-added products such as biofertilizers (Thys et al., 2006), animal feed (Odetallah et al., 2003), and biofilm (Abdel-Fattah, 2013) is very interesting for many researchers. It can be utilized in the poultry industry (Tamreihao et al., 2019).

Red spinach (*Amaranthus tricolor*) was initially known as an ornamental plant but has developed into a cheap and popular horticultural product because it is rich in nutrients and anthocyanins. In addition, red spinach is a plant that is easy to cultivate and has a relatively short harvest time (25–30 days). Generally, inorganic fertilizers are still used in cultivating red spinach in Indonesia. Continuous and inappropriate use of inorganic fertilizers for intensive crop production can lead to soil degradation, environmental pollution, and low productivity (Amoah et al., 2012). Meanwhile, using organic fertilizers in agricultural cultivation can improve soil fertility, soil structure, water holding capacity, soil exchange capacity, and biological activity and contribute to sustainable crop production (Machado et al., 2020). The use of organic fertilizers can also reduce the volume of organic residues and reduce greenhouse gas emissions while increasing carbon sequestration (Favoino and Hogg, 2008). Organic compost increases soil nutrient content and availability to plants (Boldrin et al., 2009) and reduces inorganic nitrogen fertilization, harming the environment (Graham et al., 2017). The valorization of chicken feathers into organic fertilizer for plants through bacterial fermentation of *B. cereus* and *B. subtilis*, especially for red spinach, has not been widely published. The current study explores the use of chicken feather liquid fertilizer produced by the fermentation of *B. cereus* and *B. subtilis*.

Material and Methods

Bacterial culture

Two bacterial isolates of *Bacillus subtilis* and *Bacillus cereus*, respectively, in agar slant were purchased from Microbiology Laboratories, Department of Biology, Faculty of Science and Technology, Universitas Airlangga, Surabaya,

Indonesia. Each bacterial isolate was taken aseptically using a full-ose needle, inoculated in nutrient agar (NA) media (beef extract 3.0 g, peptone 5.0 g, agar 20.0 g, NaCl 0.5 g and distilated water 1,000 ml), and incubated for 48 h at 45ºC. The growth temperature of Bacillus bacteria depends on the strain. *B.subtilis* is a thermophilic bacterium that can grow at a temperature of 45-55°C with an optimal growth temperature of 60-80^{o}C (Andriani et al., 2017) and *B.cereus* 4-48^{o}C with an optimum of 28-35^{o}C (Schneider et al., 2015). Colonies of each bacterium growing on NA media were scratched using a fully sterile ose needle and subcultured into 10 mL of nutrient broth (NB) media with a pH of 8.0 and incubated at 45ºC for 5 d. The resulting cultures were entirely transferred into 90 mL of NB medium and incubated for 48 h at 45^{o}C. The concentration of each bacterium was calculated employing serial dilutions. One ml of each culture was serially diluted in sterile 0.1 M sodium chloride (NaCl). From the last three dilutions, 10^{-5}, 10^{-6}, and 10^{-7}, one mL of each was taken and poured into sterile Petri dishes containing 15 ml of NA medium and incubated at 45ºC for 48 h. The Total Plate Count (TPC) method counted the number of colonies.

Determination of keratinase activity

Determination of the keratinase activity of both species of *Bacillus* was carried out on chicken feather meal as a source of keratin. Chicken feathers were obtained from a local poultry slaughterhouse in the Batang district, Surabaya, Indonesia. The chicken feathers are washed thoroughly with running water to separate the chicken feathers from the remnants of blood and other impurities attached and dried in the sun for 7 d. The dried chicken feathers were ground using a milling machine and sieved to a 50 mm mesh. A total of 7.5 g of chicken feather meal was divided into 2 parts of 3.75 each, and each was dissolved in 1 l of distilled water, then sterilized at 121ºC and 1 atm pressure for 15 min. After cooling, potassium hydroxide (KOH) was added so that the mixture reached a pH of 8.5 and added water to a final volume of 1,000 ml. Fermentation of chicken feather flour by both *Bacillus* bacteria was carried out in a liquid manner. For each chicken feather meal media substrate added, 10 ml of inoculant containing 10^{6} cells of *B. cereus* and 10 ml of inoculant containing 10^{6} cells of *B. subtilis*. After being inoculated, both were incubated in a shaker incubator at 55ºC, agitation speed of 60 rpm for 72 h. The keratinase activity of each bacterium followed the method described by Cai et al. (2008). Briefly, as much as 50 ml of chicken feather meal hydrolyzate was centrifuged at 10,000 rpm, a temperature of 4ºC for 10 min. The supernatant was taken and used as a crude keratinase enzyme. A total of 1.0 ml of the crude enzyme was diluted in Tris-HCl buffer solution (0.05 mol/l, pH 8.0) and incubated with 1.0 ml of 1% keratin solution in phosphate buffer at 50ºC in a water bath for 10 min, then 2.0 mL trichloroacetic acid (TCA) was added. After stirring, the mixture was centrifuged at 1,450 rpm for 30 min. The supernatant was taken, and its absorbance was measured with a spectrophotometer at a wavelength of 280 nm against the control. Controls were made by incubating the enzyme solution with 2.0 mL of TCA without adding keratin solution. One unit (U/ml) of gelatinolytic activity was defined as an increase in corrected absorbance of 280 nm (Gradišar et al., 2000) with a control of 0.01 per min. Keratinase activity is calculated by the following equation= Keratinase activity is calculated by the following equation=$4\times n\times A_{280}/(0.01\times 10)$,
where n is the dilution rate, 4 is the final reaction volume (ml), 10 is the incubation time (min).

Chicken feather degradation

The percentage of chicken feather flour degradation by both *Bacillus* species was measured by weighing the lost weight or the difference before and after fermentation. After fermentation, the chicken feather meal media was filtered through Whatman No. 1 filter paper. The residue was washed with 70% alcohol, dried in an oven at 60ºC for 48 h, and weighed.

Determination of nitrogen

The concentration of nitrogen (NH4-N) in the liquid chicken fertilizer was determined using the method of the American Society of Agronomy and Soil Science Society of America (1982), as described by Sopandi et al. (2012). Briefly, 100 ml of hydrolyzed chicken feather meal was evaporated at 100ºC for 2 h to obtain a dry meal. A total of 50 mg of the dry hydrolyzate sample was put into a digestion tube, and then 1 g of the selenium mixture was added, which had been prepared from 1.55 g $CuSO_4$, 96.9 g Na_2SO_4, and 155 g selenium. After adding 3 ml of 97% H_2SO_4, the mixture was stirred and digested at 350ºC for 4 h until a colorless extract was obtained, cooled to room temperature, diluted with 50 ml of

distilled water, shaken vigorously, and allowed to stand for 12 h. Two milliliters were taken and put in a new borosilicate glass test tube, where 4 mL of tartrate buffer (mix 50 g NaOH and 50 g $KNaC_4H_4O_6$ in 1.0 liter distilled water) and sodium phenate solution (mix 100 g NaOH and 125 g phenol in 1.0 liter distilled water) were added. The mixture was stirred and left for 10 min, then 4 mL of 5% NaOCl was added and stirred again. After being left for 10 min, the absorbance was measured at a wavelength of 636 nm. Ammonium sulfate $(NH_4)_2SO_4$ was used as the standard nitrogen.

Determination of phosphorus

The phosphorus concentration in liquid chicken feather fertilizer was determined using the UV-Vis spectrophotometer method based on the Indonesian National Standard (SNI, 2005). Briefly, 300 ml of hydrolyzed chicken feather meal was evaporated at 100ºC for 2 h to obtain a dry meal. A total of 1.0 g of dry hydrolyzed chicken feather meal was crushed, and put into a beaker glass (250 ml), added 20 ml of concentrated HNO_3 was simmered for 30 min until brown gas appeared and then cooled at room temperature. After cooling, 20 ml of 70–72% $HClO_4$ was added, slowly boiled for 30 min, and then cooled at room temperature. After being cooled until the solution is colorless and white smoke appears, 50 ml of distilled water is added and slowly brought to a boil, then cooled and diluted to 500 ml with distilled water. The solution was then filtered, taken to 5 mL, put into a measuring flask, and diluted with 10 ml of molybdovanadate. The absorbance was measured at a wavelength of 420 nm, and the P_2O_5 solution was used as the standard.

Determination of potassium

The potassium concentration in the liquid chicken feather fertilizer was determined using the atomic absorption spectrophotometer method based on Standard Nasional Indonesia (SNI, 2009). Briefly, 300 ml of chicken feather meal hydrolyzate was evaporated at 100ºC for 2 h to obtain a dry meal. A total of 1.0 g of the sample was put into a 250 ml beaker glass, 5 ml of concentrated HNO_3 and 15 ml of concentrated HCl were added, the glass was covered with a watch glass, and it was heated on a hot plate until the brown smoke disappeared. The mixture was cooled to room temperature and transferred to a volumetric flask (500 ml), then diluted with distilled water to the mark. The solution was homogenized in a magnetic stirrer for 15 minutes and filtered using Whatman No. 40 filter paper. 25 ml of the filtrate was pipetted, put into a 50 ml volumetric flask, added 5 ml of 1% CsCl suppressor, and homogenized. The homogenate was measured by an atomic absorption spectrophotometer (AAS) of type AA30.

Preparation of chicken feather meal to make liquid fertilizer

The liquid fertilizer used in this study consists of liquid fertilizer of non-fermented chicken feather (NF) with a concentration of 0.375 g/100 ml, commercial chemical liquid fertilizer (FC) with a concentration of 0.30 g/100 ml, liquid fertilizer of fermented chicken feather *B. cereus* (FBC) with 4 concentrations, and liquid fertilizer of fermented chicken feather *B. subtilis* (FBS) with 4 concentrations. A total of 8.75 g of chicken feather meal was divided into 5 portions of 1.25 g, 1.875 g, 2.5 g, and 3.125 g, respectively. Furthermore, from each part divided by 5, 0.25 g (FBC1), 0.375 g (FBC2), 0.5 g (FBC3), and 0.625 g/100 ml (FBC4) were put into a 150 ml Erlenmeyer flask containing 90 distilled waters. Each mixture was sterilized at 121°C and 1.0 atm pressure for 15 min, then cooled. After cooling, KOH was added until the mixture had a pH of 8.5, and water was added to a final volume of 90 ml. Ten ml of inoculants containing 10^6 *B. cereus* cells were added to the mixture for further incubation in a shaker incubator at 55ºC and agitated at 60 rpm for 72 h. The same procedure and concentration were also carried out for the fermentation of chicken feather meal by *B. subtilis*, namely liquid fertilizer *B. subtilis* 0.25 g (FBS1), 0.375 g (FBS2), 0.5 g (FBS3), and 0.625 g/100 ml (FBS4).

Application of liquid fertilizer on plants

Application of liquid fertilizer to fermented chicken feathers by both bacteria was carried out using a completely randomized experimental design with 10 treatments of liquid fertilizer, and each repeated 12 times. Commercial red spinach (Amaranthus tricolor) seeds were obtained from certified seed sellers (Trubus) in the local market of Bratang, Surabaya, Indonesia. The seeds were soaked in a beaker (1,000 ml) containing 500 ml water for 12 h. The seeds floating in the water were removed, and the seeds immersed in the water are taken for sowing. Approximately 2-3 seeds are inserted into a rock wool hole measuring 2.5x2.5 cm and placed in a tray.

The seeds were watered twice a day for 7 d. After the seed height reached 4 cm with 3 leaves, it was transferred to polybags with a size of 30x30 cm containing a mixture of soil, organic fertilizer, and rice husks in a ratio of 2:1:1. A total of 120 red spinach plants that had the same height (4 cm) were divided into two parts of 60 plants each. The first part was taken randomly and divided into six parts to be applied to liquid chicken feather fertilizer fermented by *B. cereus* (FBC). The second part was applied to liquid chicken feather fertilizer fermented by *B. subtilis* (FBS). Watering with 300 ml was done twice daily, in the morning and evening. Liquid fertilizer is applied when red spinach is 2 weeks old and applied once a week by injection at about 10 cm near the plant stem, as much as 10 ml per plant. Red spinach was maintained for 30 d at an ambient temperature of 28–31ºC with a relative humidity of 55–60%. The height and number of red spinach leaves were observed every week from the third week to the sixth week. Observations of the wet weight of the harvest and the total anthocyanin content were carried out at 30 d.

Determination of total anthocyanin content

The total anthocyanin content of red spinach was determined using the differential pH method described by Lee et al. (2005). A total of 30 g of fresh red spinach leaves were weighed, crushed in a blender until smooth, immersed in 60 mL of a mixture of ethanol and 0.1 M HCl (85:15), and stirred using a magnetic stirrer for 1 h. The immersion was filtered by vacuum, and the supernatant was taken and divided by 2. One part of the supernatant was added gradually with a buffer solution of 0.025 M potassium chloride to pH 1.0, and another part of the supernatant was added with a buffer solution of 0.4 M sodium acetate to a pH of 4.5. After stirring, each was incubated at room temperature for 20 min and centrifuged at 4ºC and 7,000 rpm for 15 min. The supernatant was taken, and absorbance was measured at 520 and 700 nm. Standard solutions of cyanidin-3-glucoside (C3G) and pelargonic chloride (PG) were prepared by dissolving 4 and 0.67 mg/l, respectively, in a mixture of methanol (0.1%) and stored at -25ºC until further use. Both were used as internal standards for scanning the absorbance spectrum wavelengths, and distilled water was used as the blank. The formula calculates the anthocyanin content.

$$Anthocyanin\ pigments\ \left(\frac{mg}{100g}\right) = \frac{A\ x\ MW\ x\ DF\ x\ V\ x\ 1000}{s\ x\ l\ x\ e}$$

where A = (A 520 nm – A 700 nm) pH 1.0 – (A 520 nm – A 700 nm) pH 4.5; MW (molecular weight) for cyanidin-3-glucoside = 449.2 g/mol; DF = dilution factor; V = volume of solvent; s = sample weight (g); e = molar absorptivity (26,900/mol/cm); 1 = pathlength (1 cm)

Statistical analysis

Data from observations of keratinase activity and nitrogen, phosphorus, and potassium concentrations in chicken feather hydrolysate were statistically analyzed using the t-student test at 0.05 to see the difference between *B. cereus* and *B. subtilis*. Data from observations of height, amount, wet weight of harvest, and anthocyanin content of red spinach fertilizer were analyzed using a one-way analysis of variance at 0.05 significance. Data on the number of leaves before the analysis of variance was transformed into a log number of log+0.5. The Tukey difference test was carried out to determine the differences between treatments if the treatment has a significant effect ($P<0.05$) on the research variables.

Results

Keratinase activity

The results of the current study (Figure 1) showed that the two species of *Bacillus* bacteria showed keratinase activity in chicken feather meal hydrolysate liquid media with different values. The keratinase activity of *B. subtilis* (87.3±2.8 U/ml) was significantly ($P<0.05$) higher than *B. cereus* (64.6±4.7 U/ml).

Percentage of degradation

The percentage degradation of feathers by *B. cereus* and *B. subtilis* in this study is presented in Figure 2, which shows that the two bacteria have different degradation abilities. The percentage degradation of chicken feathers by *B. subtilis* (56.4%) was significantly ($P<0.05$) higher than *B. cereus* (45.6%).

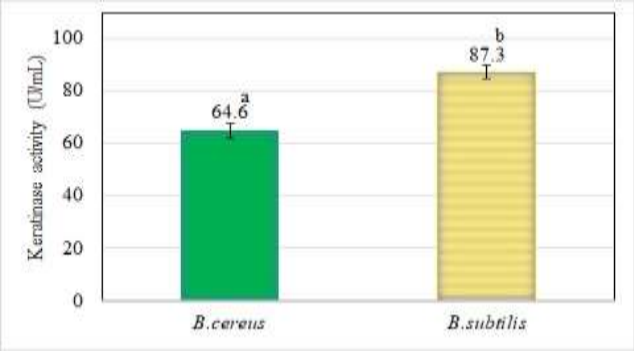

Figure-1. The bacterial keratinase activity of *B. cereus* and *B. subtilis* on chicken feather meal medium. The values of means with error bars marked with different letters from 10 independent observations were determined by a t-student test and a significant difference (P<0.05).

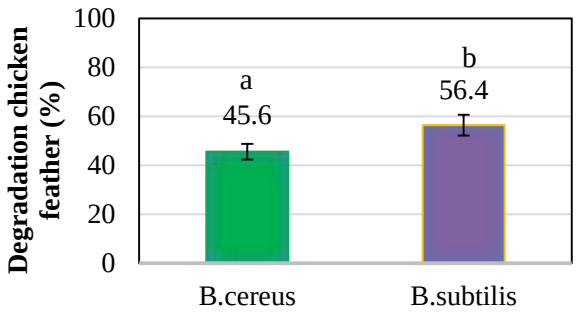

Figure-2. Percentage of degradation of chicken feather meal by *B. cereus* and *B. subtilis*. The values of means with error bars marked with different letters from 10 independent observations were determined by a t-student test and a significant difference (P<0.05).

The chemical composition of chicken feather liquid fertilizer

The nitrogen, phosphorus, and potassium contents of chicken feather liquid fertilizer fermented by *B. cereus* and *B. subtilis* are presented in Figure 3. The nitrogen content of chicken feather liquid fertilizer fermented by *B. cereus* (0.273±0.02%) and *B. subtilis* (0.376±0.03%) was both significantly (P<0.05) lower than a liquid non-fermented chicken feather (0.471±0.05%). The nitrogen content of liquid fertilizer chicken feathers produced by *B. cereus* fermentation was significantly (P<0.05) lower than *B. subtilis*. This study also showed no significant difference (P>0.05) in phosphorus and potassium content between liquid fertilizer and liquid unfermented chicken feathers.

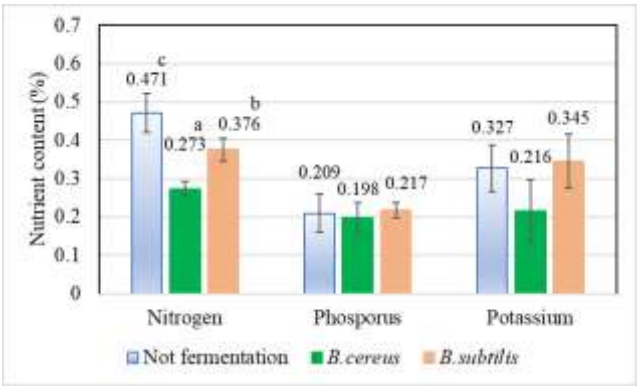

Figure-3. The nutritional content of chicken feather liquid fertilizer is fermented by *B. cereus* and *B. subtilis*, and liquid chicken feather is not fermented. The values of means with error bars marked with different letters from 10 independent observations were determined by a t-student test and a significant difference (P<0.05).

Plant height

The height of red spinach fed with liquid chicken feather not-fermented (NF), commercial chemical liquid fertilizer (FC), liquid fertilizer *B. cereus* (FBC), and liquid fertilizer *B. subtilis* (FBS) in the current study are presented in Table 1. The results showed that the plant height of red spinach nurtured with FBC and FBS increased with increasing concentration. From the third week (1 week after fertilizer application) to the sixth week, the average height of red spinach nurtured FBC_3, FBC_4, FBS3, and FBS_4 was significantly (P<0.05) higher than NF and FC. Meanwhile, the height of red spinach nurtured to FBC_1, FBC2, and FBS_1 was not significantly different (P>0.05) compared to FC, but all three were significantly (P<0.05) higher than NF. In the fourth to sixth weeks, the height of the red spinach that was nurtured with FBS_4 was significantly (P<0.05) higher than all concentrations of liquid fertilizer.

The results of the current study (Figure 4) also showed that the growth rate of red spinach applied to FBC_4, FBS_2, FBS3, and FBS_4 was significantly (P<0.05) higher than NF and FC. The growth rate of red spinach that was nurtured with FBS_3 and FBS_4 was significantly (P<0.05) higher than FBC at all concentrations. The highest growth rate of red spinach was found in PBS_4.

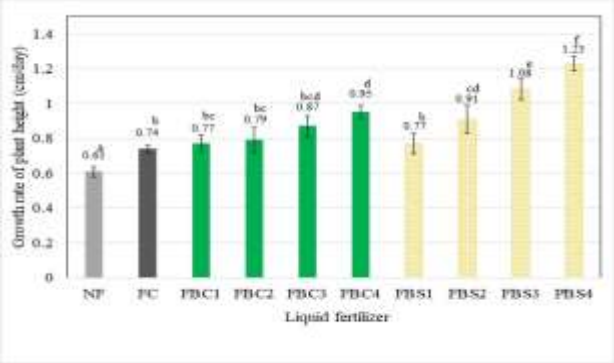

Figure-4. The growth rate of red spinach was nurtured with liquid chicken feather not-fermented (NF), commercial chemical liquid fertilizer (FC), liquid chicken feather fertilizer *B. cereus* (FBC), and liquid chicken feather fertilizer *B. subtilis* (FBS) at several concentrations. The Tukey test determined the values of means with error bars marked with different letters from 5 independent observations and a significant difference (P<0.05).

Table-1. Plant height of red spinach (cm) nurtured with liquid fertilizer of chicken feather not-fermented (NF), commercial chemical liquid fertilizer (FC), liquid chicken feather fertilizer *B. cereus* (FBC), and liquid fertilizer chicken feather *B. subtilis* (FBS) pada beberapa konsentrasi. The Tukey test was used to calculate the values, which reflect means and standard deviation with distinct superscript letters in the same column and show a substantially different (P<0.05).

Fertilizers	Plant height (cm/plant) of red spinach at week					
	1	2	3	4	5	6
NF	3.53±0.54	6.64±0.65	10.60±0.24^{a}	14.13±1.30^{a}	19.96±0.30^{a}	20.58±0.62^{a}
FC	4.15±0.62	6.52±0.91	12.6±0.96^{b}	15.61±1.08^{ab}	20.74±0.94^{b}	23.34±0.91^{b}
FBC_1	4.18±0.59	6.26±0.83	13.30±0.61^{b}	17.42±0.59^{bc}	22.66±1.50^{bc}	23.46±1.34^{b}
FBC_2	4.38±0.66	6.44±1.17	13.66±1.93^{bc}	17.74±0.72^{bc}	23.28±2.07^{bc}	24.44±2.50^{b}
FBC_3	5.05±0.58	6.36±0.51	16.1±1.82^{cde}	18.81±0.84^{bcd}	24.44±1.07^{cd}	26.26±1.63^{c}
FBC_4	4.31±0.65	6.42±0.31	17.40±0.96^{e}	20.31±0.27^{cd}	27.12±0.65^{ef}	28.26±0.75^{d}
FBS_1	3.89±0.63	6.42±0.58	12.06±0.81^{ab}	21.52±0.19^{de}	22.18±0.42^{abc}	23.60±2.35^{b}
FBS_2	4.21±0.76	6.22±0.39	14.26±0.67^{bcd}	24.52±0.24^{ef}	26.02±1.23^{de}	26.86±1.84^{c}
FBS_3	4.35±0.64	6.34±0.43	16.92±0.69^{de}	26.54±0.96^{f}	29.16±0.89^{fg}	31.22±1.68^{de}
FBS_4	4.32±0.59	6.36±0.27	17.66±0.59^{e}	28.44±0.27^{f}	31.08±1.27^{g}	34.68±1.03^{e}

Table-2. The number of red spinach leaves that were nurtured with liquid fertilizer of chicken feather not-fermented (NF), commercial chemical liquid fertilizer (FC), liquid chicken feather fertilizer *B. cereus* (FBC), and liquid chicken feather fertilizer *B. subtilis* (FBS) at several concentrations. The Tukey test was used to calculate the values, which reflect means and standard deviation with distinct superscript letters in the same column and show a substantially different (P<0.05).

Fertilizers	The number of leaves (strands/plants) of red spinach at week					
	1	2	3	4	5	6
NF	3	3.8±0.45	4.2±0.45^{a}	4.6±0.55^{a}	6.4±0.55^{a}	7.0±0.71^{a}
FC	3	3.6±0.55	4.8±0.45^{a}	6.0±1.00^{b}	6.8±0.45^{ab}	7.6±0.55^{ab}
FBC_1	3	3.6±0.55	4.4±0.89^{a}	6.6±0.55^{bc}	7.2±1.0.9^{abcd}	8.0±0.71^{abc}
FBC_2	3	3.8±0.45	5.0±0.71^{a}	6.8±0.83^{bc}	8.0±0.71^{bcde}	8.6±0.55^{bcd}
FBC_3	3	3.6±0.55	5.8±0.45^{ab}	7.0±1.00^{bc}	7.0±0.07^{abc}	9.0±0.71^{bcd}
FBC_4	3	3.6±0.55	6.0±0.00^{b}	7.4±0.55^{bc}	8.4±0.55^{cdef}	9.4±0.89^{cd}
FBS_1	3	3.8±0.45	4.2±0.45^{a}	6.6±0.55^{bc}	7.6±0.55^{abcde}	7.6±0.55^{ab}
FBS_2	3	3.8±0.55	5.0±0.00^{ab}	6.4±0.89^{bc}	8.6±0.55^{def}	9.2±0.45^{cd}
FBS_3	3	3.8±0.45	5.0±0.71^{ab}	6.4±0.54^{bc}	8.8±0.45^{ef}	9.6±0.55^{d}
FBS_4	3	3.6±0.55	5.2±0.45^{ab}	7.8±0.45^{c}	10.0±1.22^{e}	11.4±1.14^{e}

Number of leaves

The results of the current study (Table 2) showed that the number of red spinach leaves was significantly (P<0.05) affected by the application of liquid fertilizer. In the fifth to sixth weeks, the number of leaves of red spinach plants nurtured with FBC4,

FBS2, FBS3, and FBS4 was significantly higher (P<0.05) compared to NF and FC.

Yields

The results of the current study (Figure 5) showed that the yield of red spinach was significantly (P< 0.05) affected by the use of liquid fertilizer. The yield of red spinach nurtured by FBC and FBS was significantly (P<0.05) higher than NF.

The yield of red spinach nurtured to FBC_1 and FBC_2 was not significantly (P>0.05) different compared to FC, but the yield of FBC_3 and FBC_4 was significantly (P<0.05) higher than FC. There was no significant (P>0.05) difference between the yields of red spinach nurtured to FBC_3 and FBC_4. The yield of red spinach nurtured to FBS_1 was not significantly (P>0.05) different from FC; however, FBS_2, FBS3, and FBS_4 were significantly (P<0.05) higher than FC. The yield of red spinach to which FBS_3 and FBS4 were nurtured was significantly (P<0.05) higher than FBC at all concentrations applied. There was no significant (P>0.05) difference between the yields of red spinach nurtured with FBS_3 and FBS_4.

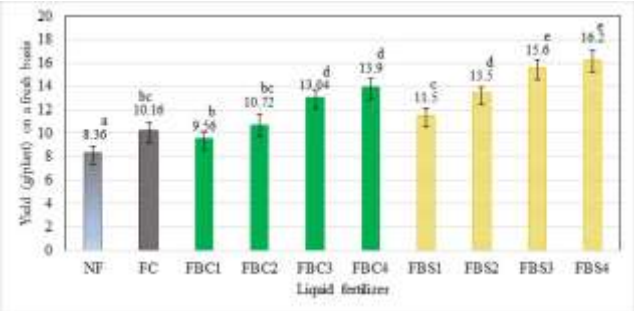

Figure-5. The yield of red spinach was applied liquid fertilizer of chicken feather not-fermented (NF), commercial chemical liquid fertilizer (FC), liquid chicken feather fertilizer *B. cereus* (FBC), and liquid chicken feather fertilizer *B. subtilis* (FBS). The Tukey test determined the values of means with error bars marked with different letters from 5 independent observations and a significant difference (P<0.05).

Anthocyanin content

The results of the current study (Figure 6) showed that the anthocyanin content of red spinach was significantly (P 0.05) affected by the use of liquid fertilizer. The anthocyanin content of red spinach nurtured to FBC_2, FBC3, and FBC_4, as well as FBS_1, FBS_2, FB3, and FBS_4, was significantly higher (P<0.05) compared to NF. The anthocyanin content of red spinach nurtured to FBC_1 was not significantly different (P>0.05) compared to NF and FC. The anthocyanin content of red spinach was highest in FBS_4 but was not significantly different (P>0.05) from FBS_3.

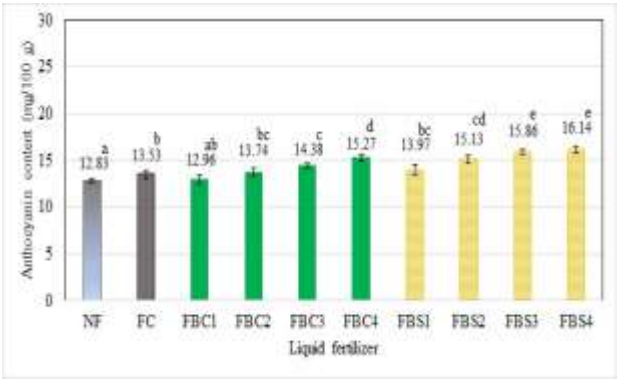

Figure-6. Anthocyanin content of red spinach leaves nurtured with liquid chicken feather not-fermented (NF), commercial chemical liquid fertilizer (FC), liquid chicken feather fertilizer *B. cereus* (FBC), and liquid chicken feather fertilizer *B. subtilis* (FBS). The Tukey test determined the values of means with error bars marked with different letters from 5 independent observations and a significant difference (P<0.05).

Discussion

The current study indicates that *B. cereus* and *B. subtilis* can release the extracellular keratinase enzyme and degrade chicken feather meal in media. The current study aligns with several researchers, such as Jeong et al. (2010) and Nilegaonkar et al. (2007), who reported that *B. subtilis* and *B. cereus* could produce keratinase and degrade chicken feathers. In the current study, the keratinase activity of *B. subtilis* in chicken feather meal media was higher than *B. cereus*. The current study aligns with Kim et al. (2001), who reported that the keratinase activity of *B. subtilis* on chicken feather media was higher than *B. cereus*. However, in contrast with Nagal and Jain (2010), who reported that the keratinase activity of *B. cereus* on chicken feather media was higher than *B. subtilis*. The current study indicates that the liquid fermented conditions of chicken feather meal at 55ºC, pH 8.5, and 60 rpm agitation for 72 h were thought to be more suitable for *B. subtilis* than *B. cereus*. Several factors can affect bacterial keratinase activity, including

incubation time and temperature (Dhiva et al., 2020), supplementation components, and the initial pH of the media (Kalaikumari et al., 2019). Several researchers have reported the activity of keratinases *B. cereus* and *B. subtilis* in chicken feather media as a source of keratin under various fermentation conditions. Adelina et al. (2019) reported that *B. subtilis* showed keratinase activity of 273.33 U/ml in chicken feather media fermented at 50ºC, pH 8, for 72 h. Nagal and Jain (2010) reported that *B. cereus* KB043 and *B. subtilis* KB099 showed keratinase activity of 39,100.4 U/ml[1] and 25,400.1 U/ml, respectively, in basal media supplemented with 1% chicken feather fermented at 37ºC, pH 7.0, and agitated at 150 rpm for 6 d. Akhter et al. (2020) reported that *B. cereus* had a keratinase activity of 74,661.52 U/ml in chicken feather flour media fermented at 37ºC for 48 h. de Paiva et al. (2019) reported that the keratinase activity of *B. subtilis* was 127 U/ml on chicken feather media supplemented with 0.1% yeast extract and fermented at 37ºC, pH 8.0, for 24 h. The effect of substrate size on *Bacillus* keratinase activity has also been reported by Mazotto et al. (2011), who found that the production of the enzyme keratinase by *B. subtilis* in chicken feather meal media as a carbon and nitrogen source was higher than that of whole chicken feathers.

The current study showed that the percentage of degradation of chicken feather meal fermented by *B. subtilis* was higher than *B. cereus*, in contrast to Nagal and Jain (2010), who reported that the percentage of feather degradation by *B. cereus* was higher than *B. subtilis*. The percentage of chicken feather degradation aligns with the keratinase activity, which in this study was higher in *B. subtilis* than in *B. cereus*. Peng et al. (2019) suggested that chicken feathers' degradation rate increased along with keratinase and protease activity. Several researchers have reported that the ability of microorganisms to degrade keratin and the levels of keratinase produced varied depending on the species, chemical composition, the molecular structure of the keratin substrate, and culture conditions (Son et al., 2008; Cai and Zheng, 2009).

The current study showed that fermentation by *B. cereus* and *B. subtilis* reduced the nitrogen content of chicken feather meal media. The decrease in nitrogen content is likely due to the formation of volatile components during keratin degradation. Several researchers reported that deamination occurs during keratin degradation, producing ammonia from proteins, peptides, and amino acids (Lakshmi et al., 2013; Trivedi et al., 2008; Bhange et al., 2016). Keratinase-producing bacteria such as *B. subtilis* have been reported to produce indole-3-acetic acid (IAA) and hydrogen cyanide (HCN) in chicken feather media (Bhange et al., 2016). The current study also showed that the nitrogen content in the liquid fertilizer chicken feather fermented by *B. subtilis* was higher than that of *B. cereus*, which indicated that the volatile components during degradation in *B. cereus* were higher than those in *B. subtilis*.

The current study shows that the response of growth and yield of red spinach to liquid chicken feather fertilizer fermented with *B. subtilis* was higher than that of liquid chicken feather fertilizer that was not fermented. This increase is due to the liquid chicken feather fertilizer providing nutritional components such as nitrogen, phosphorus, and potassium, which plants easily absorb. Gupta et al. (2013) reported that feathers are a source of protein (80–90%), nitrogen (19%), 19 kinds of amino acids, minerals such as calcium and phosphorus, trace elements, and other growth factors. However, the source of nutrients contained in chicken feathers is difficult for plants to utilize (Nurdiawati et al., 2019) because the presence of keratin in chicken feathers with a supercoiled and complex structure is very resistant and not easily degraded (de Oliveira et al., 2019). Several studies reported that chicken feather protein hydrolyzate containing low-molecular-weight peptides and free amino acids was quickly absorbed by plant tissues (Morales-Payan and Stall, 2003; Cerdán et al., 2009). Nagarajan et al. (2018) suggested that nitrogen is a constituent component of nucleic acids needed by plants for cell division and reproduction. Available nitrogen, which is easily absorbed, is required for plant growth (Tsavkelova et al., 2012). Phosphorus is an essential nutrient that supplies energy for plant growth and maintenance (Ahmad and Kibret, 2014). Meanwhile, potassium is a cation essential in plant growth and development as an activator of enzymes such as protein synthesis, sugar transport, nitrogen metabolism, and carbon metabolism (Hepler et al., 2001). Potassium also plays a role in photosynthesis, stimulates and controls ATPase in the plasma membrane to produce acid stimulation, which then triggers cell wall loosening and hydrolase activation (Oosterhuis et al., 2014), and is involved in the regulation of stomata opening and closing, cell elongation, and other critical physiological processes

(Xu et al., 2020).

The current study showed that the growth and yield of red spinach fed with liquid fertilizer made from chicken feather meal fermented by *B. subtilis* were higher than those of *B. cereus*, presumably because the dissolved nitrogen content was higher in the hydrolyzate of *B. subtilis*. In addition to providing nutrients that plant easily absorb, several researchers have reported the effect of chicken feather fertilizer on plant metabolism. Protein hydrolyzate can stimulate nitrogen and carbon metabolism, regulate plant nitrogen assimilation (du Jardin, 2015; Nardi et al., 2016), and increase nutrient absorption and nutrient use efficiency (Colla et al., 2015; Ertani et al., 2009; Halpern et al., 2015). Keratinase-producing bacteria have plant growth-promoting properties because they produce indole-3-acetic acid (IAA) phytohormones and inorganic dissolution (Tamreihao et al., 2019). *B. subtilis has been* reported to produce IAA (Jeong et al., 2010), which is responsible for cell division, elongation, differentiation, and pattern formation in plants (Dastager et al., 2010).

Several researchers have reported the response of plant growth and yield to the application of chicken feather fertilizer. Paul et al. (2013) reported that chicken feather fertilizer increases the number and length of root hairs, shoot weight and plant growth. Adelina et al. (2021) reported that the application of chicken feathers fermented by *B. subtilis* increased the growth of mung beans (*Vigna radiata*) because *B. subtilis* produced IAA and dissolved potassium in the media. Joardar and Rahman (2018) reported that giving chicken feather compost increased the height, number of leaves, and weight of water spinach (*Ipomoea aquatica*) plants. Sobucki et al. (2019) reported that the application of chicken feather fertilizer fermented by *Bacillus* sp. CL18 increased the lettuce's leaf and root dry weight (*Lactuca sativa*).

The current study showed that applying liquid chicken feather meal fertilizer fermented by *Bacillus* increased the anthocyanin content in red spinach. The increase in anthocyanin content was thought to be due to the concentration of nitrogen and potassium available in the liquid chicken feather fertilizer. Delgado et al. (2006) reported that nitrogen and potassium in fertilizers increased the total anthocyanin content because they played a role in phenolic ripening. Hilbert et al. (2003) reported that high nitrogen availability in vine fertilizers affects anthocyanin metabolic pathways, increasing polyphenol degradation during the final stage of berry ripening. Delgado et al. (2006) also suggested that adequate potassium nutrition in fertilizers can compensate for the adverse effects of excess nitrogen. It stimulates sugar translocation to fruit, indirectly benefiting the synthesis of phenolic components during ripening because it is closely related to carbohydrates. Al-Mohammad et al. (2021) reported that biofertilizers increased the total anthocyanin content in roselle plants.

Conclusion

The current study concluded that both *B. cereus* and *B. subtilis* could release extracellular keratinase and degrade the keratin in chicken feather meals. Keratinase activity, degradation, and nitrogen content in fermented chicken feather meal *B. subtilis* hydrolyzate were higher than those of *B. cereus*. Applying liquid chicken feather fertilizer fermented by *B. cereus* and *B. subtilis* can increase red spinach's growth, yield, and anthocyanin content. The current study recommends using chicken feathers fermented by *B. subtilis* as a liquid fertilizer for plants, especially red spinach, at a dose of 0.05 g/plant every week to replace commercial chemical liquid fertilizers. Optimum conditions for chicken feather fermentation by several species of *Bacillus* bacteria and their effect on soil fertility need further investigation.

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Contribution of Authors

Wardah: Conceived idea, designed research methodology, data collection and article write up
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Sopandi T: Conceptual framework, data collection and article write up