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In vitro Screening of Sunflower Associated Endophytic Bacteria With Plant Growth-Promoting Traits

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Harnessing endophytic microbes as bioinoculants promises to solve agricultural problems and improve crop yield. Out of fifty endophytic bacteria of sunflowers, 20 were selected based on plant growth-promoting. These plant growth-promoting bacteria were identified as *Bacillus, Pseudomonas,* and *Stenotrophomonas*. The qualitative screening showed bacterial ability to produce hydrogen cyanide, ammonia, siderophore, indole-3-acetic acid (IAA), exopolysaccharide, and solubilize phosphate. The high quantity of siderophore produced by *B. cereus* T4S was 87.73%. No significant difference was observed in the *Bacillus* sp. CAL14 (33.83%), *S. indicatrix* BOVIS40 (32.81%), *S. maltophilia* JVB5 (32.20%), *S. maltophilia* PK60 (33.48%), *B. subtilis* VS52 (33.43%), and *P. saponiphilia* J4R (33.24%), exhibiting high phosphate-solubilizing potential. *S. indicatrix* BOVIS40, *B. thuringiensis* SFL02, *B. cereus* SFR35, *B. cereus* BLBS20, and *B. albus* TSN29 showed high potential for the screened enzymes. Varied IAA production was recorded under optimized conditions. The medium amended with yeast extract yielded high IAA production of 46.43 μg/ml by *S. indicatrix* BOVIS40. Optimum IAA production of 23.36 and 20.72 μg/ml at 5% sucrose and 3% glucose by *S. maltophilia* JVB5 and *B. cereus* T4S were recorded. At pH 7, maximum IAA production of 25.36 μg/ml was obtained by *S. indicatrix* BOVIS40. All the isolates exhibited high IAA production at temperatures 25, 30, and 37°C. The *in vitro* seed inoculation enhanced sunflower seedlings compared to the control. Therefore, exploration of copious endophytic bacteria as bioinoculants can best be promising to boost sunflower cultivation.

Keywords: bioinoculants, *Helianthus annuus*, plant growth promotion, seed inoculation, South Africa, sustainable agriculture

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INTRODUCTION

The environmental problems posed by the use of agrochemicals on farmlands have necessitated the need to search for ecofriendly and sustainable approaches by harnessing endophytic bacteria as the best alternative bio-input (Basu et al., 2021; Bhutani et al., 2021). Devising suitable methodologies for the characterization of agriculturally important endophytic microbes from economic crops, however, promise to avert future food insecurity. The microbes found inhabiting the internal tissue of plants are called endophytic microbes and their mutual interdependence with the host

plants contributes to plant growth and health (Mukherjee et al., 2021). Hence, the overview of the multifaceted functions of endophytic microbes can systematically bring new insights into agriculture biotechnology by maximum exploration and applications.

Endophytic microbes employed direct and indirect mechanisms to ensure sustainable plant nutrition (Zaman et al., 2021). Nitrogen fixation, IAA production, phosphate solubilization, and ACC deaminase activity contribute to soil nitrogen pool, root development, plant growth, and resilience to abiotic drought stress. The antibiosis, induced systemic resistance, hydrogen cyanide, siderophore, and enzyme production by endophytic microbes protect plants from pathogen attack, so indirectly enhance plant growth (Adeleke and Babalola, 2022). An increase in plant growth and crop yield upon inoculation with plant growth-promoting endophytic bacteria in the genera *Bacillus, Enterobacter, Klebsiella, Pantoea,* and *Rhizobium* has been documented (Nascimento et al., 2020; Mowafy et al., 2021; Preyanga et al., 2021). In addition, some endophytic bacteria isolated from oilseed crops have been reported to enhance plant growth due to their plant growth-promoting traits (Lally et al., 2017; Abdel-Latef et al., 2021). Nevertheless, scant information is available on endophytic bacteria isolated from sunflower cultivated in Southern Africa. Hence, research findings into the sunflower microbial world for maximum exploration as bioinoculants will help improve crop yield sustainably.

Aside from maize, cowpea, wheat, and sorghum, sunflowers are one of the most edible and economic crops cultivated in the North West Province of South Africa and other countries of the world (Adeleke and Babalola, 2020). Sunflower cultivation promise to ensure food security, and the supply of nutritional and healthy food for both livestock and human beings (Seiler et al., 2017). The economic value of sunflowers is enormous, such that, sunflower oil is widely distributed and available in South African markets. The seed inoculation and optimization of endophytic bacteria under different growth conditions remain fundamental to testing their effects on sunflower seed germination. Furthermore, harnessing copious endophytic microbes on a large scale can potentially boost sunflower oil production in South Africa. Hence, this research was designed to isolate, characterize and screen sunflower-associated endophytic bacteria with plant growth-promoting traits *in vitro*.

MATERIALS AND METHODS

Helianthus annuus Sampling

The roots and stems of *H. annuus* cultivar PAN 7160 CLP were sourced from commercial farmland in Lichtenburg, North West Province, South Africa in February 2020. The climatic conditions of this region were characterized by an annual rainfall of 360 mm and a temperature range of 3–21°C during winter and 22–34°C during summer. The healthy sunflower samples were carefully uprooted, labeled, placed inside sterile zip-lock bags, and transported to the Microbial Biotechnology Research Group laboratory, North-West University, South Africa at 4°C for further analysis. A total of 24 samples were randomly collected in triplicates from four points within the field for the isolation of endophytic bacteria.

Root and Stem Surface Sterilization and Isolation of Endophytic Bacteria

The sunflower roots and stems were cut into small sizes with a sterile scalpel and then washed in sterile distilled water. The samples were surface sterilized by soaking in 70% ethanol for 3 min, followed by 3% sodium hypochlorite for 3 min, 70% ethanol for 30 s, and lastly rinsed 5 times with sterile distilled water. The sterilization level of the samples was assessed by plating the last rinse on Luria Bertani (LB) media (Miller, Sigma Aldrich, USA). Five grams of plant material were weighed (Radwag weighing machine; Lasec; Poland), suspended in 1 M phosphate-buffered solution (FBS), and manually macerated in a mortar and pestle until smooth suspensions were obtained. One gram of the macerated samples was weighed and aseptically dispensed into sterile test tubes containing 9 ml sterile distilled water and mixed properly. Then, 1 ml from the mixture was aseptically pipetted and serially diluted up to 10^{-9} dilutions. From dilutions 10^{-5} and 10^{-6}, 0.1 ml of the suspension were gently dispensed into Petri plates in triplicates. The plates were pour-plated with molten sterilized Luria Bertani (LB) media and incubated at 28 ± 2°C for 24 h. Distinct bacterial colonies formed on the plates were counted and recorded. Colonies from each plate were observed and selected based on morphological characteristics. The pure bacterial cultures were obtained by repeated streaking on fresh LB agar plates. Pure bacterial colonies were preserved on LB medium amended with 30% (v/v) glycerol at −20°C.

Biochemical Characterization of Pure Bacterial Cultures

Gram staining and various biochemical tests were performed to characterize the bacterial isolates. Oxidase, urease, starch hydrolysis, citrate, and sugar fermentation tests were performed following the modified methods of Majeed et al. (2018). Other biochemical tests performed include Voges-Proskauer, hydrogen sulfide production, citrate test, nitrate utilization, methyl red test, and indole production test. All the chemical reagents used for these tests were procured from Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa.

Screening of Bacterial Isolates for Plant Growth-Promoting Traits

Ammonia Production Test

The ability of bacterial isolates to produce ammonia was tested according to the methods described by Alkahtani et al. (2020). Briefly, ammonia production was performed as follows: 0.1 ml of 24-h old bacterial culture (10^6 CFU/ml) was aseptically inoculated into test tubes containing 10 ml sterile peptone broth (peptone 0.2 g, 10 ml sterile distilled water) and incubated on a rotary shaker (SI-600, LAB Companion, Korea) at 120 rpm, at ambient temperature for 96 h. After incubation, a 0.5 ml Nessler's reagent was gently dispensed into each test tube, then allowed

to stand for 5 min for color development. A color change from yellow to dark brown indicated a positive reaction. A tube without bacterial inoculation served as a control.

Phosphate Solubilization

The phosphate solubilization potential of the bacterial isolates was evaluated according to the methods described by Premono et al. (1996). The qualitative test was performed on modified Pikovskaya agar composed of (g/L; tricalcium phosphate ($Ca_3(PO_4)_2$) 5, glucose ($C_6H_{12}O_6$) 10, manganese sulfate ($MnSO_4{\cdot}H_2O$) 0.002, sodium chloride (NaCl) 0.2, potassium chloride (KCl) 0.2, magnesium sulfate ($MgSO_4$) 0.1, ammonium sulfate ($(NH4)_2SO_4$) 0.5, yeast extract 0.5, agar 15, at pH 7. Twenty-four-hour-old bacterial cultures were spot inoculated directly at the center of each Pikovskya's agar (PA) plate. The plates were incubated at 28°C for 5 days for the visible zone of clearance (ZOC). The ZOC (mm) around the colony on the cultured plates indicated positive results for phosphate solubilization, while the un-inoculated plate served as control. The ZOC (mm) around the colony was measured, and colony diameter measurements (mm) were summarized as low (+), medium (++), and high (+ + +). The phosphate-solubilizing index (PSI) was enumerated as:

$$\text{PSI} = \frac{\text{colony diameter (mm)} + \text{ZOC (mm)}}{\text{colony diameter (mm)}}$$

PSI was grouped as low (PSI $<$ 2.00), intermediate (2.00 $\leq$ PSI $<$ 4.00), or high (PSI $\geq$ 4.00) based on Marra et al. (2011) methods.

For the quantitative assay, the phosphate solubilizing-producing ability of the bacterial isolates was performed by inoculating 10 ml sterile Pikovskaya broth in 50 ml Falcon tubes with 0.1 ml (10^6 CFU/ml) freshly grown bacterial culture, incubated at 30°C for 5 days at 180 rpm on a rotary shaker (SI-600, LAB Companion, Korea). The supernatant was obtained after cold centrifugation of 10 ml bacterial cultures at 10,000 rpm for 5 min at 4°C. Four milliliters of the color reagent (1:1:1:2 ratio of 3 M H_2SO_4, 10% (w/v) ascorbic acid, 2.5% (w/v) ammonium molybdate, and distilled water) were added to 10% (w/v) of 5 ml trichloroacetic acid inside test tubes. The inoculated tubes were allowed to stand for 15 min at room temperature. The quantity of phosphate content was measured according to phosphomolybdate, a blue method, at an absorbance of 820 nm. The phosphate solubilization potential of endophytic bacteria in the Pikovskaya broth was determined from the phosphate (KH_2PO_4) standard curve. Medium without bacterial inoculation served as control.

Siderophore Screening

The siderophore production ability of the bacterial isolates was performed according to the methods of Khan et al. (2020) with few modifications. Each bacterial isolate was aseptically inoculated into a sterilized medium amended with CAS, i.e., chrome azurol S. Preparation of CAS solution was performed by weighing 60.5 mg CAS into 10 ml of 1 mM iron (III) solution ($FeCl_3.6H_2O$) (a). The iron (III) solution was diluted in 10 mM HCl. The solution (a) was gently mixed with 0.0729 g hexadecyltrimethylammonium (HDTMA, Merck, SA) bromide suspended in 40 ml sterile distilled water (b) on the magnetic stirrer. From the mixture of "a" and "b," solution, 100 ml was measured and added to 900 ml sterilized LB medium at pH 6.8. The sterilized medium at 121°C for 15 min was allowed to cool and pour plated into sterile Petri dishes. Each bacterial culture was spot-inoculated at the mid-point of the solidified agar plates and incubated at 28°C for 5 days. The development of yellow ring coloration around the bacterial colonies on the plates indicated positive reactions for siderophore production. Un-inoculated plates served as control.

The quantity of siderophore production was determined by inoculating LB broth solution containing CAS with 0.1 ml of 24-h old bacterial culture and incubated at 180 rpm on a rotary shaker (SI-600, LAB Companion, Korea) for 7 days. The grown bacterial culture was centrifuged at 8,000 × g for 10 min. From the filtrate, 0.5 ml was added to 0.5 ml CAS reagent, mixed, and incubated for 2 min at room temperature. The quantity of siderophore produced was measured at 630 nm using a spectrophotometer (Thermo Spectronic, Merck Chemicals, SA). The siderophore values were obtained from the regression equation of the standard curve. The experiment was carried out in triplicate.

Test for Hydrogen Cyanide

The test for HCN production by bacterial endophytes was determined according to the modified method of Igiehon et al. (2019). Nine milliliters of LB broth amended with 0.4% (w/v) glycine was aseptically dispensed into test tubes, sterilized, allowed to cool, and then inoculated with 0.1 ml (10^6 CFU/ml) fresh bacterial inoculum. Whatman filter paper No.1 was dipped in 0.5% (w/v) picric acid and subsequently in 2% (w/v) sodium carbonate. Then, the filter paper was plugged into each test tube (without touching the broth solution), then screw-cap and stop-up with parafilm and incubated at 28°C for 5 days. The test tubes were examined daily for color changes in the filter paper. A color change from yellow to brown indicated a positive result. The un-inoculated tube served as control. The experiment was carried out in triplicate.

Screening of Extracellular Enzymes

Screening for enzyme production was performed using plate assay techniques. The enzymes screened include, mannanase, cellulase, amylase, xylanase, and protease.

Mannanase

Screening of bacterial isolates for mannanase production was performed as described by Blibech et al. (2020) with few modifications. Briefly, media composition of g/L; Locust bean gum (3), dipotassium hydrogen phosphate (K_2HPO_4) 1, iron sulfate ($FeSO_4$) 0.001, ammonium chloride (NH_4Cl) 1, sodium chloride (NaCl) 0.5, calcium chloride ($CaCl_2$) 0.1, magnesium sulfate ($MgSO_4$) 0.5, and agar 13 at pH 7.2 was sterilized at 121°C for 15 min, allowed to cool. The media were poured plated and allowed to solidify. A 24-h old bacterial culture was gently inoculated in the middle of the agar plates and then incubated at 28°C for 48 h. Each cultured plate was flooded with iodine solution and observed for 15 min. The staining solution was poured off and further treated by flooding with 1 M sodium

chloride (NaCl) for 15 min for the visible ZOC around the colonies. The colonies with a ZOC (mm) indicated mannanase production. The un-inoculated plate served as control. The experiment was carried out in triplicate for each bacterial isolate.

Cellulase

The qualitative screening of endophytic bacteria for cellulase production was performed using plate assay techniques according to Alkahtani et al. (2020) with little modifications. Freshly grown pure bacterial cultures were inoculated by single streaking on carboxymethyl cellulose (CMC) amended media composed of g/L; dipotassium hydrogen phosphate (K_2HPO_4) 1, sodium nitrate ($NaNO_3$) 3, iron sulfate ($FeSO_4$) 0.01, CMC 1, potassium chloride (KCl) 0.5, magnesium sulfate ($MgSO_4$) 0.5, and agar 20 at pH 7.0. The inoculated CMC plates were incubated at 28°C for 48 h and then flooded with 1% (w/v) Congo red (CR) for 10 min. The CR on the plates was gently washed off and the plates were further washed with 1 M NaCl for 15 min. The ZOC (mm) encircling the colonies indicated a positive result for cellulase production. The un-inoculated plate served as control. Negative result plates were further flooded with 5% acetic acid solution for 2 min and then washed with sterile distilled water. A clear ZOC (mm) around the colony was determined and recorded. The experiment was carried out in triplicate for each bacterial isolate.

Amylase

The amylase production was tested on starch agar according to the methods of Alkahtani et al. (2020) with little modifications. A 24-h bacterial culture was spot-inoculated on sterilized starch agar medium composed of peptone 5 g, magnesium sulfate ($MgSO_4$) 0.5 g, yeast extract 5 g, iron sulfate ($FeSO_4$) 0.01 g, soluble starch 10 g, agar 15 g, and sodium chloride (NaCl) 0.01 g in 1,000 ml sterile water and then incubated at 37°C for 48 h. After that, Lugol's iodine solution (iodine 0.4%, potassium iodide 0.8%, distilled water−200 ml) was poured on the plates for 10 min. The formation of a ZOC (mm) around each bacterial isolate on the plate indicated amylase production. The un-inoculated plate served as control. The experiment was carried out in triplicate for each bacterial isolate.

Xylanase

Screening of bacterial isolates for xylanase production on mineral salt medium (MSM) supplemented with 0.5% xylan (beech-wood) was performed according to the methods described by Alkahtani et al. (2020) with minor modifications. The MSM composition include, agar 2%, peptone 0.5%, yeast extract 0.3%, and sodium chloride (NaCl) 0.5%. The media solution was adjusted to pH 9 before sterilization at 121°C for 15 min. The media were allowed to cool and pour plating. Plates were inoculated with fresh 24-h old bacterial culture by straight streak at the mid-point of the plates and then incubated at 28°C for 24 h. After that, plates were flooded with 0.4% Congo red and incubated for 10 min, then washed with 1 M NaCl to determine the ZOC. The ZOC around the bacterial isolates on each plate was considered positive for xylanase production. The un-inoculated plate served as control. The experiment was carried out in triplicate for each bacterial isolate.

Protease

The primary screening for each endophytic bacterium for protease production was performed on LB agar plates supplemented with skim milk powder according to the methods described by Alkahtani et al. (2020) with little modifications. The media composition (g/L) includes, skim milk powder 28, dextrose 1, casein 5, yeast extract 2.5, and agar 15 at pH 7. The media were prepared, sterilized, and then allowed to cool before pour-plating. Consequently, fresh bacterial culture was inoculated on each plate and incubated at 28°C for 48 h. The bacterial isolates exhibiting a circular ZOC (mm) indicated a positive result for protease production. An un-inoculated plate served as control. The experiment was performed in triplicate.

Indole Acetic Acid

The IAA production was tested according to the modified method of Gutierrez et al. (2009). Ten milliliters of LB broth supplemented with tryptophan were aseptically inoculated with 0.1 ml freshly grown bacterial culture (10^6 CFU/ml) and incubated at 28°C for 7 days at 120 rpm in a rotary shaker (SI-600, LAB Companion, Korea). The bacterial cultures were cold centrifuged at 4°C for 10 min at 8,000 rpm. IAA from the crude extract was measured by transferring 1 ml of the supernatant into a clean tube and one drop of orthophosphoric acid (10 mM) and Salkowski reagent (2 ml) (1:30:50 ratio of 0.5 M $FeCl_3$ solution: 95% w/w sulfuric acid: distilled water) was added. The mixture was allowed to stand (incubation) for 10 min at room temperature. The appearance of pink coloration in the tubes after incubation in the dark indicated a positive result. An un-inoculated plate served as control. Color development by the bacterial strains was grouped as low, average, and high. The IAA production of the reacting mixture after incubation was determined at 530 nm using UV-spectrophotometer (ThermoFisher Scientific, USA). The IAA concentration of each bacterial isolate was evaluated from the IAA gradient standard curve (SC). The experiment was performed in triplicate for mean value calculation. Furthermore, three bacterial isolates were optimized under different growth conditions: nitrogen source, carbon source, pH, and temperature.

Media Preparation and Optimization Process for IAA Production

Varied concentrations of carbon and nitrogen sources were supplemented into an IAA production medium (IPM) composed of (g/L); yeast extract 6, L-tryptophan 1, peptone 10, and NaCl 5 at pH 7.6 (Chandra et al., 2018). Other optimized conditions include incubation time, temperature, and pH. For incubation time, a 200 ml IPM inside 500 ml conical flasks was sterilized at 121°C for 15 min, allowed to cool, and then inoculated with fresh 24-h grown *S. indicatrix* BOVIS40, *B. cereus* T4S, and *S. maltophilia* JVB5 with 0.5 optical density at 630 nm. The culture medium was incubated at 37°C, and 180 rpm for 11 days.

TABLE 1A | Biochemical characterization of endophytic bacteria from sunflowers.

Strain	SP	GR	CT	Gel	OX	SH	H_2S	VP	MR	MT	NT		IND	CS	UR	Isolation sources	
											Clr	Gas					
B. cereus SFR35	Rod	+	+	+	+	+	+	+	+	+	+	−	+	−	−	Root	Ten (10) bacterial isolates
B. wiedmannii FTL29	Rod	+	−	+	+	+	+	+	+	+	+	−	−	+	−	Root	
Bacillus sp. CAL14	Rod	+	+	−	+	+	+	+	+	+	−	+	−	+	−	Root	
B. cereus T4S	Rod	+	+	−	+	+	+	+	+	+	+	−	−	+	−	Root	
P. lini BS27	Rod	−	+	−	+	+	+	+	+	+	+	−	−	+	−	Root	
S. indicatrix BOVIS40	Rod	−	+	−	+	−	+	+	+	+	+	−	−	−	−	Root	
S. maltophilia JVB5	Rod	−	+	−	+	+	+	+	+	+	+	−	−	+	−	Root	
B. albus TSN29	Rod	+	+	−	+	+	+	+	+	+	+	+	−	+	−	Root	
B. cereus BLBS20	Rod	+	+	−	+	+	+	+	+	+	−	+	−	−	−	Root	
B. thuringiensis SFL02	Rod	+	+	−	+	+	+	+	+	+	+	+	+	+	−	Root	
S. maltophilia PK60	Rod	−	+	+	+	+	+	+	+	+	+	−	+	+	−	Stem	Ten (10) bacterial isolates
B. subtilis VS52	Rod	+	+	+	+	+	+	+	+	+	+	−	−	+	−	Stem	
B. thuringiensis BAAG44	Rod	+	+	+	+	−	+	+	+	+	+	−	−	−	−	Stem	
B. pseudomycoides SFS19	Rod	+	−	−	+	−	+	+	+	+	+	−	−	+	−	Stem	
B. toyonensis OLT2020	Rod	+	−	−	+	−	+	+	+	+	+	+	+	+	−	Stem	
B. thuringiensis BSA123	Rod	+	+	+	+	+	+	+	+	+	−	+	−	+	−	Stem	
B. paramycoides LS11	Rod	+	−	+	+	+	+	+	+	+	−	+	+	+	−	Stem	
P. saponiphilia J4R	Rod	−	−	+	+	+	+	+	+	+	+	+	−	+	−	Stem	
B. cereus VEJU7080	Rod	+	+	+	+	−	+	+	+	+	+	−	−	−	−	Stem	
Pseudomonas sp. FOBS21	Rod	−	+	−	+	+	+	+	+	+	+	−	−	+	−	Stem	

+, positive; –, negative; SP, shape; GR, Gram reaction; Clr, color; CT, citrate; Gel, gelation; OX, oxidase; SH, starch hydrolysis; H2S, hydrogen sulfide; VP, vogue Proskauer; MR, methyl red; NT, nitrate; IND, indole; CS, casein; UR, urease; MT, motility.

One ml of the cultured medium was constantly withdrawn at 24-h intervals and assayed for IAA production by Salkowski's reagent. Furthermore, similar incubation conditions were used to monitor the effects of other parameters under the same IAA assay conditions. The ability of bacterial isolates to utilize carbon as substrate and their effects on IAA production was tested using 5 sugars; namely, maltose, fructose, sucrose, glucose, and galactose, at different concentrations (1, 3, and 5%) as described by Khan et al. (2020). Additionally, the ability of bacterial isolates to utilize nitrogenous-base compounds, such as peptone, potassium nitrate, casein, yeast extract, and urea as substrates were tested at different concentrations of 1, 3, and 5% (Chandra et al., 2018). The pH of the medium ranging from 4 to 10 was examined. The pH of the IAA-producing medium was adjusted using 1 M of NaOH or HCl. IPM was optimized at varied temperatures; 25, 30, 37, 45, and 60°C. After sterilization, the IPM for each optimized parameter was allowed to cool, inoculated with 0.1 ml (10^6 CFU/ml) of each selected bacterium, and incubated at 28 ± 2°C for 7 days on a rotary incubator machine at 180 rpm. After incubation, the supernatant was subjected to an IAA assay and IAA concentration was measured at 630 nm for pH and temperature, and 590 nm for carbon and nitrogen, respectively, using a spectrophotometer (Thermo Spectronic; Meck, South Africa). The common reagents used for the plant growth-promoting screening were procured from Merck Chemicals (Pty) Ltd, Gauteng, South Africa, and Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa.

Molecular Identification of Plant Growth-Promoting Endophytic Bacteria

DNA Extraction Process

The genomic content of pure bacterial isolates was extracted using a commercial Quick-DNATM Miniprep Kit specific for fungi or bacteria (Zymo Research, Irvine, CA, USA; Cat. No. D6005), following the manufacturer's guide. The quantity of the extracted DNA (ng/μl) was measured using a NanoDrop ND-2000 UV-Vis spectrophotometer (ThermoFisher Scientific, USA) and stored at −80°C.

Polymerase Chain Reaction and Sequence Analysis

The determination of 16S rRNA nucleotide sequences of the identified bacterial isolates was achieved using the amplified PCR products. The specific forward and reverse primers, 27F (5′-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5′-TACGGTTACCTTGTTACGACTT-3') were purchased from Inqaba Biotechnological Industrial (Pty) Ltd, Pretoria, South Africa. A total of 25 μl reaction volume for each bacterial isolate composed of 12.5 μl OneTaq 2X MasterMix with the Standard Buffer, 1 μM for each primer, ∼5 ng genomic DNA, and 9.5 μl nuclease-free water were used for PCR amplification on DNA Engine DYAD^{TM} Peltier Thermal Cycler

TABLE 1B | Sugar utilization by endophytic bacteria from sunflowers.

Isolate code.	Sugars used									Isolation sources	
	Mal	Gal	Glu	Arab	Suc	Fru	Raff	Mann	Xyl		
B. cereus SFR35	+A	+AG	+Ag	+A	+Ag	+A	+A	+A	+A	Root	Ten (10) bacterial isolates
B. wiedmannii FTL29	+A	+Ag	+Ag	+A	+Ag	+A	–a	+A	+A	Root	
Bacillus sp. CAL14	+A	+Ag	+AG	+A	+Ag	+A	+A	+A	+A	Root	
B. cereus T4S	+A	+AG	+AG	+A	+Ag	+A	+A	+A	+A	Root	
Pseudomonas lini BS27	+A	+Ag	+AG	+A	+Ag	+A	+A	+A	+A	Root	
S. indicatrix BOVIS40	+A	+Ag	+Ag	+A	+Ag	+A	–a	+A	+A	Root	
S. maltophilia JVB5	+A	+Ag	+Ag	+A	+Ag	+A	+A	+A	+A	Root	
B. albus TSN29	+A	+Ag	+AG	+A	+Ag	+A	–a	+A	+A	Root	
B. cereus BLBS20	+A	+AG	+Ag	+A	–ag	+A	+A	+A	+A	Root	
B. thuringiensis SFL02	+A	+Ag	+Ag	+A	+Ag	+A	+A	+A	+A	Root	
S. maltophilia PK60	+A	+Ag	+Ag	+A	+Ag	+A	+A	+A	+A	Stem	Ten (10) bacterial isolates
B. subtilis VS52	+A	+AG	+AG	+A	+AG	+A	–a	+A	+A	Stem	
B. thuringiensis BAAG44	+A	–ag	+AG	+A	+Ag	+A	+A	+A	+A	Stem	
B. pseudomycoides SFS19	+A	–ag	+Ag	+A	–ag	+A	+A	+A	+A	Stem	
B. toyonensis OLT2020	+A	+AG	+AG	+A	+AG	+A	–a	+A	+A	Stem	
B. thuringiensis BSA123	+A	+AG	+AG	+A	+Ag	+A	+A	+A	+A	Stem	
B. paramycoides LS11	+A	+Ag	+Ag	+A	+Ag	+A	+A	+A	+A	Stem	
P. saponiphilia J4R	+A	–ag	+Ag	+A	+Ag	+A	+A	+A	+A	Stem	
B. cereus VEJU7080	+A	+AG	+AG	+A	+Ag	+A	+A	+A	+A	Stem	
Pseudomonas sp. FOBS21	+A	+AG	+Ag	+A	+Ag	+A	+A	+A	+A	Stem	

ag, no acid and gas production; AG, acid and gas production; +, positive; –, negative; Suc, sucrose; Glu, glucose; Fru, fructose; Arab, arabinose; Mann, mannose; Xyl, xylose; Mal, maltose; Raff, raffinose.

(BIO-RAD, USA, C1000 TouchTM). The PCR cycle parameters were programmed as follows: initial denaturation at 94°C for 5 min; 35 cycles of amplification. Also, the denaturation for 30 s at 94°C, annealing for 30 s at 50°C, extension for 1 min at 68°C; and a final overall extension for 10 min at 68°C. After running PCR, the PCR product was determined on agarose gel electrophoresis. Subsequently, the gel was carefully removed, and confirmation of the expected size of the product was visualized on a UV trans-illuminator. The resulting outcome was captured in a Chemidoc^{TM} imaging system (BIO-RAD Laboratories, California, USA). Finally, 20 μl of the PCR product for each bacterial isolate was placed in an ice-box pack and sent for sequencing at Inqaba Biotechnical Industries (Pty) Ltd, Pretoria of South Africa. 16S rRNA sequences for each bacterium were submitted to GenBank on the NCBI online server and were assigned with accession numbers. The twenty identifiable endophytic bacteria deposited on GenBank of the National Center for Biotechnology Information (NCBI) web server can be accessed from the links provided in the data availability section.

Sequence Alignment and Construction of Phylogenetic Tree

The Basic Local Alignment Search Tools (BLAST) program of the nucleotide sequences on the National Center for Biotechnology Information (NCBI) was employed to determine bacterial isolate sequence similarities and identities. The sequenced data were further analyzed by subjecting to multiple sequence alignment by ClustalW using a Bio-Edit program. MEGA-X online program was used to construct the phylogenetic tree from the resulting ClustalW sequences and the maximum likelihood method of the taxa with the Tamura-Nei model. The phylogeny test of the aligned sequences was achieved by the bootstrap method (Tamura et al., 2013).

Sunflower Seed Inoculation and *in vitro* Effect of Endophytic Bacteria on Seedling Growth

The effectiveness of sunflower seed inoculation was performed based on the methods described by Ullah et al. (2017). The bacterial inoculum size in LB broth at 24-h incubation was standardized to 0.5 (10^6 CFUml^{-1}) at OD_{600}. The three bacterial isolates, namely, *S. indicatrix* BOVIS40, *B. cereus* T4S, and *S. maltophilia* JVB5 were selected based on the most promising plant growth-promoting properties. A seed inoculation assay was used to facilitate bacterial adherence to the disinfected sunflower seeds. Cleaning of the seeds was performed by washing in sterile distilled water to remove floating-unhealthy seeds and dirt, and disinfected in 70% ethanol for 3 min, followed by 3% hypochlorite for 3 min, then immersed in 70% alcohol for 30 s, and lastly rinsed 5 times with sterile distilled water. Prepared LB broth inoculated with fresh bacterial culture was incubated at room temperature in a rotary incubator machine (SI-600, LAB

TABLE 2 | Identification of endophytic bacteria based on 16S rRNA gene sequences.

Strain	Identity	% Similarity	GBAN	Homologous accessions	Isolation sources
SFR35	*B. cereus*	100	MW265416	MW092893	Root
FTL29	*B. wiedmannii*	99	MW265418	MZ292345	Root
CAL14	*Bacillus* sp.	95	MW265422	MK554656	Root
T4S	*B. cereus*	100	MW265423	MW115619	Root
BS27	*Pseudomonas lini*	94	MW265425	JQ833637	Root
BOVIS40	*S. indicatrix*	100	MW265419	MW116366	Root
JVB5	*S. maltophilia*	100	MW265431	MT605498	Root
TSN29	*B. albus*	99	MW265420	MT636856	Root
BLBS20	*B. cereus*	100	MW265427	MT543036	Root
SFL02	*B. thuringiensis*	99	MW265413	MK743981	Root
PK60	*S. maltophilia*	97	MW265415	MK588914	Stem
VS52	*B. subtilis*	100	MW265429	MT613731	Stem
BAAG44	*B. thuringiensis*	98	MW265424	MK743981	Stem
SFS19	*B. pseudomycoides*	99	MW265430	MK999393	Stem
OLT2020	*B. toyonensis*	100	MW265417	MT605503	Stem
BSA123	*B. thuringiensis*	100	MW265426	JX994096	Stem
LS11	*B. paramycoides*	100	MW265414	MW090883	Stem
J4R	*P. saponiphilia*	100	MW265421	MT501808	Stem
VEJU7080	*B. cereus*	99	MW265428	MH231418	Stem
FOBS21	*Pseudomonas* sp.	100	MW261910	MT561438	Stem

GBAN, GenBank accession number.

Companion, Korea) at 180 rpm for 24 h. The bacterial cells in the broth culture were harvested by centrifugation at 8,000 × g for 10 min to obtain the pelletized cells and then washed in 0.85% normal saline solution. The centrifugation and washing of the pellets were performed under sterile conditions. The surface-sterilized seeds were suspended in a bacterial suspension containing 1% (v/w) carboxymethyl cellulose (CMC) as an adhesive (binder) in a 250 ml flask for 60 min. The seeds suspended in sterile distilled water containing 1% (v/w) CMC without bacterial inoculum served as control.

Under sterile conditions, 10 coated seeds (of each bacterial strain) were placed inside Petri dishes lined with moistened sterile absorbent cotton and sealed with parafilm. The plates were kept at 28°C in the growth chamber and seedling growth was monitored daily for 5 days. Each treatment was performed in triplicates. The plates were carefully taken out for seedling growth assessment. The fresh weight and dry weight of seedlings after oven drying at 60°C were measured. The seedling's fresh and dry weight obtained was expressed in gram (g) per triplicate.

Statistical and Data Analysis

The analysis of data from this study was performed using SPSS - Statistical Package for the Social Sciences (version 6.0) and Microsoft Excel. A significant difference among the treatment groups was calculated using ANOVA - one-way analysis of variance. The mean difference was determined by Duncan's tests at a 5% level of significance. Data obtained were presented as mean ± standard deviation. All experiments were performed in triplicates.

Data Availability

The sequenced dataset associated with this study can be accessed at https://www.ncbi.nlm.nih.gov/nuccore/MW261910, and https://www.ncbi.nlm.nih.gov/nuccore/?term=MW265413:MW265431[accn].

RESULTS

Bacterial Endophytes Isolation and Biochemical Characterization

A total of twenty-seven bacterial isolates from the roots and twenty-three bacterial isolates from the stems were isolated and characterized. However, ten (10) bacteria isolated from the stems and ten (10) bacteria isolated from the roots were further characterized and selected for plant growth-promoting screening based on the distinct morphological characterization, Gram staining, and biochemical tests (see **Table 2** below). The cultural, biochemical characterization, and sugar utilization by endophytic bacteria from sunflowers were presented in **Tables 1A**,**B**.

Based on Gram reaction, 70% of the bacterial isolates were Gram-positive, whereas 30% were Gram-negative. The Gram-negative bacterial endophytes identified include *Pseudomonas* sp. FOBS21, *S. maltophilia* PK60/JVB5, *S. indicatrix* BOVIS40, *P. saponiphilia* J4R, and *P. lini* BS27, while Gram-positive bacterial isolates include the genus *Bacillus*. All the bacterial isolates were rod-like with positive results for oxidase, hydrogen sulfide production, Voges-Proskauer, and methyl red. *B. thuringiensis* BSA123 was positive for the citrate test, while nine isolates were positive for gelatin liquefaction. *B. cereus* SFR35 utilizes indole

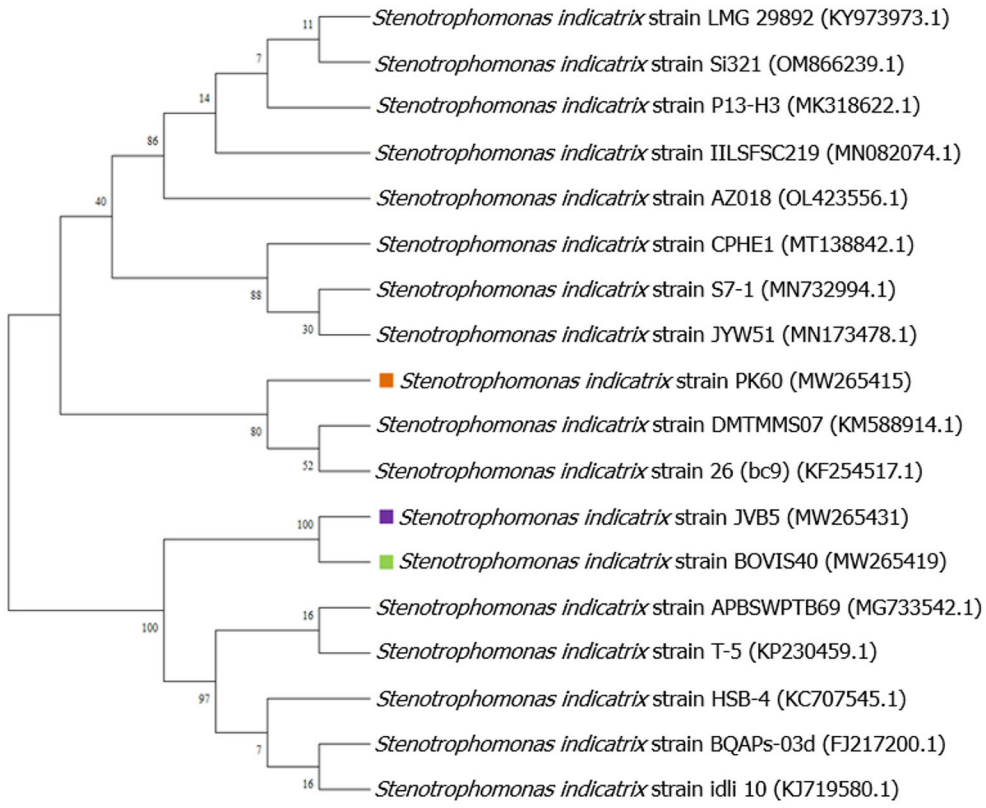

FIGURE 1 | Evolutionary relationships of taxa tree based on partial 16S rRNA sequences using maximum likelihood-based on the Tamura-Nei model showing relationships between the endophytic *Stenotrophomonas* species and its closely related strains from NCBI GenBank.

while *B. thuringiensis* BSA123 showed a positive reaction to casein hydrolysis. For nitrate utilization, eight bacterial isolates produced gas (N_2), while sixteen reduced nitrates. All the isolates were urease negative. For sugar fermentation tests, all bacterial isolates fermented glucose, fructose, arabinose, mannitol, xylose, and maltose, respectively. Based on molecular identification, *Bacillus* species were the most common identifiable bacteria in the stem and root samples (**Table 2**). Each bacterial strain was designated as SFR35, FTL29, T4S, CAL14, BS27, BOVIS40, JVB5, TSN29, BLBS20, SFL02, PK60, VS52, BAAG44, SFS19, OLT2020, BSA123, LS11, J4R, VEJU7080 and FOBS21 (**Table 2**).

Identification of Selected Endophytic Bacteria by 16S rRNA Gene Sequencing and Phylogeny Analysis

The identification of endophytic bacteria based on 16S rRNA gene sequences was presented in **Table 2**. The phylogeny information of the identifiable bacteria genera and bacterial sequences of related genera recovered from the GenBank database is shown in **Figures 1**–**3**.

Plant Growth-Promoting Traits

The plant growth-promoting traits of endophytic bacteria from sunflowers are presented in **Table 3**. The qualitative screening revealed the ability of the bacterial isolates to produce ammonia, siderophore, exopolysaccharide, hydrogen cyanide, IAA, and solubilize phosphate. Nine bacterial isolates exhibited high siderophore production, while five exhibited medium and six exhibited low activity for the siderophore production. The quantitative results revealed a high siderophore value of 87.73 % by *B. cereus* T4S.

Screening of Extracellular Enzymes

The qualitative screening of endophytic bacteria for enzyme production; namely, amylase, cellulase, xylanase, mannanase, and protease was presented in **Table 4**. *S. indicatrix* BOVIS40, *B. weidmannii* FTL29, *B. subtilis* VS52, and *B. thuringiensis* BSA123, exhibited a positive reaction to all enzymes assayed. Except for amylase, bacterial isolates *B. cereus* VEJU7080 and *B. cereus* T4S were positive for other screened enzymes. Summarily, bacterial isolated designated *B. weidmannii* FTL29, *B. albus* TSN29, *B. thuringiensis* BSA123, and *B. thuringiensis* BAAG44 displayed amylase, xylanase, mannanase, and protease production tendencies, respectively.

Optimization of Process Parameters for IAA Production

All the bacterial isolates displayed varied IAA activities at different L-tryptophan concentrations. The medium

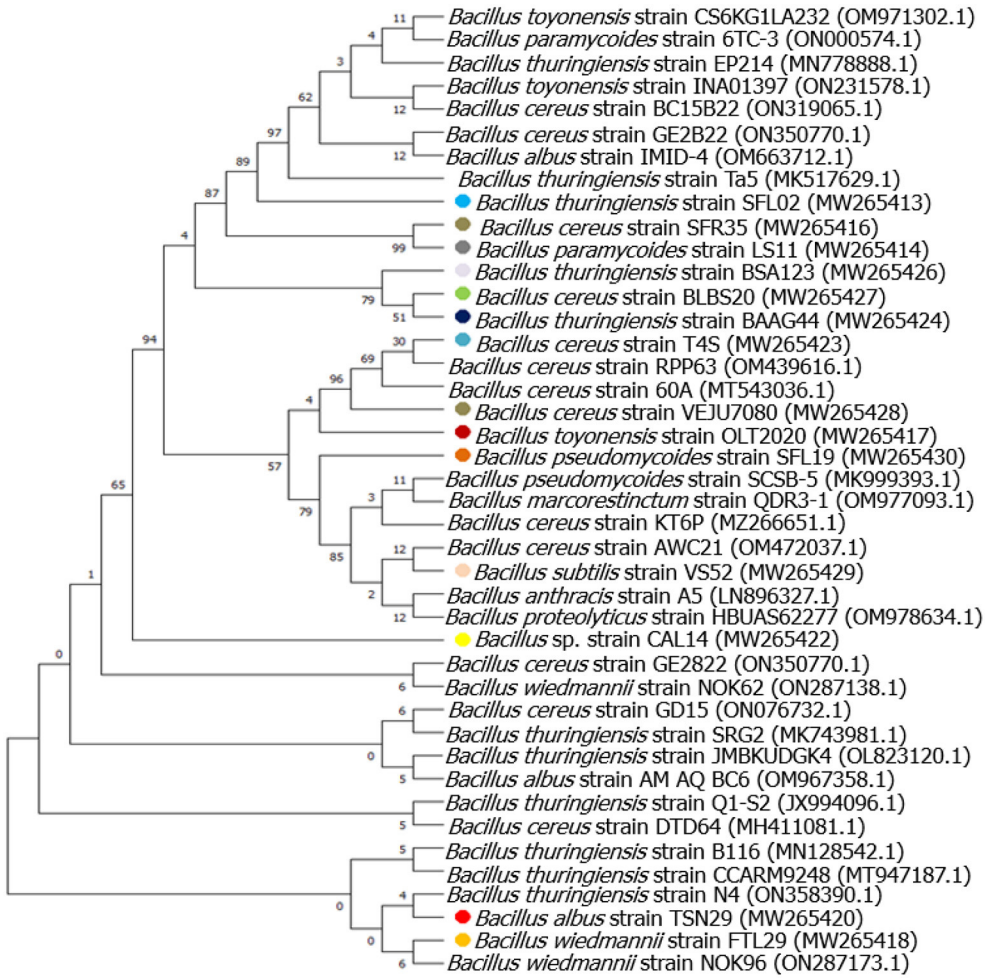

FIGURE 2 | Evolutionary relationships of taxa tree based on partial 16S rRNA sequences using maximum likelihood-based on the Tamura-Nei model showing relationships between the endophytic *Bacillus* species and its closely related strains from NCBI GenBank.

supplemented with L-tryptophan yielded higher IAA production compared to the control (**Figure 4**). Bacterial strains, *S. indicatrix* BOVIS40, *B. cereus* T4S, and *S. maltophilia* JVB5 from the roots of sunflower exhibited high IAA production compared with other isolates. In contrast, the lowest IAA production was observed in *B. cereus* SFR35 compared with the control.

Effect of Incubation Time and pH on IAA Production

The time course for IAA production by the bacterial isolates was presented in **Figure 5**. An increase in IAA production with an increase in incubation time was recorded. Optimum IAA production was attained at 168 h and beyond this point, there was a decline. The optimum IAA production of 16.94, 11.76, and 9.92 μg/ml at 168 h of incubation were recorded by *S. maltophilia* JVB5, *B. cereus* T4S, and *S. indicatrix* BOVIS40, respectively. The results of IAA produced by the bacterial isolates monitored between pH 4–10 were presented in **Figure 6**. The IAA production increased from pH 4–7, and beyond this point, there was a decline from pH 8–10. *S. indicatrix* BOVIS40 showed maximum IAA production of 25.36 μg/ml, followed by *B. cereus* T4S of 12.34 μg/ml and *S. maltophilia* JVB5 of 5.46 μg/ml at pH 7. Similarly, maximum IAA production of 11.83 μg/ml by *S. maltophilia* JVB5 at pH 6, 10.74 μg/ml at pH 8, with the least IAA production of 2.83 μg/ml at pH 4 were obtained. At pH 9 and 10, no significant difference was observed in the IAA production of *B. cereus* T4S and *S. maltophilia* JVB5.

Effect of Temperature on IAA Production

The effect of incubation temperature on IAA production by endophytic bacteria ranging from 25 to 60°C was presented in **Figure 7**. Maximum IAA production of 34.40 μg/ml by *B. cereus* T4S at 37°C was recorded. The amount of IAA produced at temperatures 45 and 60°C was lower compared with other temperatures. Bacterial strains showed an increase in IAA production from temperature 25−37°C before it declined. At 30 and 37°C, there was no significant difference in the IAA production by *S. maltophilia* JVB5.

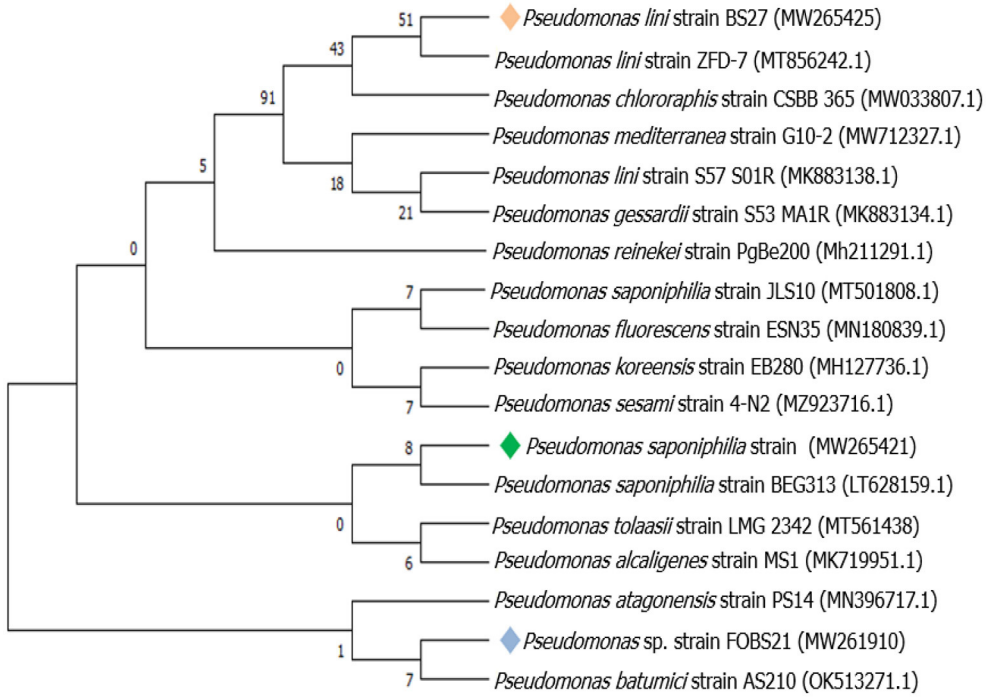

FIGURE 3 | Evolutionary relationships of taxa tree based on partial 16S rRNA sequences using maximum likelihood-based on the Tamura-Nei model showing relationships between the endophytic *Pseudomonas* species and its closely related strains from NCBI GenBank.

Effect of Carbon Source on IAA Production

Figure 8 showed the effect of carbon source on IAA production by endophytic bacteria from sunflowers. The amount of IAA produced in the growth medium varied with the sugar concentration. A maximum IAA production of 23.36 and 20.72 μg/ml were recorded from *S. maltophilia* JVB5 and *B. cereus* T4S at 5% sucrose and 3% glucose, respectively.

Effect of Nitrogen Source on IAA Production

The effect of nitrogen source on IAA production by endophytic bacteria was presented in **Figure 9**. All the bacterial isolates exhibited IAA production >20 μg/ml in a medium amended with casein and yeast extracts. Maximum IAA production of 19.31 and 17.70 μg/ml were recorded from *S. maltophilia* JVB5 at 3 and 5% peptone. Similarly, *B. cereus* T4S exhibited a maximum IAA production of 17.94 μg/ml at 3% peptone. A high IAA production of 14.97 μg/ml was obtained from *B. cereus* T4S at 5% potassium nitrate. There was no significant difference in the IAA production by *S. maltophilia* JVB5 at 5% yeast extract. Similar results were obtained of *S. indicatrix* BOVIS40 and *B. cereus* T4S at 1% and *B. cereus* T4S and *S. maltophilia* JVB5 at 3% yeast extract, respectively. An increase in the amount of IAA production with an increase in yeast extract concentration was recorded. *S. indicatrix* BOVIS40 exhibited an IAA production increase from 21.71 to 45.34 μg/ml, while *B. cereus* T4S showed an increase in IAA production from 21.00 to 42.89 μg/ml and *S. maltophilia* JVB5 from 25.58 to 45.82 μg/ml, respectively. There was no significant difference in the amount of IAA produced by *S. maltophilia* JVB5 at 1 and 5% urea. *B. cereus* T4S displayed high IAA production of 5.30 μg/ml at 1% urea.

In vitro Effect of IAA-Producing Endophytic Bacterial Isolates on Sunflower Seedling Growth

The effect of IAA-producing bacteria *S. indicatrix* BOVIS40, *B. cereus* T4S, and *S. maltophilia* JVB5 on sunflower seeds by inoculation were tested (**Table 5**). The percent increase of the number of lateral roots of 5.13, 6.97, and 1.58% were obtained from the inoculated sunflower seedling with bacterial strain *S. indicatrix* BOVIS40, *B. cereus* T4S, and *S. maltophilia* JVB5 compared to the un-inoculated sunflower seeds (control). A significant difference in the shoot and root length of sunflower seedlings compared with the control was recorded. The percentage increase of 9.09, 3.7, and 20.88% of root length and 6.23, 5.23, and 2.88% of shoot length were obtained from the inoculated sunflower seedlings compared with the un-inoculated sunflower seeds.

DISCUSSION

The need to ensure a safe environment for improved crop production has been a major concern, as insights into plant-microbe interactions remain crucial in developing eco-friendly agriculture. Due to the complex dynamics of biodiversity in the plant root environment, this can facilitate the recruitment

TABLE 3 | Plant growth-promoting traits of endophytic bacteria.

	Qualitative						Quantitative		
Strain	**HCN**	**AM**	**SDR**	**IAA**	**EPS**	**PS**	**PSI (mm)**	**%PS (v/v)**	**%SDR (v/v)**
B. cereus SFR35	++	+	+	+	++	+	4.09 ± 0.08^{l}	29.96 ± 0.03^{e}	64.93 ± 0.04^{k}
B. wiedmannii FTL29	+	+	++	+	+++	+	2.65 ± 0.04^{e}	30.96 ± 0.03^{e}	15.30 ± 0.04^{d}
Bacillus sp. CAL14	++	+	++	+	+++	+	3.14 ± 0.05^{j}	33.83 ± 0.04^{g}	52.96 ± 0.04^{i}
B. cereus T4S	++	+	+++	+	+++	+	2.62 ± 0.02^{e}	30.54 ± 0.05^{e}	87.73 ± 0.05^{q}
P. lini BS27	++	+	+	+	++	+	3.07 ± 0.12^{hij}	30.62 ± 0.03^{e}	36.90 ± 0.02^{f}
S. indicatrix BOVIS40	++	+	+++	+	+++	+	2.10 ± 0.10^{a}	32.81 ± 0.18^{g}	77.33 ± 0.05^{n}
S. maltophilia JVB5	++	+	+++	+	+++	+	2.95 ± 0.05^{g}	32.20 ± 0.17^{g}	$79.81 \pm 0.17^{\circ}$
B. albus TSN29	++	+	+	+	+	+	3.50 ± 0.02^{k}	26.11 ± 0.03^{d}	80.50 ± 0.02^{p}
B. cereus BLBS20	+	+	+	+	+	+	2.50 ± 0.04^{d}	31.82 ± 0.14^{f}	0.53 ± 0.03^{a}
B. thuringiensis SFL02	+	+	+++	+	+	+	2.41 ± 0.01^{c}	26.48 ± 0.03^{c}	77.72 ± 0.04^{n}
S. maltophilia PK60	++	+	+++	+	+++	+	3.03 ± 0.06^{ghi}	33.48 ± 0.03^{g}	58.50 ± 0.02^{j}
B. subtilis VS52	++	+	+++	+	+	+	2.82 ± 0.01^{f}	33.43 ± 0.04^{g}	17.73 ± 0.04^{e}
B. thuringiensis BAAG44	++	+	+++	+	++	+	2.43 ± 0.03^{cd}	15.76 ± 0.21^{a}	70.12 ± 0.03^{m}
B. pseudomycoides SFS19	+	+	+++	+	+++	+	2.17 ± 0.03^{a}	27.64 ± 0.04^{d}	68.89 ± 0.09^{l}
B. toyonensis OLT2020	+	+	+	+	++	+	3.01 ± 0.02^{gh}	22.64 ± 0.03^{b}	5.72 ± 0.02^{b}
B. thuringiensis BSA123	++	+	++	+	++	+	2.79 ± 0.03^{f}	24.95 ± 0.04^{c}	64.92 ± 0.04^{k}
B. paramycoides LS11	++	+	++	+	+	+	2.80 ± 0.03^{f}	29.75 ± 0.22^{e}	44.53 ± 0.03^{g}
P. saponiphilia J4R	++	+	+	+	+	+	3.11 ± 0.02^{ij}	33.24 ± 0.05^{g}	12.91 ± 0.01^{c}
B. cereus VEJU7080	++	+	+++	+	+	+	2.66 ± 0.02^{e}	24.55 ± 0.04^{c}	46.10 ± 0.02^{h}
Pseudomonas sp. FOBS21	+	+	++	+	+	+	2.31 ± 0.02^{b}	31.32 ± 0.02^{b}	53.34 ± 0.04^{i}

IAA, indole-3-acetic acid; AM, ammonia; HCN, hydrogen cyanide; EPS, exopolysaccharide; %PS, percentage of phosphatase; %SDR, percentage of siderophore production. The values of triplicate readings represented as mean ± standard deviation with different alphabets down the column show a significant difference.

of soil-root microbes to established microbial biomass in the endosphere (Liu et al., 2021). Plants harbor diverse agriculturally important endophytic microbes with notable plant growth-promoting traits and their exploration has been proven efficient in enhancing crop yield (Alkahtani et al., 2020). The ability of endophytic microbes to withstand drought stress or climate-induced abiotic stress for plant survival and nutrition can suggest their future exploration as a suitable candidate for formulating bioinoculants for sustainable agriculture (Khalil et al., 2021). The presence of endophytic bacteria in host plants and their ability to synthesize growth hormones can significantly enhance seedling growth, development, and elongation of lateral roots and cell differentiation (Shahzad et al., 2017).

In this study, the combination of culturing and molecular techniques in the characterization of sunflower roots and stem-associated endophytic bacteria has been reported (Tiwari and Thakur, 2014; Bahmani et al., 2021; Shah et al., 2022). The biochemical characterization reflected the most identifiable Gram-positive compared to the Gram-negative bacterial isolates. The screened twenty bacterial isolates showed multifunctional PGP traits. The bacteria identified in this study showed similarities to the previous studies by Ambrosini et al. (2016) and Schmidt et al. (2021), who reported similar bacteria from sunflowers. A study by Forchetti et al. (2007) reported the isolation of *Bacillus* from sunflower plants. Recent genomics has revealed plant growth promotion and stress tolerance attributes of *Stenotrophomonas* strain 169 (Ulrich et al., 2021). The bacteria identified agreed with the findings of Ambrosini et al. (2012), who isolated Stenotrophomonas spp. and Pseudomonas spp. from the root of sunflower.

The ability of endophytic bacteria to solubilize phosphate was evident from the work of researchers (Khamwan et al., 2018; Sánchez-Cruz et al., 2019; Alkahtani et al., 2020). Several endophytic bacterial genera, such as *Stenotrophomonas, Bacillus,* and *Pseudomonas* have been reported as phosphate solubilizers (Pandey et al., 2013). In this study, all the bacterial isolates displayed phosphate-solubilizing traits. Hence, these bacteria with greater potential can be harnessed as bio-input in both present and future agriculture. Diverse phosphate-solubilizing endophytic bacteria have been reported to increase phosphate levels in the soil (Alkahtani et al., 2020; Varga et al., 2020). Shahid et al. (2015) reported phosphate-solubilizing endophytic *Bacillus* sp. Ps-5 and *Alcaligenes faecalis* Ss-2, contribute to the sunflower yield. The results obtained from this study corroborate with Pandey et al. (2013) and Vandana et al. (2021), who reported phosphate-solubilizing endophytic *Stenotrophomonas*, *Bacillus,* and *Pseudomonas* from the root of sunflower, and soybean. The phosphate solubilization potential of bacterial isolates might depend on suitable growth conditions, genetic make-up, and limited nutrient supply (Youseif, 2018). Furthermore, the results here compared to previous studies confirmed the phosphate-solubilizing potential of sunflower-associated endophytic bacteria with promises in ensuring the bioavailability of soluble minerals in soils for plant nutrition.

TABLE 4 | Qualitative screening of endophytic bacteria for enzyme production.

Isolate	Zone of clearance (mm)				
	AM	XL	PR	CL	MN
B. cereus SFR35	0.00 ± 00^{a}	0.00 ± 00^{a}	36.24 ± 0.40^{m}	44.03 ± 0.06^{a}	43.06 ± 0.05^{g}
B. wiedmannii FTL29	20.05 ± 0.05^{c}	40.02 ± 0.03^{f}	18.03 ± 0.03^{e}	47.06 ± 0.05^{b}	42.04 ± 0.04^{f}
Bacillus sp. CAL14	0.00 ± 00^{a}	0.00 ± 00^{a}	10.06 ± 0.06^{b}	59.05 ± 0.05^{g}	0.00 ± 00^{a}
B. cereus T4S	0.00 ± 00^{a}	41.02 ± 0.04^{g}	35.05 ± 0.04^{l}	57.02 ± 0.02^{e}	45.00 ± 0.01^{h}
P. lini BS27	0.00 ± 00^{a}	32.96 ± 0.06^{c}	30.00 ± 0.11^{i}	58.07 ± 0.11^{f}	18.02 ± 0.55^{b}
S. indicatrix BOVIS40	44.05 ± 0.05^{e}	35.04 ± 0.04^{e}	16.03 ± 0.02^{d}	50.10 ± 0.10^{d}	43.05 ± 0.05^{g}
S. maltophilia JVB5	0.00 ± 00^{a}	0.00 ± 00^{a}	25.06 ± 0.05^{d}	58.08 ± 0.11^{f}	0.00 ± 00^{a}
B. albus TSN29	0.00 ± 00^{a}	0.00 ± 00^{a}	0.00 ± 00^{a}	48.04 ± 0.05^{c}	54.04 ± 0.05^{k}
B. cereus BLBS20	0.00 ± 00^{a}	0.00 ± 00^{a}	0.00 ± 00^{a}	69.10 ± 0.10^{i}	0.00 ± 00^{a}
B. thuringiensis SFL02	0.00 ± 00^{a}	47.05 ± 0.05^{j}	0.00 ± 00^{a}	60.03 ± 0.03^{h}	30.05 ± 0.06^{e}
S. maltophilia PK60	18.04 ± 0.04^{b}	0.00 ± 00^{a}	31.01 ± 0.03^{j}	59.03 ± 0.06^{g}	18.04 ± 0.55^{b}
B. subtilis VS52	20.05 ± 0.04^{c}	35.04 ± 0.04^{d}	22.03 ± 0.06^{f}	59.02 ± 0.07^{g}	51.06 ± 0.06^{j}
B. thuringiensis BAAG44	0.00 ± 00^{a}	0.00 ± 00^{a}	30.06 ± 0.06^{i}	58.06 ± 0.06^{f}	18.02 ± 0.04^{b}
B. pseudomycoides SFS19	0.00 ± 00^{a}	0.00 ± 00^{a}	0.00 ± 00^{a}	58.02 ± 0.02^{f}	0.00 ± 00^{a}
B. toyonensis OLT2020	0.00 ± 00^{a}	43.05 ± 0.06^{h}	0.00 ± 00^{a}	60.03 ± 0.03^{h}	20.05 ± 0.05^{c}
B. thuringiensis BSA123	18.01 ± 0.05^{b}	40.06 ± 0.06^{f}	35.02 ± 0.03^{l}	47.04 ± 0.05^{b}	42.02 ± 0.04^{f}
B. paramycoides LS11	25.04 ± 0.04^{d}	0.00 ± 00^{a}	29.04 ± 0.04^{h}	58.04 ± 0.05^{f}	45.03 ± 0.04^{h}
P. saponiphilia J4R	0.00 ± 00^{a}	0.00 ± 00^{a}	33.10 ± 0.10^{l}	59.08 ± 0.10^{g}	18.06 ± 0.06^{b}
B. cereus VEJU7080	0.00 ± 00^{a}	32.03 ± 0.03^{b}	15.03 ± 0.05^{c}	48.04 ± 0.04^{c}	50.01 ± 0.02^{i}
Pseudomonas sp. FOBS21	0.00 ± 00^{a}	0.00 ± 00^{a}	32.03 ± 0.03^{k}	50.24 ± 0.40^{d}	25.02 ± 0.03^{d}

AM, amylase; CL, cellulose; XL, xylanase; MN, mannanase; PR, protease. The values of triplicate readings represented as mean ± standard deviation with different alphabets down the column show a significant difference.

Siderophore-producing microbes can protect plants by mitigating the effect of induced biotic and abiotic stresses (Ferreira et al., 2019). In this study, endophytic bacteria displayed varied siderophore production. The differences observed may be due to the bacterial viability and genetic make-up. Siderophore producing ability of endophytic bacteria associated with *Vitis vinifera* has been reported to increase mineral elements in the soil (Andreolli et al., 2016). Pourbabaee et al. (2018) reported the potential contribution of siderophore-producing bacteria to the growth and Fe ion concentration of sunflower under water stress.

The HCN production by bacteria can inhibit cell metabolism and electron transport chain, thus causing cell death. The HCN and siderophores production by endophytic bacteria can provide a competitive advantage by exploring them as biocontrol agents in plant disease suppressiveness (Igiehon et al., 2019). The results obtained in this study revealed HCN production by the endophytic bacteria. The ability of endophytic bacteria to produce ammonia with the underlining antibiosis activities has been reported (Khan et al., 2020). All the bacterial isolates produce ammonia and HCN, thus suggesting their possible use as a biocontrol agent. The HCN and ammonia production by the bacterial isolates conformed with the findings of Pandey et al. (2013) and Moin et al. (2020) who reported HCN, ammonia, and volatile antifungal metabolites biosynthesis by the endophytic bacterium *Pseudomonas* isolated from healthy sunflower plants. Additionally, the production of exopolysaccharides, signal molecules, multilayered cell wall structures, extracellular enzymes, and stress-resistant endospores by *Bacillus* spp., however, can contribute to their survival and ecological functions in diverse environments (Lyngwi et al., 2016).

Endophytic microbes can stand as a potential source of extracellular enzymes for industrial purposes due to catalytic activity, thermostability, low cost, organic substrates availability, etc. (Toghueo and Boyom, 2021). Screening of extracellular enzymes, such as cellulases, proteases, xylanases, chitinases, and xylanases from plant microbes has been documented (Alkahtani et al., 2020; Blibech et al., 2020). The substrate level and growth conditions may influence the enzyme production ability of the bacterial isolates in the growth medium (Yadav, 2017). With the biotechnological views, sunflower endophytic bacteria can be harnessed as a source of enzymes in the degradation of complex organic compounds and derivation of desirable bio-products.

IAA is considered the most important phytohormone that enhances plant root development and the rate of nutrient absorption for plant growth promotion (Ahmad et al., 2020). The ability of microbes to produce growth hormones can underline their multifunctional effects on improving agricultural productivity (Choudhury et al., 2021). Different bacterial species have been implicated in the synthesis of IAA depending on their ability to utilize the precursory substance L-tryptophan in the growth medium (Mustafa et al., 2018). The increase in the amount of IAA produced by the bacterial isolates in the IAA production medium (IPM) conformed with the findings

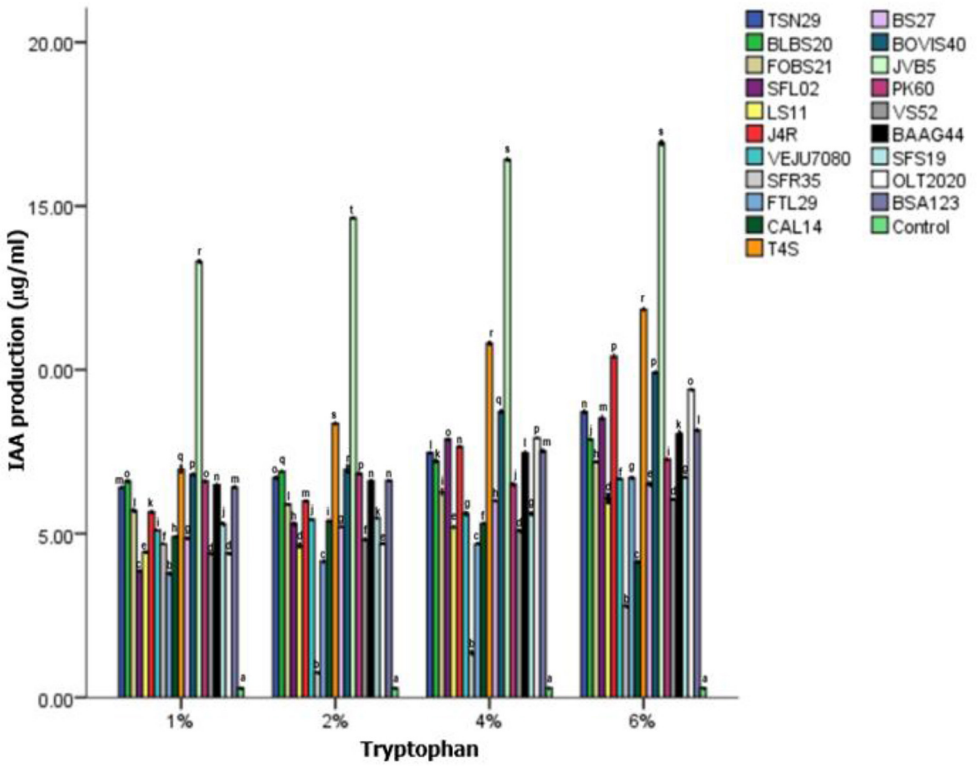

FIGURE 4 | Qualitative screening of endophytic bacteria for indole acetic acid production. Bacterial isolate codes are represented in **Table 2** and different alphabets indicate significant differences in triplicate readings. SFR35, *B. cereus*; FTL29, *B. wiedmannii*; CAL14, *Bacillus* sp.; T4S, *B. cereus*; BS27, *P. lini*; BOVIS40, *S. indicatrix*; JVB5, *S. maltophilia*; TSN29, *B. albus*; BLBS20, *B. cereus*; SFL02, *B. thuringiensis*; PK60, *S. maltophilia,* VS52, *B. subtilis,* BAAG44, *B. thuringiensis,* SFS19, *B. pseudomycoides,* OLT2020, *B. toyonensis,* BSA123, *B. thuringiensis,* LS11, *B. paramycoides,* J4R, *Pseud. saponiphilia*; VEJU7080, *B. cereus*; FOBS21, *Pseudomonas* sp.

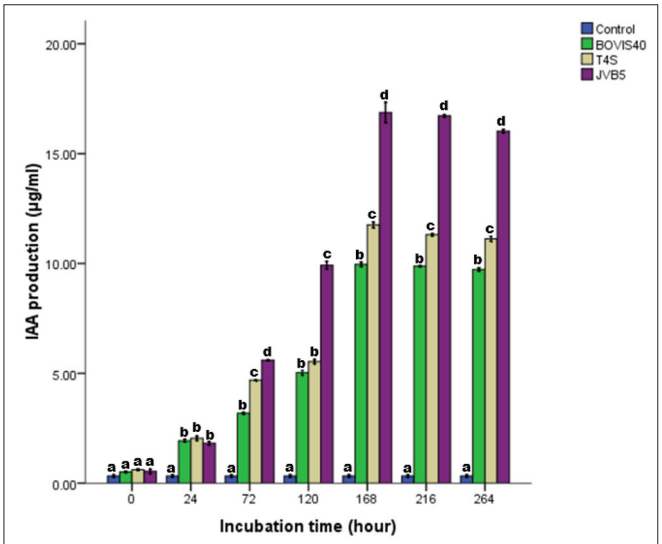

FIGURE 5 | IAA production by endophytic bacteria in the growth medium after 11 days of incubation. The different alphabets indicate significant differences in triplicate readings.

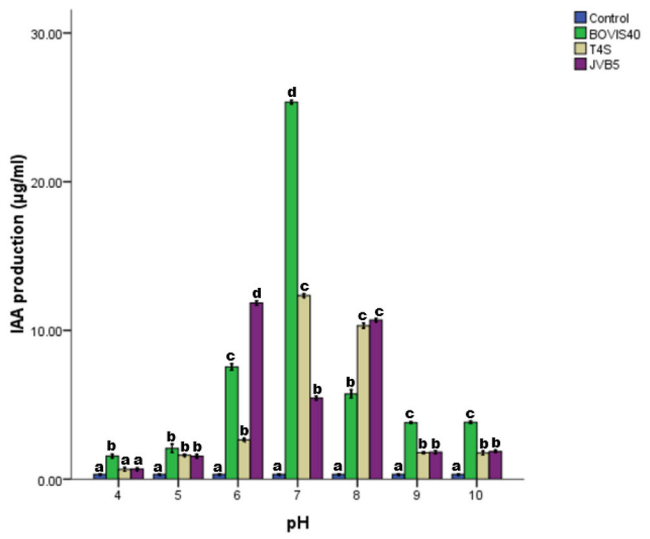

FIGURE 6 | Effect of pH on IAA production by endophytic bacteria. The different alphabets indicate significant differences in triplicate readings.

of Chukwuneme et al. (2020), who reported the enhancement of IAA production by the addition of tryptophan to the IPM. IAA production by *B. amyloliquefaciens* FZB42 in a tryptophan-dependent medium and its effect on plant growth promotion

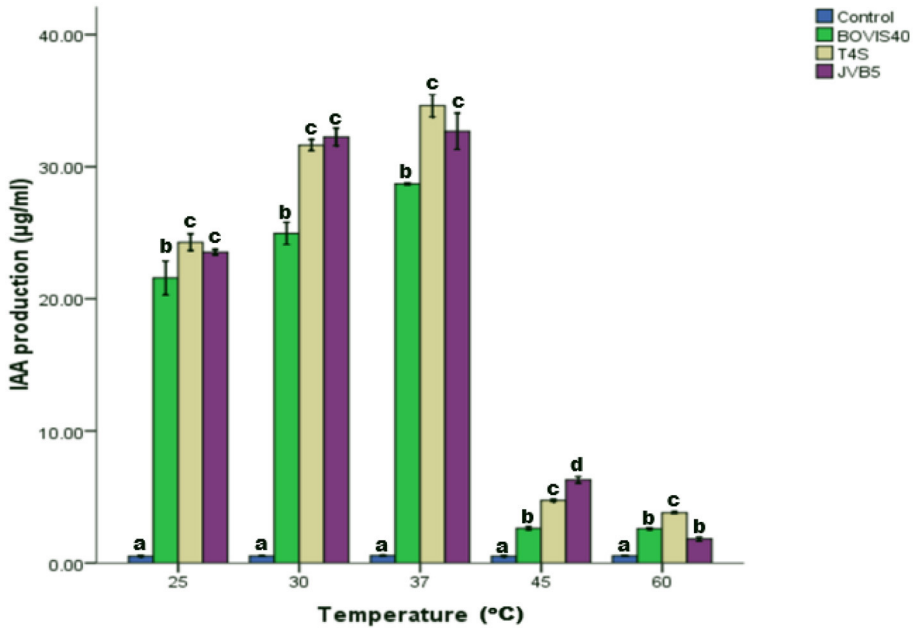

FIGURE 7 | Effect of temperature on IAA production by endophytic bacteria. The different alphabets indicate significant differences in triplicate readings.

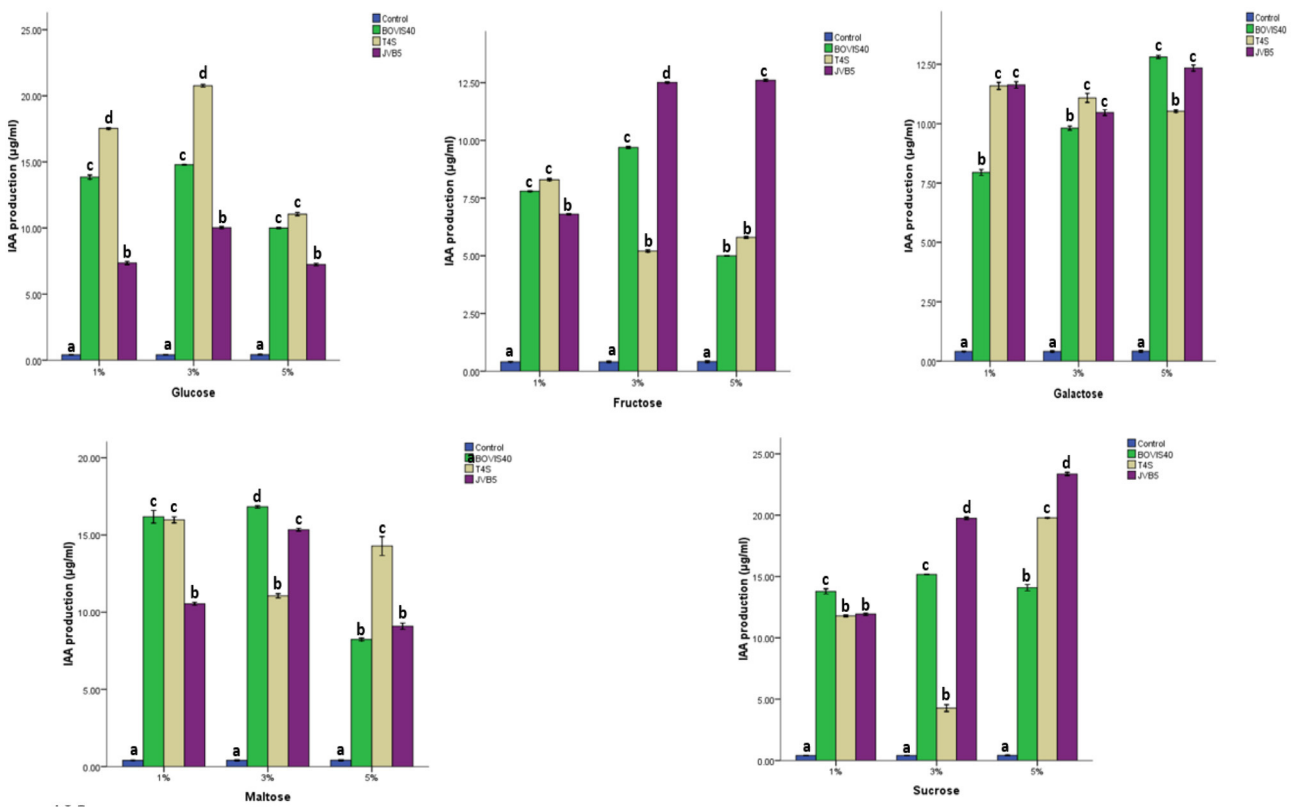

FIGURE 8 | Effect of different carbon source on IAA production by endophytic bacteria. The different alphabets indicate significant differences in triplicate readings.

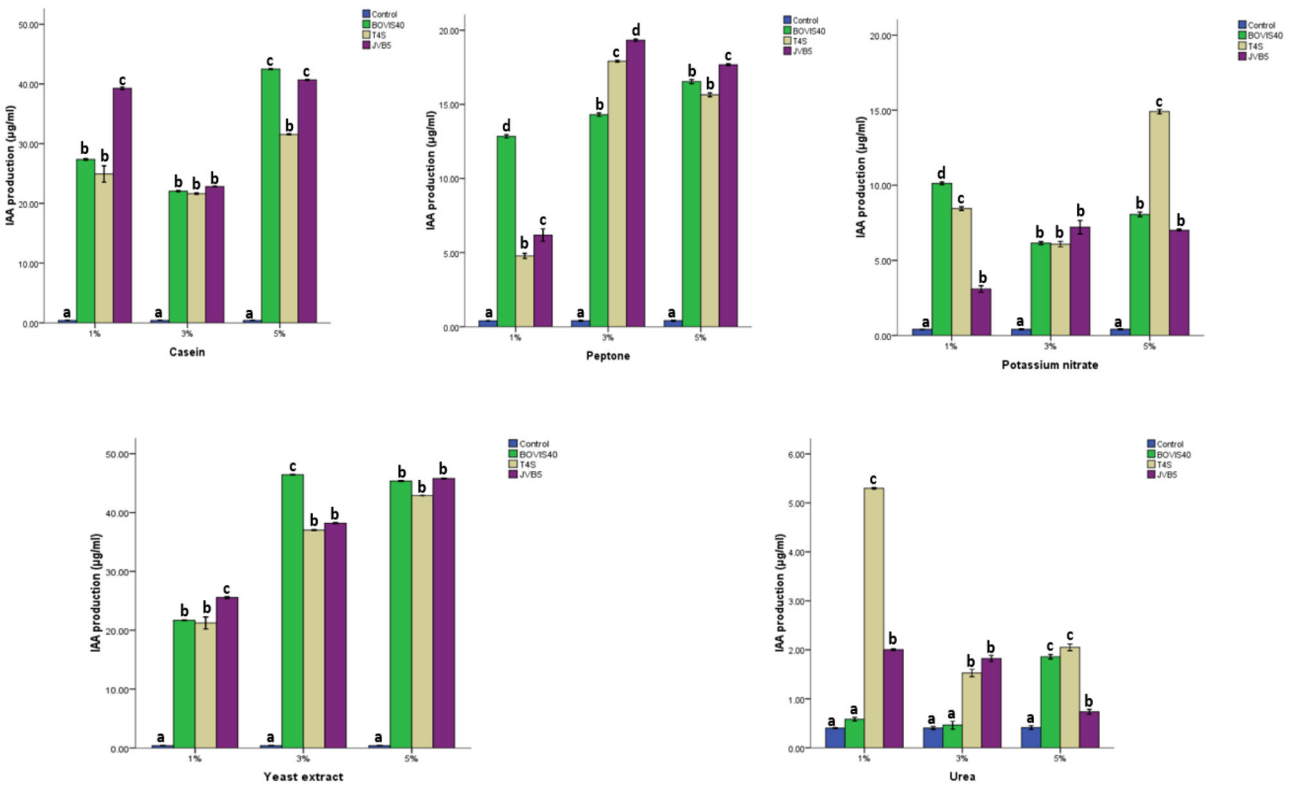

FIGURE 9 | Effect of different nitrogen sources on IAA production by endophytic bacteria. The different alphabets indicate significant differences in triplicate readings.

have been reported (Idris et al., 2007). The IAA potential displayed by the bacterial isolates corroborates the findings of Bashir et al. (2020), who reported IAA production by *Bacillus* spp. isolated from sunflowers. Furthermore, endophytes, *P. stutzeri, B. subtilis, S. maltophilia, B. cereus,* and *B. thuringiensis* native to the sunflower with IAA-producing potential have been reported to improve sunflower growth, seed germination, root elongation, and crop yield (Pandey et al., 2013; Singh et al., 2019).

Importantly, the time monitoring in a culture medium for metabolite biosynthesis is crucial in determining the biological activity of endophytic bacteria in the growth medium. Growing bacterial isolates in a medium amended with L-tryptophan as a precursor enhanced the IAA production based on their ability to utilize substrate in the medium through diverse IAA metabolic pathways (Hoseinzade et al., 2016). The geometric increase in IAA concentration with incubation time can be attributed to the ability of bacterial isolates to adjust and metabolize the substrate in the growing medium for maximum productivity. At low concentrations, a limited supply of substrate in the growth medium may affect the IAA-producing ability of the endophytic bacteria. In this study, a strong correlation between bacterial biomass and IAA production exists. A decrease in IAA concentration beyond the optimum level might be linked to the reduction in the amount of substrate or synthesis of lytic enzymes, such as IAA peroxidase and oxidase in the growing medium (Lebrazi et al., 2020). Nevertheless, bacterial growth under shaking conditions may influence IAA production due to agitation that allows the free flow of oxygen in the medium. Oxygen availability in the medium facilitates the conversion of tryptophan into auxins. Research findings on sunflower root endophytic bacteria and their optimization with incubation time have not been documented. The results obtained corroborate the conclusions of Myo et al. (2019) who reported IAA production of 82.36 µg/ml by *Streptomyces fradiae* NKZ-259 after 6 days of incubation. Interestingly, endophytic *Rhizobium* spp., *Bacillus subtilis* KA(1)5r and *Pseudomonas mosselii* with high IAA production at 216 and 96 h incubation have underlined their ability in promoting the growth of the medicinal herb *Aconitum heterophyllum* and wheat (*Triticum* spp.) (Emami et al., 2019; Lebrazi et al., 2020; Minakshi et al., 2020). Furthermore, IAA synthesis by actinomycetes in an IPM supplemented with suitable precursor L-tryptophan has been reported to occur *via* a tryptophan-dependent pathway or other similar pathways (Samaras et al., 2020).

The pH is an important factor that influences growth and microbial metabolism. At low or high pH, microbial activities may be affected (Alkahtani et al., 2020). Adjustment of pH in the growth medium to suitably favor bacterial growth can facilitate IAA biosynthesis. The differences observed in IAA production can be attributed to the pH of the medium and media composition (Widawati, 2020). A study by Myo et al. (2019) has reported maximum IAA production by *Pantoea glomerans* PVM,

TABLE 5 | Growth parameters of inoculated and un-inoculated sunflower seedlings.

Bacterial strain	Root length (mm)	Shoot length (mm)	Number of lateral roots	Fresh weight (g)	Dry weight (g)
Un-inoculated (control)	$16.00 \pm 0.0a^{a}$	22.03 ± 0.06^{b}	5.09 ± 0.19^{a}	0.14 ± 0.00^{b}	0.03 ± 0.00^{a}
S. indicatrix BOVIS40	25.00 ± 0.02^{c}	27.98 ± 0.08^{c}	10.17 ± 0.17^{c}	0.15 ± 0.00^{b}	0.06 ± 0.00^{a}
B. cereus T4S	19.66 ± 0.05^{b}	26.99 ± 0.02^{c}	11.99 ± 0.01^{c}	0.17 ± 0.00^{b}	0.04 ± 0.00^{a}
S. maltophilia JVB5	36.67 ± 0.02^{d}	24.66 ± 0.03^{a}	6.65 ± 0.05^{b}	0.18 ± 0.00^{b}	0.05 ± 0.00^{a}

Values are represented as means ± standard deviation in replicates. The different alphabets down the column show a significant difference.

Klebsiella pneumoniae K8, and *Streptomyces viridis* CMU-H009 at pH ranging between pH 7 and 8. Here, the results obtained were similar to the findings of Widawati (2020), who reported optimum IAA production by *B. siamensis* at pH 7 and 8, respectively. Furthermore, changes in the temperature of the growth medium may influence IAA synthesis. A study by Emami et al. (2019) reported optimum IAA production of 23.62 μg/ml by *Pseudomonas mosselii* isolated from the root of wheat at 32°C.

Different carbon sources amended in the IAA-producing medium can serve as energy sources to enhance the overall efficiency of recycling co-factor in the cells for IAA biosynthesis (Myo et al., 2019). The differences observed in the IAA production by the bacterial isolates can be attributed to the carbon source, concentration, and utilization of the substrate (Khan et al., 2020). Usually, a growing medium amended with monosaccharide sugar compared to di-or-polysaccharides can contribute to high IAA production based on the ability of endophytic microbes to assimilate. In addition, the utilization of monosaccharide sugars by most bacteria has been linked to high IAA production (Emami et al., 2019). However, the results from this study revealed high IAA concentration in IPM amended with sucrose, thus suggesting sucrose as a sole carbon source. The differences observed in the IAA concentrations may depend on the sugar source and the ability of the bacteria to utilize them for growth (Oliveira et al., 2021). An increase in IAA production in the IPM amended with sucrose agrees with the findings of Huu et al. (2015) and Payel et al. (2017), who reported an increase in IAA production by *B. subtilis* and *Pantoea agglomerans* on sucrose amended media. Also, results from this study corroborate the findings of Bharucha et al. (2013) who reported maximum IAA production by *P. putida* UB1 in a medium amended with sucrose. Lactose and glucose have also been reported as preferred sugars for maximum IAA production by *Enterobacter* sp. and *Rhizobium* (Basu and Ghosh, 2001; Nutaratat et al., 2017). Similarly, reports on maximum IAA production by root endophytic bacteria, such as *Rhizobium* P2, *Bacillus* spp., *Pantoea* spp., and *Pseudomonas mosselii* in a medium amended with sucrose, glucose, and maltose have been documented to enhance IAA production (Apine and Jadhav, 2011; Kucuk and Cevheri, 2016; Emami et al., 2019).

The addition of various nitrogen sources increased IAA production compared to the control medium. The soluble nitrogen source in the growth medium remains the key factor for bacterial growth and metabolite biosynthesis (Khan et al., 2017). The addition of various nitrogen sources to the IPM influences the rate of IAA production (Shahzad et al., 2017). Casein and yeast extract yielded high IAA production (>20 μg/ml) compared to other nitrogen sources. Like other parameters tested, varying concentrations of nitrogen source added to the growing medium can influence the amount of IAA biosynthesis (Emami et al., 2019). IAA production by rhizobacteria inhabiting the root of leguminous plants in a growing medium amended with glutamic acid and L-asparagine as a nitrogen source has been reported (Zhao et al., 2020). The results from this study corroborate the findings of Balaji et al. (2012), who reported yeast extract as the best nitrogen source for *Pseudomonas* species with an IAA concentration of 210 μg/ml. Also, Emami et al. (2019) reported IAA production of 23.66 μg/ml by *Pseudomonas* in a yeast extract amended medium. Furthermore, the addition of tryptone, beef extract, and peptone with varied IAA production can contribute to the bacterial lifestyle in the synthesis of phytohormones (Widawati, 2020).

In this study, endophytic bacteria with promising phytostimulant activities, i.e., *B. cereus* T4S, *S. maltophilia* JVB5, and *S. indicatrix* BOVIS40 were selected and their *in vitro* effect was assessed on sunflower seedlings growth. Inoculation of *Sesbania aculeate, Brassica campestris, Vigna radiate,* and *Pennisetum americanum* with endophytic bacteria *Azotobacter* spp., *Bacillus* spp., *Azospirillum brasilense,* and *Pseudomonas putida*, which increase adventitious root development, shoot, root length, and chlorophyll pigmentation has experimented (Khan Latif et al., 2016). The observed variation in the weight, root, and shoot length of rice and maize inoculated with *Bacillus* and *Pseudomonas* has been presumed to be influenced by IAA production (Karnwal, 2017, 2018). Most *Bacillus* spp. isolated from the root endosphere has been implicated in nitrogen fixation in legumes with a positive influence on seedling's growth (Bertani et al., 2016). An increase in rice shoot, root, and leaf length due to phytohormone production by the bacterial isolates contributes to crop production. Furthermore, the rooting potential of *Agrobacterium rhizogenes* in jujube's root has been reported (Lebrazi et al., 2020).

CONCLUSIONS

This study provides information on the *in vitro* screening of endophytic bacteria associated with sunflower. The PGP traits, such as IAA, ammonia production, exopolysaccharide, hydrogen cyanide, siderophore, and enzyme production exhibited by the endophytic bacteria, can underline their potential in plant growth promotion and protection from the biotic and abiotic stresses. The IAA production by the identifiable endophytic bacteria can contribute to sunflower rooting for nutrient and water absorption from the soil. The significant differences observed

in the inoculated sunflower seedling compared to un-inoculated showed the tendencies of these bacteria in plant growth promotion. Hence, based on the high siderophore potential of *B. cereus* T4S among the screened bacterial isolates with multifunctional attributes, this bacterium can be explored for sunflower cultivation.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI (accession: MW265413-MW265431 and MW261910).

AUTHOR CONTRIBUTIONS

BSA, ASA, and OOB designed the study. BSA managed the literature searches, carried out the laboratory work, interpreted the results, wrote the first draft of the manuscript, and revised and formatted the manuscript. ASA assisted in the result analysis and review of various drafts. OOB provided academic input, thoroughly critiqued the manuscript, proofread the draft, and secured funds for the research. All authors approved the article for publication.

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