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Exploration of bacteria isolated from black soldier fly larvae for plant growth-promotion and organic acid production

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ABSTRACT: Bacteria isolated from Black Soldier Fly (BSF) larvae have significant potential as effective sources of beneficial microorganisms. The objective of this research was to investigate and classify the utility of bacteria isolated from the BSF larvae. The isolation process involved examining the microorganisms' morphology and performing Gram staining, followed by classification using the *16S rRNA* gene. The activities of the isolates—including ammonium release, phosphate solubilization, plant hormone production, and organic acid generation—were thoroughly analyzed. A total of twelve bacterial isolates were obtained from the BSF larvae. Selected isolates with notable activity levels and non-pathogenic characteristics were subjected to further investigation. Among these, isolate BSFL07 was identified as *Bacillus siamensis*, which produced 25.12 µg/ml of indole-3-acetic acid (IAA) and exhibited the highest production of isovaleric acid (22.73 µg/ml). Isolate BSFL08 was identified as *Bacillus stercoris*, producing 14.38 µg/ml of IAA. Isolate BSFL11 was classified as *Bacillus wiedmannii*, producing 18.96 µg/ml of IAA, and the highest levels of acetic acid (137.1 µg/ml) and isobutyric acid (21.08 µg/ml). All three isolates exhibited phosphate-solubilizing activity and limited nitrogen-fixation ability, which are key traits associated with plant growth promotion. Future studies will evaluate the effects of growth stage, cultivation time, and medium C/N ratio on metabolite production to optimize their application as biofertilizers.

KEYWORDS: organic acid, black soldier fly, nitrogen fixation, plant growth-promoting bacteria

INTRODUCTION

Black Soldier Fly (BSF), *Hermetia illucens* L., belongs to the family Stratiomyidae within the order Diptera. This insect is non-pathogenic and is not considered a pest, as it does not transmit diseases. The BSF is commonly found in temperate and tropical climates [1]. The BSF undergoes a life cycle comprising five stages: egg, larva, pupa, pre-pupa, and adult [2]. Previous research identified pathogenic bacteria from the BSF larvae showing soft rot symptoms that inhibit larval development [3]. In contrast, Fouillaud and Dufossé reported the isolation of cellulolytic bacteria from the gut of BSF larvae reared on rice straw diets, which could be utilized for organic waste decomposition [4]. The BSF larvae can efficiently convert industrial residues into high biomass, a process facilitated by the adaptability of their gut microbiota. Studies have shown that the type of substrate and larval developmental stage significantly influence the bacterial community. The hindgut, in particular, hosts a high abundance of bacteria with low diversity, maintaining a stable core microbiota [5]. These results suggest that the BSF larvae could serve as a valuable microbial reservoir.

Numerous bacteria isolates were obtained from the BSF larvae fed with chicken feed or fiber-rich substrates, revealing 172 species with potential for symbiosis and enzyme production [6]. Among the bacteria isolated from the gut of BSF larvae on nutrient agar were *Morganella morganii*, *Providencia rettgeri*, *Bacillus halodurans*, *Proteus mirabilis*, *Providencia alcalifaciens* and *Providencia* sp. [7]. These dominant genera are likely crucial for maintaining the health of the BSF larvae gut. However, there is limited research investigating the activity of bacteria isolated from the BSF larvae for promoting plant growth. Such activities include: 1) The nitrogen fixation process in bacteria is a crucial biological mechanism mediated by the enzyme nitrogenase, which enhances nitrogen availability for plants [8], 2) the production of hormones by plant growth-promoting bacteria, which thrives in soil and plant tissues, is a key factor in their ability to enhance plant growth [9, 10], and 3) phosphate solubilization by bacteria, such as *Pseudomonas* sp., *Bacillus* sp., and *Xanthomonas* spp., involves the production of acidic substances that transform phosphorus into plant-usable form. These compounds encompass organic acids such as propionic, citric, and succinic acid, as well as inorganic acids like nitric acid. This process converts insoluble phosphate into soluble forms (HPO_4^{2-} or $H_2PO_4^-$), making phosphorus accessible to plants [11, 12]. In addition, microbial secondary metabolites (toxins, gibberellins, pigments, alkaloids, antibiotics, enzymes, organic acids, and biopolymers)

play important ecological roles, supporting the survival and health of diverse microorganisms in nature while also offering potential industrial applications [13, 14]. Applying beneficial microorganisms to develop soil with disease-resistant properties supports sustainable agriculture by reducing pesticide use, increasing crop yields, and strengthening plant immunity to pathogens for long-term sustainability [15]. This research aimed to investigate the activities of beneficial bacteria isolated from the BSF larvae, which consume organic materials. The study focused on their potential to promote plant growth and their capacity to produce valuable secondary metabolite.

MATERIALS AND METHODS

Isolation and characteristic of microorganisms from BSF larvae

This study was approved by the Institutional Animal Care and Use Committee of Kasetsart University, Thailand (ID: ACKU64-AGR-022). The BSF larvae were provided by the Department of Animal Science, Faculty of Agriculture, Kasetsart University, Thailand. A total of 500 BSF larvae were reared in plastic trays containing 150 g of coconut coir with organic materials added as feed every 3–5 days and moisture maintained at 70%.

The larvae were weighed to 10 g and placed in a sterilized bag, then finely ground. Next, 90 ml of sterilized distilled water was added. This procedure was conducted within a laminar airflow cabinet. After thoroughly shaking the mixture, 1 ml was transferred into 9 ml of sterile distilled water to prepare a series of dilutions. Each dilution was then spread on HiCrome™ Bacillus Agar (Himedia, India) plates, with three replicates per dilution, and incubated at room temperature (approximately 30 °C) and shaking at 120 rpm for 24 h. Colony characteristics were observed, and bacterial cells were examined under a microscope at 1,000× magnification using the Gram staining method.

Classification of microorganisms

Microorganisms were cultured on the nutrient agar (Himedia) at 24 h. DNA was extracted from bacterial colonies using the NucleoSpin® Tissue kit (Macherey-Nagel, Germany) following the manufacturer's instructions. DNA purity and concentration were assessed using a 1% agarose gel electrophoresis and a Nanodrop spectrophotometer (Maestro; Taiwan), respectively. The DNA was then amplified by polymerase chain reaction (PCR) using *16S rRNA* gene primers: 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) [16]. The resulting nucleotide sequences were analyzed for similarity using the EzBioCloud database (https://www.ezbiocloud.net/) [17], and phylogenic classification was performed with Molecular Evolutionary Genetics Analysis (MEGA) software package version 11 [18]. Sequences were deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank/index.html) under the accession numbers [GenBank: PV611481–PV611492].

Organic acids analysis

Microorganisms were cultured in nutrient broth (NB) (Himedia) for 24–48 h. Organic acids were analyzed using gas chromatography (GC) (Chrompack CP 9001, Middelburg, Netherlands) following the method described by Zhong et al [19]. Briefly, a loopful of bacterial cells was inoculated into 50 ml of NB and incubated at 30 °C, 120 rpm for 24 h. The optical density (OD) at 600 nm was measured using a spectrophotometer (Genesys20, Massachusetts, USA) and adjusted to 0.6. Then, 1 ml of this culture was transferred into fresh NB and incubated under the same conditions for 16 h. The bacterial suspension was centrifuged at 13,587×*g* for 5 min, and 1 ml of the supernatant was transferred to a new tube. The reaction was stopped by adding 1 ml of 6 N HCl, followed by a second centrifugation. To the clarified supernatant, 20 µl of 25% metaphosphoric acid in 5 N H_2SO_4 was added and mixed thoroughly by vortex. The prepared sample was stored at 4 °C for 12 h before GC analysis, with results compared against an organic acid standard.

Release of ammonium

Each microbial isolate was cultured in 50 ml nitrogen-free medium (KH_2PO_4 1.0 g/l, $CaCl_2$ 1.0 g/l, $MgSO_4 \cdot 7H_2O$ 0.25 g/l, NaCl 0.5 g/l, $FeSO_4 \cdot 7H_2O$ 0.01 g/l, $MnSO_4 \cdot H_2O$ 0.01 g/l, Na_2MoO_4 0.01 g/l and Glucose 7.0 g/l) and was incubated at 30 °C, 120 rpm for 24 h to reach an OD of 0.6. Subsequently, 1 ml of this culture was transferred into an Erlenmeyer flask containing 50 ml of fresh nitrogen-free medium and incubated under the same condition. Samples (5 ml) were collected at 7, 14, 21, and 28 days and centrifuged at 13,587×*g* for 10 min. After centrifugation, 1 ml of the supernatant was transferred to a test tube, followed by the addition of 5 ml of reagent A (a mixture of phenol and nitroprusside) and 5 ml of reagent B (alkaline hypochlorite). The mixture was then shaken and incubated in the dark at 37 °C for 20 min until a blue color developed. The absorbance was measured using a Genesys20 spectrophotometer at 625 nm. Ammonium concentration was determined by comparison with a standard curve of ammonium chloride [20].

IAA production

The concentration of the IAA hormone was determined following the method described by Abd El-Azeem et al [21]. Bacteria were cultured in 50 ml of NB and incubated with shaking at 30 °C, 120 rpm for 24 h. The OD was adjusted to 0.6, and 1 ml of the culture was transferred into an Erlenmeyer flask containing 50 ml of NB with 100 µg/ml of tryptophan. The cultures were incubated under the same conditions for 7, 14, 21, and

28 days. After each period, 5 ml of the culture was centrifuged at 13,587×*g* for 10 min and 3 ml of the supernatant was transferred to a test tube. To this, 2 ml of Salkowski reagent was added, prepared by mixing 1 ml of 0.5 N $FeCl_3$ with 50 ml of 35% $HClO_4$. The mixture was shaken and incubated in the dark for 30 min. Absorbance was measured at 535 nm, and IAA concentrations were calculated by comparison with a standard curve of IAA.

Phosphate solubilization

Microorganisms were cultured in 50 ml of NB and incubated at 30 °C with shaking at 120 rpm for 24 h. The OD of the cultures was adjusted to 0.6, and 1 ml of the bacterial cultures was transferred into an Erlenmeyer flask containing 50 ml of Pikovskaya medium [22]. The cultures were incubated under the same conditions for 7, 14, 21, and 28 days. To analyze the available phosphorus, following the method of Bray and Kurtz [23], 5 ml of the culture medium was centrifuged at 13,587×*g* for 10 min, and 1 ml of the supernatant was collected. The supernatant was mixed with 4 ml of ammonium molybdate ascorbic acid solution and 5 ml of distilled water, and the mixture was incubated until a blue color change developed. The final volume was adjusted to 25 ml. The absorbance of the resulting solution was measured at 882 nm, and phosphorus concentrations were calculated by comparison with a standard phosphorus curve.

RESULTS

Isolation and Gram staining of microorganisms isolated from the BSF larvae

Twelve bacterial isolates were obtained from BSF larvae. Gram staining was performed and observed under a microscope after 24–48 h of incubation. Eight isolates—BSFL01, 03, 04, 05, 06, 09, 10, and 12—were identified as Gram-negative bacteria (Fig. 1A, C, D, E, F, I, J, and L). Four isolates—BSFL02, 07, 08, and 11—were identified as Gram-positive bacteria (Fig. 1B, G, H, and K).

Organic acids production of microorganisms from the BSF larvae

Microorganisms isolated from BSF larvae demonstrated their ability to produce various types and quantities of organic acids (Table 1). The production of organic acids by each isolate was statistically significant at $p < 0.05$. The BSFL11 isolate exhibited the highest concentrations of acetic acid and isobutyric acid at 137.13 µg/ml and 21.80 µg/ml, respectively. The BSFL03 and BSFL12 isolates produced the highest concentrations of propionic acid at 17.90 µg/ml and 17.20 µg/ml, respectively; however, the BSFL10 isolate did not produce this acid. The BSFL10 isolate displayed the highest production of butyric acid at 9.20 µg/ml, followed by the BSFL12 isolate at 8.23 µg/ml, while the BSFL08 and BSFL11 isolates showed no production. Additionally, the BSFL07 and BSFL10 isolates produced the highest concentrations of isovaleric acid at 22.73 µg/ml and 20.67 µg/ml, respectively, whereas the BSFL01 and BSFL08 isolates did not produce this acid.

Ammonium release of microorganisms from the BSF larvae

The release of ammonium by microorganisms isolated from BSF larvae was assessed over 7, 14, 21, and 28 days (Table 2). Ammonium production of each isolate was statistically significant at $p < 0.05$. At 7 days, the BSFL12 isolate released the highest ammonium at 2.97 µg/ml, whereas BSFL06 released the lowest amount at 0.37 µg/ml. By 14 days, BSFL12 showed the highest ammonium releasing at 3.04 µg/ml, whereas BSFL02 showed the lowest release at 0.36 µg/ml. At 21 days, the BSFL12 isolate maintained the highest ammonium production at 3.26 µg/ml, while BSFL03 exhibited the lowest production at 0.35 µg/ml. At 28 days. BSFL01 and 04 released the highest ammonium at 0.84 and 0.82 µg/ml, respectively, while BSFL02 released the lowest concentration at 0.44 µg/ml.

IAA production of microorganisms from the BSF larvae

The production of IAA by microorganisms isolated from BSF larvae was significant at $p < 0.05$ (Table 3). At 7 days, the BSFL01 isolate produced the highest IAA concentration at 3.58 µg/ml, whereas BSFL02 showed the lowest concentration at 0.22 µg/ml. At 14 days, BSFL01 maintained the highest IAA production at 31.09 µg/ml, whereas BSFL02 showed the lowest concentration at 4.63 µg/ml. At 21 days, BSFL01 continued to produce the highest IAA level at 31.44 µg/ml, while BSFL02 recorded the lowest IAA at 2.53 µg/ml. Finally, at 28 days, BSFL01 demonstrated the highest IAA production at 28.15 µg/ml, while BSFL10 exhibited the lowest level at 2.19 µg/ml.

Phosphate solubilization of microorganisms from the BSF larvae

The phosphate solubilization capacity of microorganisms isolated from BSF larvae was assessed over 7, 14, 21, and 28 days, as shown in Table 4, in term of available phosphorus. The phosphate solubilization levels for each isolate were statistically significant ($p < 0.05$). At 7 days, BSFL05 exhibited the highest available phosphorus at 15.22 µg/ml, whereas BSFL04 showed the lowest at 2.98 µg/ml. At 14 days, BSFL02 produced the maximum available phosphorus at 13.87 µg/ml, whereas BSFL07 recorded the lowest at 3.68 µg/ml. At 21 days, BSFL05 continued to show the highest available phosphorus at 14.80 µg/ml, while BSFL07 showed the lowest at 3.78 µg/ml. Finally, at 28 days, BSFL05 maintained the maximum available

Table 1 The organic acid production of microorganisms from the BSF larvae by cultured on nutrient broth at 16 h.

Isolate	Organic acid production (µg/ml)				
	Acetic acid	Propionic acid	Isobutyric acid	Butyric acid	Isovaleric acid
BSFL01	34.33 ± 1.10^{d}	8.73 ± 1.91^{bc}	5.63 ± 0.85^{ef}	4.83 ± 0.75^{c}	0.00 ± 0.00^{e}
BSFL02	59.77 ± 12.78^{c}	5.53 ± 1.16^{de}	4.03 ± 1.60^{ef}	3.93 ± 1.36^{cd}	4.43 ± 2.80^{d}
BSFL03	72.10 ± 5.50^{b}	17.90 ± 4.16^{a}	7.37 ± 3.19^{de}	7.93 ± 2.57^{ab}	13.27 ± 2.93^{b}
BSFL04	2.60 ± 0.78^{g}	6.27 ± 1.42^{cde}	4.20 ± 1.15^{ef}	1.70 ± 0.36^{de}	4.73 ± 1.10^{d}
BSFL05	5.33 ± 1.27^{fg}	7.70 ± 1.45^{bcd}	2.57 ± 1.01^{f}	5.30 ± 1.66^{bc}	4.60 ± 2.74^{d}
BSFL06	2.17 ± 0.90^{g}	4.27 ± 0.47^{e}	3.90 ± 0.92^{ef}	3.40 ± 2.52^{cd}	3.57 ± 0.72^{de}
BSFL07	24.30 ± 3.32^{e}	8.50 ± 0.50^{bcd}	12.27 ± 2.25^{c}	4.40 ± 1.87^{cd}	22.73 ± 5.42^{a}
BSFL08	2.00 ± 0.62^{g}	4.03 ± 0.67^{e}	2.23 ± 0.38^{f}	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}
BSFL09	8.77 ± 0.31^{fg}	9.90 ± 0.36^{b}	4.43 ± 0.40^{ef}	6.33 ± 0.45^{abc}	7.93 ± 1.50^{cd}
BSFL10	3.40 ± 0.70^{g}	0.00 ± 0.00^{f}	10.00 ± 0.70^{cd}	9.20 ± 1.51^{a}	20.67 ± 3.35^{a}
BSFL11	137.13 ± 7.38^{a}	8.90 ± 1.68^{bc}	21.80 ± 3.76^{a}	0.00 ± 0.00^{e}	6.70 ± 0.72^{cd}
BSFL12	12.30 ± 3.72^{f}	17.20 ± 1.42^{a}	17.93 ± 2.10^{b}	8.23 ± 3.04^{ab}	9.53 ± 0.97^{ab}
F-test	*	*	*	*	*

* Mean within a column followed by the same letter are not significantly different at $p < 0.05$.

Table 2 Quantity of ammonium released by microorganisms from the BSF larvae by cultured on nutrient broth at 7, 14, 21, and 28 days.

Isolate	Ammonium release of microorganism (µg/ml)			
	7 days	14 days	21 days	28 days
BSFL01	0.67 ± 0.02^{cde}	1.15 ± 0.37^{c}	0.56 ± 0.03^{bc}	0.84 ± 0.05^{a}
BSFL02	1.24 ± 0.03^{b}	0.36 ± 0.02^{e}	0.37 ± 0.07^{d}	0.44 ± 0.01^{e}
BSFL03	0.81 ± 0.08^{c}	1.44 ± 0.13^{b}	0.35 ± 0.04^{d}	0.69 ± 0.04^{b}
BSFL04	0.46 ± 0.02^{fg}	0.56 ± 0.01^{de}	0.61 ± 0.09^{b}	0.82 ± 0.02^{a}
BSFL05	0.55 ± 0.16^{ef}	0.47 ± 0.03^{de}	0.47 ± 0.03^{cd}	0.70 ± 0.02^{b}
BSFL06	0.37 ± 0.01^{g}	0.41 ± 0.05^{de}	0.36 ± 0.03^{d}	0.47 ± 0.01^{de}
BSFL07	0.42 ± 0.06^{fg}	1.22 ± 0.03^{bc}	0.38 ± 0.10^{d}	0.49 ± 0.03^{de}
BSFL08	0.42 ± 0.06^{fg}	1.23 ± 0.06^{bc}	0.38 ± 0.03^{d}	0.50 ± 0.01^{d}
BSFL09	0.52 ± 0.14^{efg}	0.46 ± 0.03^{de}	0.40 ± 0.04^{d}	0.51 ± 0.02^{d}
BSFL10	0.72 ± 0.09^{cd}	1.26 ± 0.07^{bc}	0.47 ± 0.07^{cd}	0.49 ± 0.05^{de}
BSFL11	0.63 ± 0.07^{de}	0.61 ± 0.06^{d}	0.46 ± 0.04^{cd}	0.58 ± 0.04^{c}
BSFL12	2.97 ± 0.09^{a}	3.04 ± 0.07^{a}	3.26 ± 0.18^{a}	0.52 ± 0.05^{d}
F-test	*	*	*	*

* Mean within a column followed by the same letter are not significantly different at $p < 0.05$.

Table 3 Indole-3-acetic acid production of microorganisms from the BSF larvae by cultured on nutrient broth with tryptophan at 7, 14, 21, and 28 days.

Isolate	Indole-3-acetic acid concentration (µg/ml)			
	7 days	14 days	21 days	28 days
BSFL01	3.58 ± 0.05^{a}	31.09 ± 3.85^{a}	31.44 ± 1.92^{a}	28.15 ± 0.05^{a}
BSFL02	0.22 ± 0.04^{h}	4.63 ± 1.15^{f}	2.53 ± 0.27^{j}	3.85 ± 0.02^{h}
BSFL03	2.51 ± 0.10^{b}	30.82 ± 3.89^{a}	28.75 ± 0.55^{bc}	22.44 ± 0.05^{c}
BSFL04	1.74 ± 0.00^{e}	10.98 ± 0.23^{e}	15.8 ± 0.27^{h}	13.28 ± 0.02^{g}
BSFL05	1.78 ± 0.03^{e}	17.2 ± 0.83^{bc}	22.91 ± 0.27^{e}	19.12 ± 0.00^{e}
BSFL06	1.34 ± 0.02^{g}	13.1 ± 0.40^{de}	19.91 ± 0.00^{fg}	15.80 ± 0.02^{f}
BSFL07	1.73 ± 0.20^{e}	19.18 ± 0.46^{b}	25.12 ± 0.00^{g}	21.78 ± 0.02^{c}
BSFL08	1.73 ± 0.04^{e}	10.85 ± 0.61^{e}	14.38 ± 0.27^{i}	13.41 ± 0.02^{g}
BSFL09	1.96 ± 0.02^{d}	29.37 ± 1.05^{a}	29.54 ± 0.27^{bc}	23.90 ± 0.14^{b}
BSFL10	2.11 ± 0.02^{c}	19.58 ± 0.61^{b}	28.28 ± 0.27^{c}	2.19 ± 0.00^{c}
BSFL11	1.43 ± 0.06^{fg}	14.42 ± 1.50^{cd}	18.96 ± 0.00^{g}	15.80 ± 0.02^{f}
BSFL12	1.53 ± 0.00^{fg}	14.82 ± 0.23^{cd}	20.38 ± 0.47^{f}	20.45 ± 0.09^{d}
F-test	*	*	*	*

* Mean within a column followed by the same letter are not significantly different at $p < 0.05$.

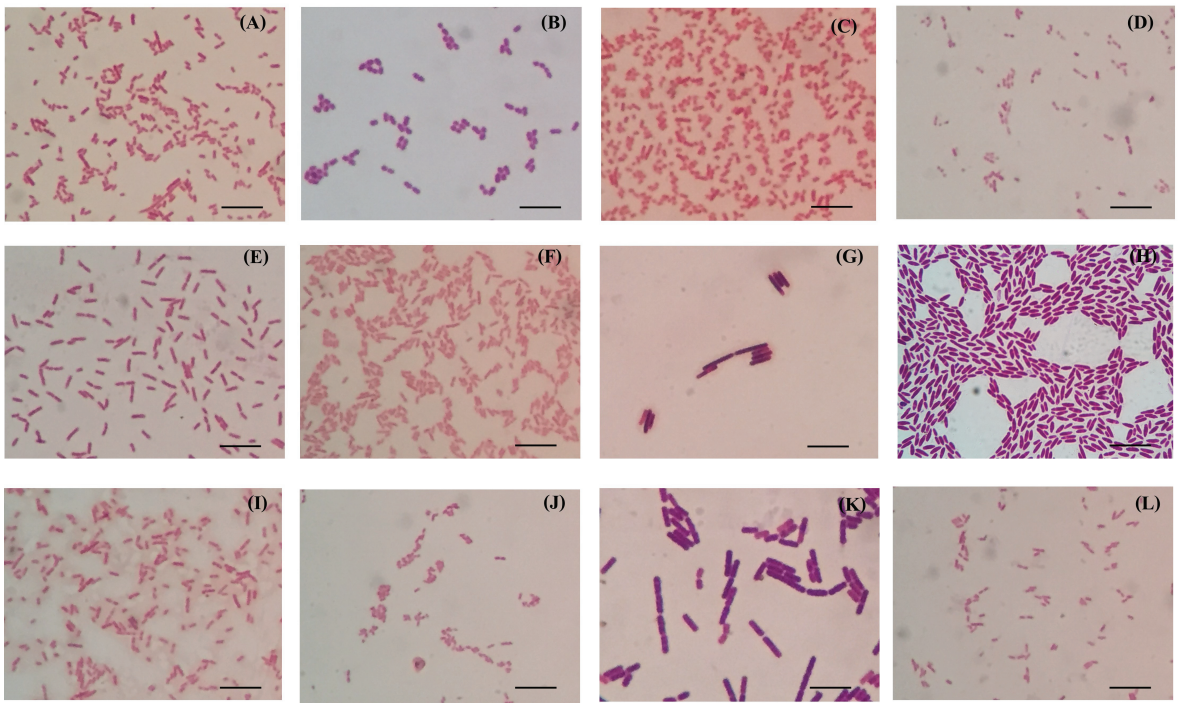

Fig. 1 Gram staining of bacterial isolates grown on nutrient agar and incubated at 30 °C. Isolates BSFL01 (A), BSFL03 (C), (BSFL04 (D), BSFL05 (E), BSFL06 (F), BSFL09 (I), BSFL10 (J), and BSFL12 (L) were Gram-negative staining. Isolates BSFL02 (B), BSFL07 (G), BSFL08 (H), and BSFL11 (K) were Gram-positive staining, bar = 20 µm.

Table 4 Available phosphorus of microorganisms from the BSF larvae by cultured on nutrient broth at 7, 14, 21, and 28 days.

Isolate	Available phosphorus (µg/ml)			
	7 days	14 days	21 days	28 days
BSFL01	4.67 ± 0.55^{b}	4.98 ± 0.05^{de}	4.74 ± 0.18^{f}	2.2 ± 0.40^{h}
BSFL02	5.03 ± 0.40^{b}	13.87 ± 0.20^{a}	14.25 ± 0.21^{b}	13.56 ± 0.33^{b}
BSFL03	4.61 ± 0.13^{b}	4.32 ± 0.04^{def}	4.96 ± 0.04^{e}	4.96 ± 0.06^{de}
BSFL04	2.98 ± 0.02^{d}	5.09 ± 0.05^{d}	5.23 ± 0.05^{d}	5.39 ± 0.11^{d}
BSFL05	15.22 ± 0.78^{a}	11.65 ± 1.88^{b}	14.8 ± 0.08^{a}	15.15 ± 0.38^{a}
BSFL06	3.73 ± 0.05^{c}	6.40 ± 0.17^{c}	6.58 ± 0.17^{c}	6.57 ± 0.13^{c}
BSFL07	3.15 ± 0.05^{d}	3.68 ± 0.04^{f}	3.78 ± 0.04^{h}	3.91 ± 0.05^{f}
BSFL08	4.61 ± 0.01^{b}	3.78 ± 0.16^{f}	3.89 ± 0.16^{h}	4.69 ± 0.04^{e}
BSFL09	4.69 ± 0.25^{b}	4.35 ± 0.21^{def}	4.36 ± 0.13^{g}	3.48 ± 0.19^{g}
BSFL10	4.71 ± 0.21^{b}	4.56 ± 0.01^{def}	4.98 ± 0.08^{e}	4.03 ± 0.13^{f}
BSFL11	5.16 ± 0.07^{b}	3.97 ± 0.18^{ef}	5.1 ± 0.06^{de}	4.2 ± 0.45^{f}
BSFL12	2.69 ± 0.24^{d}	5.03 ± 0.02^{d}	5.17 ± 0.02^{d}	5.06 ± 0.03^{de}
F-test	*	*	*	*

* Mean within a column followed by the same letter are not significantly different at $p < 0.05$.

phosphorus at 15.15 µg/ml, while BSFL01 recorded the lowest at 2.20 µg/ml.

Classification of microorganisms using *16S rRNA* gene

The most beneficial microorganism with the highest activities was selected for classification based on their *16S rRNA* gene sequences. The nucleotide sequences were compared for similarity with the EzTaxon server database, as shown in Table 5. BSFL01 and BSFL03 were identified as *Klebsiella variicola* subsp. *variicola* DSM 15968^{T} (CP010523) with 99.7% and 99.9% similarity, respectively. BSFL02 was classified as *Enterococcus pallens* ATCC BAA-351^{T} (AJAQ01000034) with 100.0% similarity, BSFL04 as *P. mirabilis* ATCC 29906^{T} (ACLE01000013) with 99.9%, BSFL05 as *Salmonella enterica* subsp. *arizonae* ATCC 13314^{T} (AF008580) with 98.0%, BSFL06 as *Providencia sneebia* DSM 19967^{T} (AKKN01000006) with 99.6%, and BSFL09 as *Enterobacter quasimori* WCHEn090040^{T} (RXSJ01000023) with 97.1%. BSFL07, BSFL08, and BSFL11 was classified as *B. siamensis* KCTC 13613^{T}

Table 5 Identification results of *16S rRNA* gene analysis of bacteria isolated from the BSF larvae.

Isolate no.	Accession number	Closely related taxa identified by using the EzTaxon server database[a]	% Similarity (nt diff./nt total)[b]
BSFL01	PV611481	*Klebsiella variicola* subsp. *varicola* DSM 15968^T (CP010523)	99.7 (4/1387)
BSFL02	PV611482	*Enterococcus pallens* ATCC BAA-351^T (AJAQ01000034)	100.0 (0/1400)
BSFL03	PV611483	*Klebsiella variicola* subsp. *varicola* DSM 15968^T (CP010523)	99.9 (1/1377)
BSFL04	PV611484	*Proteus mirabilis* ATCC 29906^T (ACLE01000013)	99.9 (1/1391)
BSFL05	PV611485	*Salmonella enterica* subsp. *arizonae* ATCC 13314^T (AF008580)	98.0 (27/1352)
BSFL06	PV611486	*Providencia sneebia* DSM 19967^T (AKKN01000006)	99.6 (6/1355)
BSFL07	PV611487	*Bacillus siamensis* KCTC 13613^T (AJVF01000043)	99.8 (3/1396)
BSFL08	PV611488	*Bacillus stercoris* JCM 30051^T (MN536904)	99.3 (9/1193)
BSFL09	PV611489	*Enterobacter quasimori* WCHEn090040^T (RXSJ01000023)	97.1 (40/1366)
BSFL10	PV611490	*Morganella morganii* subsp. *morganii* ATCC 25830^T (AJ301681)	99.7 (4/1355)
BSFL11	PV611491	*Bacillus wiedmannii* FSL W8-0169^T (LOBC01000053)	100.0 (0/1396)
BSFL12	PV611492	*Morganella morganii* subsp. *morganii* ATCC 25830^T (AJ301681)	99.7 (4/1367)

[a] (http://eztaxon-e.ezbiocloud.net)
[b] nt diff./nt total = Nucleotide difference/total nucleotide used in analysis.

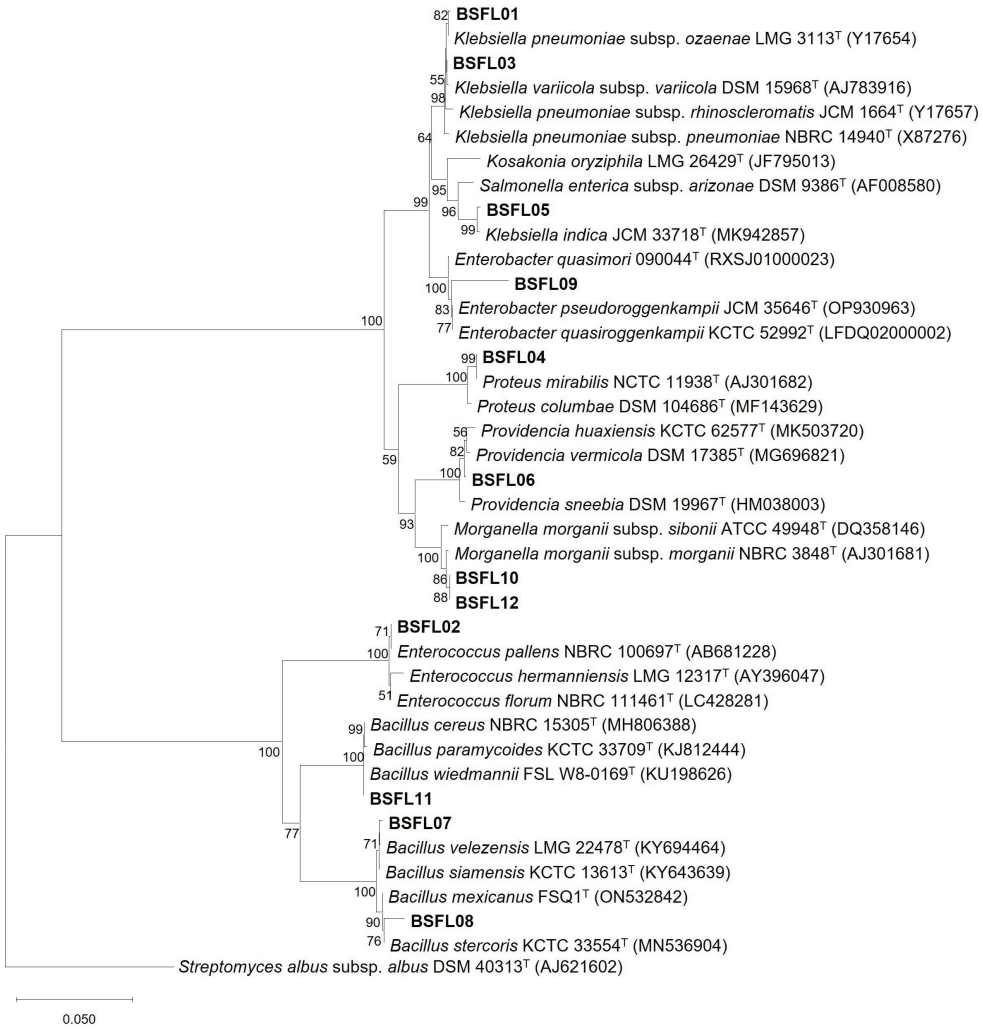

Fig. 2 Phylogenetic tree based on *16S rRNA* gene sequences obtained by the neighbor-joining (NJ) method showing the phylogenetic relationships among twelve bacteria isolated from the BSF larvae (shown in boldface) and related bacteria. Numbers at nodes indicate levels of bootstrap support (%) based on a NJ analysis of 1,000 resampled datasets, only values greater than 50% are shown.

(AJVF01000043) with 99.8% similarity, *B. stercoris* JCM 30051^{T} (MN536904) with 99.3%, and *B. wiedmannii* FSL W8-0169^{T} (LOBC01000053) with 100.0%, respectively. Meanwhile, BSFL10, and BSFL12 were classified as *M. morganii* subsp. *morganii* ATCC 25830^{T} (AJ301681) at 99.7%. The phylogenic tree (Fig. 2) presented the evolutionary relationship among the 12 isolates and their closely related nucleotide sequences. The position of these isolates confirmed their affiliation to 8 genera of *Bacillus*, *Enterobacter*, *Enterococcus*, *Klebsiella*, *Morganella*, *Proteus*, *Providencia*, and *Salmonella*.

DISCUSSION

Beneficial bacteria isolated from the BSF larvae exhibited potential applications in agricultural cropping. In the current research, the bacteria activities observed included the production of organic acids, plant hormones, nitrogen fixation, and phosphate solubilization, with each isolate producing more than one type of organic acids. Bacteria belonging to the *Bacillus* group have the greatest potential for agricultural utilization due to their non-pathogenic nature. BSFL07, BSFL08, and BSFL11 were classified as *B. siamensis*, *B. stercoris* and *B. wiedmannii*, respectively. Several *Bacillus* species have shown beneficial effects to promote plant growth and pathogen suppression. For instance, *B. siamensis* has been shown to modulate plant gene expression and alter the rhizosphere microbiome, thereby enhancing maize growth [24]. Similarly, *B. stercoris* strain B.PNR2 promoted the growth of rice and waxy corn seedling in atrazine-contaminated soil through the production of IAA [25]. Additionally, *B. wiedmannii* AzBw1 has been identified as an effective biocontrol agent against root-knot nematodes [26]. In this study, BSFL07 produced the highest production of isovaleric acid. Volatile organic compounds (e.g., acetic, propionic, butanoic, valeric, and isovaleric acids) released by *Bacillus* species have been shown to inhibit the mycelial growth of *Fusarium kalimantanense* [27]. BSFL07 and BSFL08 were found to produce IAA. According to Etesami and Glick [28], bacterial IAA plays a crucial role in promoting plant growth and development, mitigate abiotic stresses, and plays an important role in plant-microbe interactions, making these isolates highly beneficial for agricultural applications. BSFL11 isolate exhibited the highest acetic and isobutyric acid production at 16 h. Organic acids promote plant growth by increasing soil acidity, which enhances the dissolution of essential nutrients such as iron, phosphorus, potassium, and zinc. These nutrients, made available through organic acid-producing bacteria, can also stimulate plant defense mechanisms under stress conditions [29, 30].

BSFL01 and BSFL03 were identified as *K. variicola* subsp. *variicola*. BSFL02, BSFL04 to BSFL06, and BSFL09 were classified as *E. pallens*, *P. mirabilis*, *S. enterica* subsp. *arizonae*, *P. sneebia*, and *E. quasimori*, respectively. BSFL10, and BSFL12 were identified as *M. morganii* subsp. *morganii*. These microorganisms have been reported to possess pathogenic potential [31, 32]. On the contrary, some of these bacteria are known to promote plant growth. For example, *K. pneumoniae* exhibited nitrogen fixation and IAA biosynthetic activities [33, 34], while *E. asburiae* strain PS2 had activities including phosphate solubilization, indole acetic acid production, exopolysaccharides synthesis, siderophores production, as well as ammonia and hydrogen cyanide generation [35]. *M. morganii* significantly enhanced leaf chlorophyll content and antioxidant enzyme activities, thereby reducing oxidative stress in plants [36].

Consistent with these reports, the present study demonstrates that bacteria traditionally regarded as opportunistic pathogens can also possess functional traits associated with plant growth promotion, such as nitrogen fixation, phosphate solubilization, and phytohormone and organic acid production. Among the tested strains, BSFL01 exhibited the highest IAA production, reaching 31.44 µg/ml after 21 days of cultivation, which was higher than the genus *Saccharibacillus* isolated from mulberry roots, with the potential to promote plant growth and producing up to 11.04 µg/ml of IAA [37]. However, this level was lower than that reported for *B. cereus* BI-8 and *B. subtilis* BI-10, which produced 117 and 108 µg/ml of IAA, respectively [38], it nevertheless confirms the biosynthetic potential of BSFL-associated isolates. Similarly, BSFL12 produced 3.26 µg/ml of ammonium at 21 days, which was lower than the maximum ammonia production observed for *Bacillus* sp. PG-8 (6.51 µmol/ml or 117.44 µg/ml) at 2 days [39]. In contrast, BSFL05 demonstrated a comparatively strong phosphate-solubilizing capacity, achieving an available phosphorus concentration of 15.22 µg/ml after 7 days of incubation exceeding that reported for *B. subtilis* PE7 (4.32 µg/ml at 24 days) [40]. Although the metabolite levels observed in this study were generally lower than those reported for some previously characterized strains, the BSFL-associated isolates exhibited multiple plant growth-promoting traits simultaneously and were derived from a biologically relevant and sustainable source. Moreover, metabolite production observed at later incubation stages suggests the potential for sustained or slow-release activity, which may be advantageous under practical field conditions.

CONCLUSION

This study demonstrated that three *Bacillus* strains isolated from BSF larvae possess potential for promoting plant growth and producing organic acids. *B. siamensis* (BSFL07) produced five organic acids, with isovaleric acid being the most prominent. *B. stercoris* (BSFL08) synthesized three organic acids and also produced IAA. *B. wiedmannii* (BSFL11) produced four organic acids, particularly high levels of acetic and isobutyric acids,

along with IAA production. These multifunctional traits highlight the promise of these isolates for agricultural applications. However, further research is needed, including optimization of culture conditions and formulation strategies, to enhance production efficiency and support their development as effective multifunctional bioinoculants. It should also be noted that opportunistic pathogenic bacteria have been detected in BSF larvae, underscoring the importance of careful strain selection and safety evaluation prior to practical application.

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