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Production of indole-3-acetic acid by *Bacillus circulans* E9 in a low-cost medium in a bioreactor

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***Bacillus circulans* E9 (now known as *Niallia circulans*) promotes plant growth-producing indole-3-acetic acid (IAA), showing potential for use as a biofertilizer. In this work, the use of a low-cost medium containing industrial substrates, soybean, pea flour, Solulys, Pharmamedia, yeast extract, and sodium chloride (NaCl), was evaluated as a substitute for microbiological Luria Broth (LB) medium for the growth of *B. circulans* E9 and the production of IAA. In Erlenmeyer flasks with pea fluor medium (PYM), the maximum production of IAA was 7.81 ± 0.16 μg mL^{-1}, while in microbiological LB medium, it was 3.73 ± 0.15 μg mL^{-1}. In addition, an oxygen transfer rate (OTR) of 1.04 kg O_2 m^{-3} d^{-1} allowed the highest bacterial growth (19.3 ± 2.18 × 10^{10} CFU mL^{-1}) and IAA production (10.7 μg mL^{-1}). Consequently, the OTR value from the flask experiments was used to define the conditions for the operation of a 1 L stirred tank bioreactor. The growth and IAA production of *B. circulans* cultured in a bioreactor with PYM medium were higher (8 and 1.6 times, respectively) than those of bacteria cultured in Erlenmeyer flasks. IAA produced in a bioreactor by *B. circulans* was shown to induce the root system in *Arabidopsis thaliana*, similar to synthetic IAA. The results of this study demonstrate that PYM medium may be able to be used for the mass production of *B. circulans* E9 in bioreactors, increasing both bacterial growth and IAA production. This low-cost medium has the potential to be employed to grow other IAA-producing bacterial species.**

[**Key words:** Stirred tank bioreactor; Oxygen transfer rate; Indole-3-acetic acid; Low-cost industrial substrates; *Arabidopsis thaliana*]

Synthetic fertilizers are used to increase the yield and quality of crops (1,2). However, their excessive use in fields causes a reduction in soil pH and increases the contents of heavy metals, contributing to soil degradation (3,4). To solve this problem, there is a need to reduce the excessive use of synthetic fertilizers, replacing them with environmentally friendly technologies.

Biofertilizers are considered clean and sustainable, and they are formulated using one or more microorganisms that provide nutrients to crops and promote plant growth (5). Plant growth-promoting rhizobacteria (PGPR) colonize plant roots and enhance plant growth (5–7). PGPR can promote nitrogen fixation, solubilize phosphate, and produce siderophores and plant growth regulators such as cytokinins, gibberellic acid, abscisic acid, and indole-3-acetic acid (IAA). In addition, PGPR are beneficial for the control of plant pathogens by producing antibiotics and inducing systemic resistance mechanisms in plants (8,9).

IAA produced by PGPR stimulates cell division and elongation of plant tissues, and it also improves nutrient and water uptake, which enhance the quality and yield of economically important crops. PGPR genera that produce IAA include *Erwinia*, *Acinetobacter*, *Azospirillum*, *Enterobacter*, *Pseudomonas*, and *Bacillus* (10–12). *Bacillus* species produce a wide range of bioactive molecules with antimicrobial activity, low toxicity to humans, and high biodegradability (13,14). Additionally, members of this genus form spores that can withstand dry conditions, high temperatures, and UV radiation, which are desirable characteristics for these bacteria to be considered for the formulation of biofertilizers (15,16). *Bacillus circulans* E9 (now known as *Niallia circulans*) (17) is a strain isolated from *Solanum lycopersicum* in Sinaloa, Mexico. This strain is remarkable for its high IAA yield when grown in Luria Broth (LB). For the mass production of *B. circulans* or any other microorganism in a bioreactor, it is necessary to select a low-cost medium formulated with economical fermentation substrates that are capable of providing the essential nutrients for bacterial growth and maintaining their biological activity (18,19).

The stirred tank bioreactor is the configuration most commonly used to grow bacteria (20,21). For the massive growth of bacteria, a high oxygen uptake rate (OUR) is required, and the lack of appropriate oxygenation may become a limiting factor for the product concentration, yield, and volumetric productivity in bioreactors. Particular attention is required to establish appropriate operating conditions. These conditions require that the oxygen transfer rate (OTR) satisfies the bacterial OUR to achieve satisfactory growth of bacteria. Previous studies have reported the appropriate operational conditions of stirred tank bioreactors for bacterial growth (22–24). However, there are limited studies on media formulations employing low-cost industrial substrates and oxygen conditions for

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bacterial growth and IAA production in bioreactors. Therefore, the objectives of this work were (i) to define a medium that uses low-cost industrial substrates and (ii) to establish the operating conditions for a stirred tank bioreactor for the growth of *B. circulans* E9 and production of IAA.

MATERIALS AND METHODS

Fermentation substrates and biological material Six agroindustry substrates currently used in fermentation technology were selected: soybean flour (Archer Daniels Midland Company, Chicago, IL, USA), pea flour (Roquette, Lestrem, France), Solulys (Roquette), Pharmamedia (Archer Daniels Midland Company), yeast extract (Roquette) and NaCl (Roquette). Samples of these substrates were kindly provided by Biologist Roberto Gutiérrez from Fermic Mexico S.A. de C.V..

The *B. circulans* E9 used in this study was from the culture collection of Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional-Unidad Sinaloa (CIIDIR Sinaloa-IPN), Sinaloa, Mexico. The strain was isolated from *S. lycopersicum* rhizospheres and was selected because it produces a high content of IAA. The stock culture was maintained by cryopreservation in glycerol stock at 20 % (v/v) at −80 °C and subcultured on microbiological LB medium with agar (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 5 g L^{-1} NaCl and 15 g L^{-1} agar, Sigma−Aldrich, St. Louis, MO, USA). Stock cultures were stored at 4 °C and subcultured at least every 24 h before required.

Media formulation Considering the composition of microbiological LB medium (Sigma−Aldrich) in g L^{-1}: tryptone, 10; yeast extract, 5; NaCl, 5, as a reference, the media prepared with the low-cost industrial substrates in g L^{-1} were SYM (soybean flour, 10; yeast extract, 5; NaCl, 5); SolYM (Solulys, 10; yeast extract, 5; NaCl; 5); PhYM (Pharmamedia, 10; yeast extract, 5; NaCl, 5); and PYM (pea flour, 10; yeast extract, 5; NaCl, 5). The cost of each medium was calculated in dollars L^{-1} and compared with the cost of LB microbiological medium. The cost of preparing a liter of LB microbiological medium was 3.6 US dollars, but with the use of the low-cost industrial substrates, the cost was considerably reduced: PhYM medium by 25.71 times, SolYM medium by 24 times, PYM medium by 20 times, and SYM medium by 22 times (data not shown).

In all experiments, a cell suspension of *B. circulans* E9 was prepared from cultures in Petri dishes with LB medium and agar. After 24 h growth, a single colony was transferred to 5 mL of LB medium and incubated at 28 °C for 24 h at 200 rpm to obtain the pre-inoculum bacterial suspension. The pre-inoculum (1 mL) suspension was used to inoculate Erlenmeyer flasks. For stirred tank bioreactor, bacterial pre-inoculum was prepared in 100 mL Erlenmeyer flasks and incubated at 28 °C for 9 h at 200 rpm in order to obtain the inoculum in the exponential growth phase. Cell concentration in the inoculum was adjusted to 1.0 absorbance unit with a spectrophotometer, which corresponded to 10^8 colony forming units per milliliter (CFU mL^{-1}) (23). *B. circulans* E9 were cultured in 500 mL Erlenmeyer flasks with 100 mL each of SYM, SolYM, PhYM, PYM and microbiological LB media. Previously, the media were autoclaved for 15 min at 121 °C and 15 psi. The cultures were incubated on a rotary shaker (Infors AG, Bottmingen, Switzerland) at a temperature of 28 °C and 200 rpm. Cell growth and IAA production were measured from 100 mL of each culture growing in 500 mL Erlenmeyer flasks after 12 h of incubation. Three independent experiments were performed and similar value was obtained in the three experiments.

The cell growth measurement was carried out by quantifying CFU mL^{-1} according to the methodology of Sieuwerts et al. (25). Culture broth aliquots were diluted and plated on LB agar Petri dishes. The Petri dishes were incubated at 28 °C for 24 h, and viable plate counts were obtained on a colony counter (SOB-Q-20, Solbat, Mexico City, Mexico).

IAA determination Determination of IAA production was performed using the colorimetric method based on Salkowsky reagent (26,27) with a modified composition of replacing perchloric acid with sulfuric acid (28). IAA produced by *B. circulans* E9 in media prepared with the low-cost industrial substrates and microbiological LB medium was centrifuged at 10,000 rpm for 15 min at room temperature. The Salkowsky technique was adapted to a 96-well microplate format by adding 100 µL of the bacterial supernatant (BS) and 100 µL of Salkowsky reagent, and the samples were kept in the dark to allow color formation. The optical density at 530 nm was recorded after 30 min using a microplate reader (Microplate Reader, BioRad, Hercules, CA, USA). IAA was determined using three replicates for each treatment. Serial dilutions (5, 10, 15, 20, 25 and 30 µg mL^{-1}) of IAA (Sigma−Aldrich) were prepared in distilled water, and the constructed standard curve had a correlation value $R^2 = 0.9965$. Three independent experiments were performed and similar trends were obtained in the three experiments.

Growth kinetics of *B. circulans* in Erlenmeyer flasks *B. circulans* E9 were grown in 500 mL Erlenmeyer flasks containing 100 mL of LB and PYM media. Previously, inoculated as described above. Cultures were carried out at 200 rpm and 28 °C in a rotary shaker (Infors AG) for 22 h. One milliliter aliquots were taken to evaluate cell growth and IAA production. The experiment was performed in three independent experiments.

Cell growth of *B. circulans* E9 was evaluated every hour by taking serial dilutions of each preparation and spreading them on LB agar (Sigma−Aldrich) in Petri dishes, with three replicates. The Petri dishes were incubated at 28 °C for 24 h and viable cells counts were measured on a colony counter (SOB-Q-20, Solbat). Finally, CFU mL^{-1} and IAA were determined as described above.

Effect of the OTR on the *B. circulans* E9 culture For the OTR analysis, *B. circulans* E9 was grown in 500 mL Erlenmeyer flasks with 100 mL of PYM medium and three different closures: aluminum foil, cotton plugs and silicone foam (Sigma−Aldrich) according to the methodology by Orozco-Sánchez et al. (29). The medium was autoclaved for 15 min at 121 °C and 15 psi. The Erlenmeyer flasks were inoculated used as a fresh inoculum as describe above. The cultures were incubated on a rotary shaker at 200 rpm and 28 °C. Cell growth and IAA were measured after 12 h of incubation, and three independent experiments were performed for each OTR condition.

Bioreactor culture Considering that the maximal growth of *B. circulans* E9 and production of IAA were obtained in an Erlenmeyer flask with PYM media and an OTR value of 1.04 kg O_2 m^{-3} d^{-1}, *B. circulans* E9 was cultured in a 2 L stirred tank bioreactor (Applikon Biotechnology, Delft, Netherlands) using conditions to generate an OTR value similar to that obtained in the Erlenmeyer flask. The bioreactor was operated with 1 L of PYM media and stirred with a 50 mm diameter Rushton impeller at 400 rpm with a 0.1 vvm air supply, and the temperature was maintained at 28 ± 2 °C. Inoculum preparation of *B. circulans* E9 was performed in a 500 mL Erlenmeyer flask with 100 mL of PYM medium as described above. Two milliliters of culture broth were taken every 1 h to estimate cell growth and IAA production. The pH and dissolved oxygen tension (DOT) were measured using electrodes connected to an in-Control controller (Applikon Biotechnology).

The kinetic parameters determined were the doubling time (T_d) and maximum specific growth rate (μ_{max}). The parameters were calculated following the equation described by de Carvalho et al. (30).

Determination of the mass transfer coefficient (K_La) was carried out by dynamic gassing out (31). This method consists of determining the dynamic oxygen balance during the absorption and desorption of oxygen in the culture. The OTR and OUR (kg O_2 m^{-3} d^{-1}) in the stirred tank bioreactor were calculated according to the equations reported by Orozco-Sánchez et al. (29). Oxygen balance was calculated to determine whether the oxygen supply was sufficient to satisfy the demand of the bacterial culture. Three replications of each treatment were conducted and the experiment was repeated twice.

IAA activity assay for plant growth promotion in *Arabidopsis thaliana* *Arabidopsis thaliana* seeds were disinfected with 70% ethanol three times for 1 min, followed by 2% sodium hypochlorite for 1 min, and were washed three times with distilled water for 2 min. Seed dormancy was interrupted to promote germination according to Bruno et al. (32). Seeds were transferred to Petri dishes containing Murashige and Skoog (MS) medium (Sigma−Aldrich) (33) and were incubated in a vertical position at 20 ± 2 °C and 60% relative humidity in growth chambers with a photoperiod of 16 h light: 8 h darkness until cotyledon formation. The cotyledon plants were transferred to Petri dishes, and the experiment consisted of four treatments: (i) MS medium (33), (ii) MS medium supplemented with the components of PYM medium, (iii) MS medium supplemented with 10.6 µM IAA (Sigma−Aldrich), and (iv) MS medium supplemented with 10.6 µM IAA from cell-free medium of *B. circulans* E9. For each treatment, excess moisture was removed to prevent contamination problems. The length of the primary root and the total number of lateral roots were monitored every 24 h for 6 consecutive days. Root hair length and root hair density were assessed using a stereomicroscope (Olympus SZX7, Olympus, Hamburg, Germany) with an attached digital camera (Cool PIX B500, Nikon, Tokyo, Japan), and image analysis was performed using ImageJ editing software (ImageJ, version 1.8.0_112). Root hair density was determined as the number of hairs in a 1 mm root segment. Nine individual plants were used for each treatment and the experiments were repeated twice.

Statistical analysis The data were processed to obtain the central tendency measurements (means and standard deviations). Significant differences in the IAA concentration obtained for each substrate and from the biological assay performed in *A. thaliana* were analyzed by one-way analysis of variance (ANOVA) and Tukey's post hoc test ($P < 0.05$). The differences in the kinetic growth and IAA content of *B. circulans* E9 grown in LB and PYM media in Erlenmeyer flasks and a bioreactor were analyzed using Student's *t*-test, with a significance value of $P < 0.05$. All the data were checked for normality using Shapiro−Wilk's test before statistical analysis. All statistical analyses were performed using the statistical software SPSS for Windows, version 24.0 (IBM Corp., Armonk, NY, USA).

RESULTS

IAA production using media with low-cost industrial substrates The highest concentrations of IAA were obtained in PhYM and PYM media (6.37 ± 0.25 and 7.81 ± 0.16 µg mL^{-1}, respectively). These values were 1.79- and 2.1-fold higher than those obtained from microbiological LB and SYM media (Fig. 1).

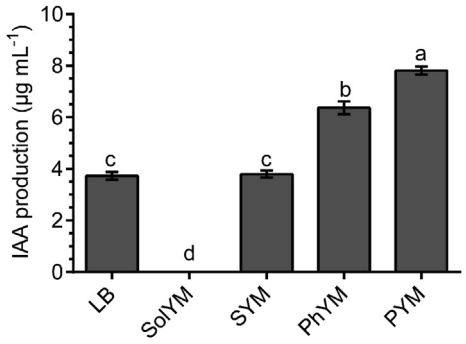

FIG. 1. IAA production by *Bacillus circulans* E9 in microbiological LB and low-cost media in an Erlenmeyer flask. The media prepared with low-cost industrial substrates in g L^{-1} were: SYM (soybean flour, 10; yeast extract, 5; NaCl, 5); SolYM (Solulys, 10; yeast extract, 5; NaCl; 5); PhYM (Pharmamedia, 10; yeast extract, 5; NaCl, 5); PYM (pea flour, 10; yeast extract, 5; NaCl, 5). The bars represent the mean ± standard deviation of three individual replicates. Different letters indicate a significant difference according to the Tukey's test ($P < 0.05$).

The concentration of IAA produced in SYM medium was not different from that produced in LB medium. Finally, IAA was not detected in SolYM medium (Fig. 1). The high concentration of IAA produced by *B. circulans* E9 in PYM media (Fig. 1) was associated with the high content of aromatic amino acids in the low-cost industrial substrates (Table S1). The total content of aromatic amino acids in the PYM and SYM media was 3.31- and 1.45-fold higher than that in microbiological LB medium, respectively. In contrast, the total aromatic amino acid content in SolYM was 3.07 times lower than that in microbiological LB medium (Table S1). PYM medium was selected for further study considering that it led to the highest production of IAA and that its cost was 20 times lower than that of microbiological LB medium.

Growth kinetics and IAA production of *B. circulans* E9 cultured in Erlenmeyer flasks with PYM medium The growth of *B. circulans* E9 in PYM medium was similar to that obtained in microbiological LB medium (Fig. 2). The bacterial growth rates (μ_{max}) in PYM and LB media were 1.20 h^{-1} and 1.03 h^{-1}, respectively. The maximum cell growth was similar between PYM (15.5 ± 2.31 × 10^{10} CFU mL^{-1}) and LB media (14.1 ± 5.62 × 10^{10} CFU mL^{-1}). *B. circulans* E9 growing in the LB media did not present a stationary phase of growth. After reaching its maximum growth (10 h), it began its cell death phase, while the cell growth of *B. circulans* E9 cultured in PYM medium presented a stationary phase (9–14 h) and then the cell death phase. However, with both medium, the lowest cell growth was observed at 22 h (Fig. 2).

IAA accumulation showed a similar profile in PYM and microbiological LB media until 10 h of culture. Similar maximal IAA concentrations were obtained in PYM and LB media, 5.87 ± 0.35 and 5.65 ± 0.4 µg mL^{-1}, respectively. The production of IAA was directly associated with cell growth (Fig. 2). The accumulation of IAA in PYM medium decreased after 10 h of culture until it was not detected after 16 h, while in microbiological LB medium, IAA was only reduced to 3.7 ± 0.44 µg mL^{-1} (Fig. 2). As shown in Fig. 2, IAA profile is consistent with the grown profile of *B. circulans* E9 in PYM medium and it is possible that the degradation of IAA is related with a decrease in carbon and nitrogen sources, that induces the catabolism in order to obtain energy for growth. However, more studies are needed to prove this hypothesis.

Effect of the OTR on the *B. circulans* E9 culture using different closures of Erlenmeyer flasks The increase in the OTR generated in Erlenmeyer flask cultures led to a progressive increase in *B. circulans* cell growth and IAA production (Table 1). Erlenmeyer flasks covered with an aluminum closure led to lower cell growth (15.3 ± 0.75 × 10^{10} CFU mL^{-1}). Cultures covered with cotton led to significantly increased cell growth, 16.9 ± 1.55 × 10^{10} CFU mL^{-1}. Notably, the *B. circulans* E9 culture covered with a silicone foam closure led to the highest cell growth (19.3 ± 2.18 × 10^{10} CFU mL^{-1}). The OTR generated in flasks with silicone foam

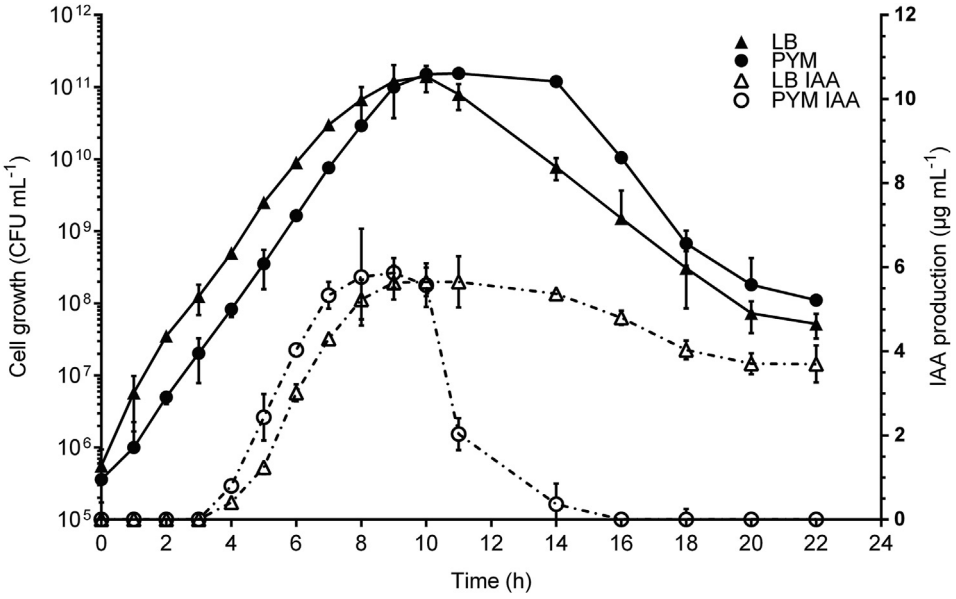

FIG. 2. Production of IAA (dashed lines) and cell growth of *Bacillus circulans* E9 (solid lines) cultured in microbiological LB and PYM media in Erlenmeyer flasks; closed triangles represent cell growth in microbiological LB medium; closed circles represent cell growth in PYM medium; open triangles represent IAA production in microbiological LB medium, and open circles represent IAA production in PYM medium. The cell growth and IAA production values are presented as the mean ± standard deviation of three individual replicates.

closures was the highest and increased cell growth by 1.26- and 1.14-fold compared to that in cultures grown in flasks with aluminum and cotton closures, respectively (Table 1).

IAA production by cultures grown in flasks with silicone foam closures was 1.32- and 1.56-fold higher than that by cultures grown in flasks with cotton and aluminum closure cultures, respectively (Table 1). The IAA yield was not different between cultures grown in flasks with aluminum and cotton closures (0.447 ± 0.068 and 0.481 ± 0.004 μg CFU^{-1}, respectively). Considering these results, an OTR value of 1.04 kg O2 m^{-3} d^{-1} was selected to grow *B. circulans* E9 in a stirred tank bioreactor.

Growth kinetics of *B. circulans* E9 in a bioreactor using PYM medium Fig. 3 shows growth kinetics of *B. circulans* in a bioreactor using PYM medium. The pH of PYM medium was 6.7 for 3 h at the beginning of fermentation. However, the pH decreased progressively to 5.8 by the end of fermentation (Fig. 3A). The DOT value decreased from 100% to 20% during the exponential phase (3–8 h), but at the end of fermentation, the DOT value was restored to 100% (Fig. 3A).

B. circulans E9 grew with a maximum growth rate of 1.81 h^{-1} and reached a maximal cell growth of 1.12 ± 0.424 × 10^{12} CFU mL^{-1} after 8 h (Fig. 3B), while IAA production was associated with the cell growth profile and reached a concentration of 6.93 ± 0.43 μg mL^{-1} at 10 h (Fig. 3B). During the exponential growth phase, the pH and DOT values decreased.

The kinetic parameters of *B. circulans* grown in an Erlenmeyer flask and a stirred tank bioreactor were determined (Table S2). The growth rate (μ_{max}) and IAA concentration of *B. circulans* grown in the bioreactor were higher than those obtained from *B. circulans* grown in an Erlenmeyer flask. The OTR value in the bioreactor was 0.5 times higher than that in an Erlenmeyer flask. *B. circulans* E9 cultured in the bioreactor had an OTR value three times higher than the OUR. Consequently, bacterial growth was not limited by oxygen. The OUR value of the *B. circulans* culture in an Erlenmeyer flask was not determined.

IAA produced by *B. circulans* E9 in a bioreactor induces root development in *A. thaliana* The effects of IAA produced by *B. circulans* in a bioreactor on the length of primary roots and the lateral density roots of *A. thaliana* were evaluated (Fig. 4). The length of primary roots of plants treated with BS was reduced by 1.64 times compared to that of plants grown in MS (Fig. 4A), while the length of primary roots of plants treated with commercial IAA decreased by 3.95 times (Fig. 4A). As expected, no effect on the length of primary roots was observed for plants treated with the components of PYM medium (Fig. 4A).

Lateral root density of *A. thaliana* was increased by treatment with MS+IAA and MS+BS (2.94 and 2.17 times, respectively) in comparison to that of control plants (MS). Interestingly, no differences were observed between the MS+IAA and MS+SB treatments (Fig. 4B).

Morphological images of *A. thaliana* roots treated with MS (Fig. 4C) and MS supplemented with the components of PYM (Fig. 4D) showed less lateral root formation than that of plants treated with commercial IAA (Fig. 4E) and with BS (Fig. 4F).

Root hair length increased significantly in *A. thaliana* plants exposed to MS+IAA and MS+BS (5.23 and 4.22 times, respectively)

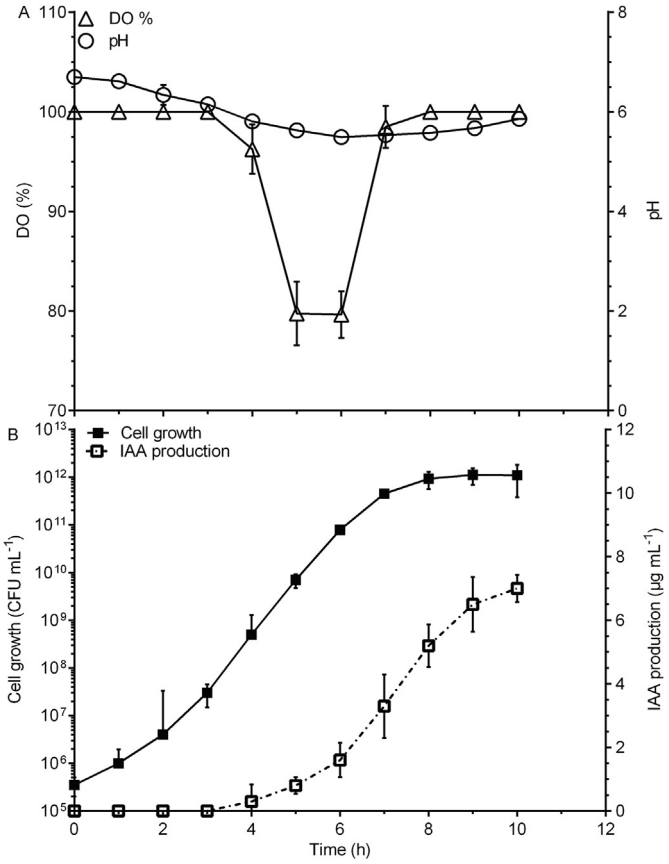

FIG. 3. Growth kinetics of *Bacillus circulans* E9 in a bioreactor using PYM medium. (A) DOT (open triangles) and pH (open circles) . (B) Cell growth (closed squares) and IAA production(open squares). The values are presented as the mean ± standard deviation of three individual replicates.

in comparison to that of control plants (MS; Fig. 5A). Consistently, the number of root hairs increased by 3.85 and 2.88 times in plants treated with SB and commercial IAA, respectively, compared with that of control plants (MS; Fig. 5B). However, for plants treated with commercial IAA, the length and the number of root hairs were higher than those for plants treated with BS (Fig. 5A and B).

Image analysis showed lower values of the length and number of root hairs in *A. thaliana* control plants (MS; Fig. 5C) and plants treated with MS supplemented with the components of PYM medium (Fig. 5D). For plants treated with commercial IAA (Fig. 5E) and BS (Fig. 5F), root hair length and density were stimulated. Taken together, these results indicate that IAA produced by *B. circulans* E9 grown in PYM medium in a bioreactor could have similar biological activity to that of commercial IAA.

DISCUSSION

The excessive use of chemical fertilizers and pesticides leads to a large number of environmental problems that directly affect crop productivity and human health (1,2,34,35).

TABLE 1. Effect of OTR generated by closure type on cell growth, IAA production, and on IAA yield of *Bacillus circulans* E9 in the PYM medium at Erlenmeyer flask.

Closure type	OTR (kg O_2 m^{-3} d^{-1})	Cell growth (10^{10} CFU mL^{-1})	IAA Production (μg mL^{-1})	IAA yield bacteriā[1] (μg CFŪ[1])
Aluminum	0.07 ± 0.00[c]	15.3 ± 0.75[c]	6.88 ± 1.37[c]	0.447 ± 0.068[b]
Cotton	0.58 ± 0.01[b]	16.9 ± 1.55[b]	8.13 ± 0.68[b]	0.481 ± 0.004[b]
Silicone	1.04 ± 0.03[a]	19.3 ± 2.18[a]	10.7 ± 0.35[a]	0.558 ± 0.045[a]

Data for each parameter (OTR, cell growth, IAA production and yield) represent mean ± standard deviations of three individual replicates. Values in the same column carrying different letters indicate a significant difference according to Tukey's test ($P < 0.05$). OTR, oxygen transfer rate; IAA, indole-3-acetic acid.

The use of microorganisms as biofertilizers has been considered an alternative to avoid the excessive use of synthetic fertilizers. However, it is necessary to establish the conditions for bacterial mass production in bioreactors and to use low-cost industrial substrates. In this work, we observed that the growth of *B. circulans* E9 and production of IAA in PYM medium formulated with low-cost industrial substrates (pea flour and yeast extract) were similar to those in microbiological LB medium. The highest bacterial growth achieved with PYM medium ($15.5 \pm 2.31 \times 10^{10}$ CFU mL^{-1}) suggested that this industrial substrate promoted cell growth influenced by the ability of microorganism to use the available carbon and nitrogen sources, similarly to other bacterial species (12,36,37). This idea supports the possibility to selecting the PYM medium as low-cost nutrient sources replacing microbiological LB to *B. circulans* E9 growth and IAA production. Similarly, several authors have proposed that the use of low-cost industrial substrates offers the possibility to replacing microbiological media as strategy to reduces the expensive operational cost and promotes the bacterial growth (19,23,38).

The content of aromatic amino acids in culture media has been proposed to play an important role in secondary metabolism, particularly in the induction of IAA biosynthesis (39,40). L-Tryptophan is the main precursor in the IAA pathway in the *Bacillus* genus (11,41). In this work, the aromatic amino acid content of the industrial substrates (soy and pea flour) used to formulate SYM and PYM media was higher than that of microbiological LB medium. Thus, the higher IAA production observed in this work in *B. circulans* cultures could be attributed to the high content of aromatic amino acids present in PYM medium, and suggest that the L-tryptophan contained in PYM medium provide a positive bacterium growth while maintain its metabolites production efficacy, as reported for other PGPR using different low-cost substrates (12,42,43). In addition, support the idea that the IAA biosynthesis in *B. circulans* E9 can be induced possibly by tyrosine and phenylalanine present in the fermentation substrates (41,44,45). Bacterial genes for IAA catabolism (*iac* and *iaa)* have been widely characterized in several bacterial genera and are related with the nutrients deficiency in the media (46,47). In this work, we observed the degradation of IAA produced by *B. circulans* E9 in the PYM medium, condition in which the stationary phase was 5 h longer than the LB media. Thus, the IAA degradation can be explained by the possible induction of catabolism genes by the deficiency in carbon and nitrogen sources in PYM medium during the stationary phase, in a similar manner to the reported by other authors (48,49).

On the other hand, oxygen is an important nutrient for bacterial metabolism (36,50). A deficiency in oxygen supply can generate stress conditions, affect cell growth and induce anaerobic conditions (29,34,51). Significant increases in cell growth and IAA yield were found in the *B. circulans* culture using a silicone foam closure compared to those of cultures using cotton or aluminum closures. These results indicate that the increase in the OTR value by the use of different closures promotes *B. circulans* E9 growth. Similarly, other authors have shown that high OTR values stimulate cell growth and secondary metabolite production by other PGPR (50–53).

In microbial processes, gas–liquid mass transfer is affected by hydrodynamic conditions (20,34,54). Stirred tank bioreactors are a biotechnological tool that allow the operating conditions to be

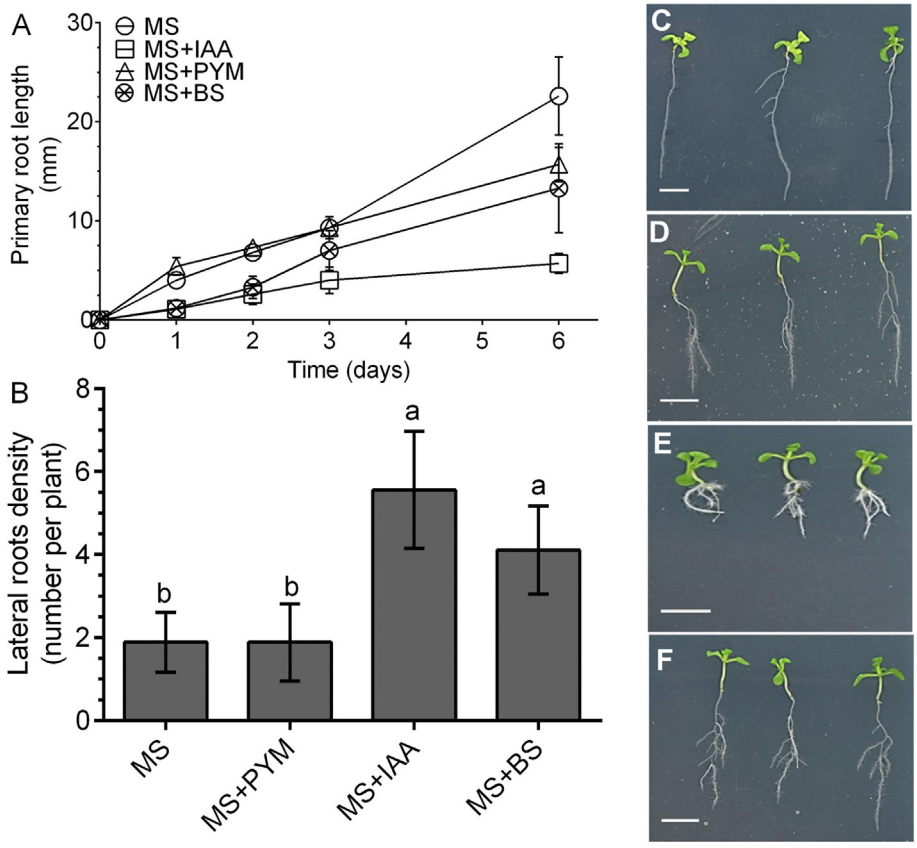

FIG. 4. Effects of IAA produced by *Bacillus circulans* E9 on primary root length (A) and on lateral root density (B) in *Arabidopsis thaliana* plants. Morphological images of primary root length are shown for control plants (MS) (C), plants treated with MS with the components of PYM (bacterium-free) (D), plants treated with MS with 10 μM IAA (Sigma–Aldrich) (E) and plants treated with MS with BS (10 μM IAA produced by *B. circulans* E9) (F). The photographs show representative plants of the nine plants examined in each experiment; scale bars: 1 mm. Different letters indicate a significant difference according to the Tukey's test ($P < 0.05$).

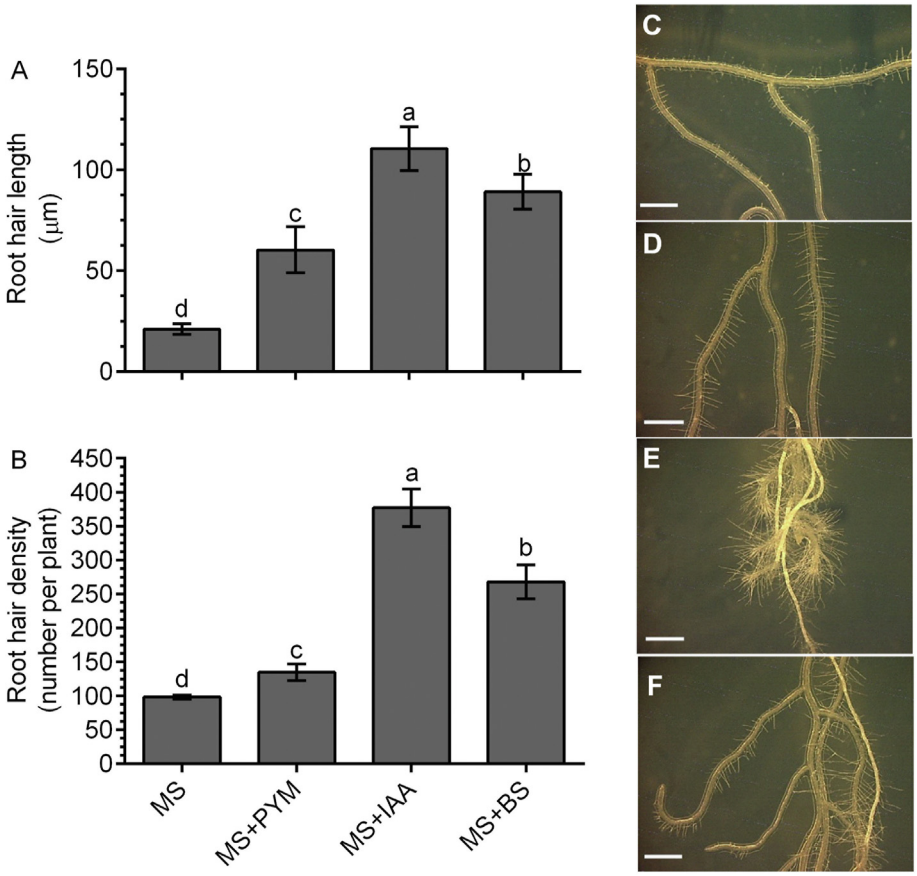

FIG. 5. Effects of IAA produced by *Bacillus circulans* E9 on the length (A) and density of root hairs (B) of *Arabidopsis thaliana*. The root hair morphology of *A. thaliana* is shown for control plants (MS) (C), plants treated with MS supplemented with the components of PYM medium (bacterium-free) (D), plants treated with MS with IAA (Sigma–Aldrich) (E) and plants treated with MS with BS (IAA produced by *B. circulans* E9) (F). Photographs show representative plants of the nine plants examined in each experiment; scale bars: 100 μm. Different letters indicate a significant difference according to the Tukey's test ($P < 0.05$).

monitored and provide high mass transfer rates for metabolite production (21,55). Our results showed that the DOT and pH values decreased greatly during the first few hours of culture, with the lowest levels found at the time *B. circulans* reached the stationary growth phase. In addition, the bacterial growth and IAA production obtained in the bioreactor were higher than those obtained in an Erlenmeyer flask. The oxygen supplied in the bioreactor system (OTR) was higher than the microbial oxygen demand (OUR), indicating that *B. circulans* E9 did not have an oxygen supply limitation. These results are consistent with those from other reports that indicate that the growth of aerobic bacteria and the production of metabolites are favored in the presence of oxygen (22,52,54,56).

In addition, to obtain massive *B. circulans* cell growth with a high content of IAA in cultures growing in a bioreactor with PYM media, it was important to demonstrate the biological efficacy of the produced IAA on roots of *A. thaliana*. IAA produced by *B. circulans* E9 in a stirred tank bioreactor led to a significant reduction in primary root elongation and promoted lateral as well as hairy root density in *A. thaliana* plants, similar to synthetic IAA. Several studies demonstrated that IAA produced by PGPR have a similar biological efficacy to promote the plant growth than commercial IAA (10,11,57–59). Taken together, these results suggest that the broth containing IAA by *B. circulans* E9 can be effectively used to stimulated the root system in plants, in comparison to commercial IAA. Also, suggest that *B. circulans* E9 is a good candidate for the inexpensive and utmost production of IAA in short period. Further studies regarding to increase IAA production adding or suturing L-tryptophan in the culture medium is underway.

These results are relevant from a bioprocess perspective and demonstrate that low-cost industrial substrates can be used to formulate and optimize specific low-cost media to produce important secondary metabolites, such as IAA, by *B. circulans* E9 in a bioreactor.

In conclusion, a low-cost medium (PYM) based on pea flour was selected for *B. circulans* E9 growth to replace microbiological LB medium. High IAA production was observed in PYM medium in comparison to that observed in conventional LB medium in Erlenmeyer flasks and was associated with a higher content of aromatic amino acids. Cell growth and IAA in the Erlenmeyer flask system were improved by the OTR increase achieved using different closures. The *B. circulans* E9 culture grown in a bioreactor with PYM medium showed better cell growth and IAA production than that grown in Erlenmeyer flasks. The IAA produced by the *B. circulans* E9 culture grown in a bioreactor induced the root system in *A. thaliana* in a similar manner to purified IAA. The results of this work suggest that PYM medium is feasible for growing *B. circulans* E9 to use it as a biofertilizer.

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References

1. **Kumar, A. and Verma, J. P.:** Does plant—microbe interaction confer stress tolerance in plants: a review? Microbiol. Res., **207**, 41–52 (2018).
2. **Perez, J. J., Francois, N. J., Maroniche, G. A., Borrajo, M. P., Pereyra, M. A., and Creus, C. M.:** A novel, green, low-cost chitosan-starch hydrogel as potential delivery system for plant growth-promoting bacteria, Carbohydr. Polym., **202**, 409–417 (2018).
3. **Grobelak, A., Kokot, P., Hutchison, D., Grosser, A., and Kacprzak, M.:** Plant growth-promoting rhizobacteria as an alternative to mineral fertilizers in assisted bioremediation - sustainable land and waste management, J. Environ. Manage., **227**, 1–9 (2018).
4. **Saleem, M., Asghar, H. N., Zahir, Z. A., and Shahid, M.:** Impact of lead tolerant plant growth promoting rhizobacteria on growth, physiology, antioxidant activities, yield and lead content in sunflower in lead contaminated soil, Chemosphere, **195**, 606–614 (2018).
5. **Etesami, H. and Maheshwari, D. K.:** Use of plant growth promoting rhizobacteria (PGPRs) with multiple plant growth promoting traits in stress agriculture: action mechanisms and future prospects, Ecotoxicol. Environ. Saf., **156**, 225–246 (2018).
6. **Ahemad, M. and Kibret, M.:** Mechanisms and applications of plant growth promoting rhizobacteria: current perspective, J. King Saud Univ. Sci., **26**, 1–20 (2014).
7. **Grobelak, A., Napora, A., and Kacprzak, M.:** Using plant growth-promoting rhizobacteria (PGPR) to improve plant growth, Ecol. Eng., **84**, 22–28 (2015).
8. **Gouda, S., Kerry, R. G., Das, G., Paramithiotis, S., Shin, H. S., and Patra, J. K.:** Revitalization of plant growth promoting rhizobacteria for sustainable development in agriculture, Microbiol. Res., **206**, 131–140 (2018).
9. **Liotti, R. G., da Silva Figueiredo, M. I., da Silva, G. F., de Mendonça, E. A. F., and Soares, M. A.:** Diversity of cultivable bacterial endophytes in *Paullinia cupana* and their potential for plant growth promotion and phytopathogen control, Microbiol. Res., **207**, 8–18 (2018).
10. **Chowdappa, P., Mohan-Kumar, S. P., Jyothi-Lakshmi, M., and Upreti, K. K.:** Growth stimulation and induction of systemic resistance in tomato against early and late blight by *Bacillus subtilis* OTPB1 or *Trichoderma harzianum* OTPB3, Biol. Control., **65**, 109–117 (2013).
11. **Dawwam, G. E., Elbeltagy, A., Emara, H. M., Abbas, I. H., and Hassan, M. M.:** Beneficial effect of plant growth promoting bacteria isolated from the roots of potato plant, Ann. Agric. Sci., **58**, 195–201 (2013).
12. **Lobo, C. B., Juárez-Tomás, M. S., Viruel, E., Ferrero, M. A., and Lucca, M. E.:** Development of low-cost formulations of plant growth-promoting bacteria to be used as inoculants in beneficial agricultural technologies, Microbiol. Res., **219**, 12–25 (2019).
13. **Ongena, M. and Jacques, P.:** Bacillus lipopeptides: versatile weapons for plant disease biocontrol, Trends Microbiol., **16**, 115–125 (2008).
14. **Ali, B., Sabri, A. N., Ljung, K., and Hasnain, S.:** Quantification of indole-3-acetic acid from plant associated *Bacillus* spp. and their phytostimulatory effect on *Vigna radiata* (L.), World J. Microbiol. Biotechnol., **25**, 519–526 (2009).
15. **Connor, N., Sikorski, J., Rooney, A. P., Kopac, S., Koeppel, A. F., Burger, A., Cole, S. G., Perry, E. B., Krizanc, D., Field, N. C., Slaton, M., and Cohan, F. M.:** Ecology of speciation in the genus *Bacillus*, Appl. Environ. Microbiol., **76**, 1349–1358 (2010).
16. **Pérez-García, A., Romero, D., and de Vicente, A.:** Plant protection and growth stimulation by microorganisms: biotechnological applications of Bacilli in agriculture, Curr. Opin. Biotechnol., **22**, 187–193 (2011).
17. **Gupta, R. S., Patel, S., Saini, N., and Chen, S.:** Robust demarcation of 17 distinct *Bacillus* species clades, proposed as novel *Bacillaceae* genera, by phylogenomics and comparative genomic analyses: description of *Robertmurraya kyonggiensis* sp. nov. and proposal for an emended genus *Bacillus* limiting it only to the members of the Subtilis and Cereus clades of species, Int. J. Syst. Evol. Microbiol., **70**, 5753–5798 (2020).
18. **Matsumoto, T., Sugiura, Y., Kondo, A., and Fukuda, H.:** Efficient production of protopectinases by *Bacillus subtilis* using medium based on soybean flour, Biochem. Eng. J., **6**, 81–86 (2000).
19. **Yánez-Mendizábal, V., Zeriouh, H., Viñas, I., Torres, R., Usall, J., de Vicente, A., Pérez-García, A., and Teixidó, N.:** Biological control of peach brown rot (*Monilinia* spp.) by *Bacillus subtilis* CPA-8 is based on production of fengycin-like lipopeptides, Eur. J. Plant Pathol., **132**, 609–619 (2012).
20. **Garcia-Ochoa, F. and Gomez, E.:** Bioreactor scale-up and oxygen transfer rate in microbial processes: an overview, Biotechnol. Adv., **27**, 153–176 (2009).
21. **Tajsoleiman, T., Mears, L., Krühne, U., Gernaey, K. V., and Cornelissen, S.:** An industrial perspective on scale-down challenges using miniaturized bioreactors, Trends Biotechnol., **37**, 697–706 (2019).
22. **De Souza, C. F. V., Rodrigues, R. C., and Ayub, M. A. Z.:** Effects of oxygen volumetric mass transfer coefficient on transglutaminase production by *Bacillus circulans* BL32, Biotechnol. Bioprocess Eng., **14**, 571–576 (2009).
23. **Yánez-Mendizábal, V., Viñas, I., Usall, J., Torres, R., Solsona, C., and Teixidó, N.:** Production of the postharvest biocontrol agent *Bacillus subtilis* CPA-8 using low cost commercial products and by-products, Biol. Control., **60**, 280–289 (2012).
24. **Fahim, S., Dimitrov, K., Vauchel, P., Gancel, F., Delaplace, G., Jacques, P., and Nikov, I.:** Oxygen transfer in three phase inverse fluidized bed bioreactor during biosurfactant production by *Bacillus subtilis*, Biochem. Eng. J., **76**, 70–76 (2013).
25. **Sieuwerts, S., De Bok, F. A. M., Mols, E., De Vos, W. M., and Van Hylckama Vlieg, J. E. T.:** A simple and fast method for determining colony forming units, Lett. Appl. Microbiol., **47**, 275–288 (2008).
26. **Gordon, S. A. and Weber, R. P.:** Colorimetric estimation of indoleacetic acid, Plant Physiol., **26**, 192–195 (1951).
27. **Tsavkelova, E. A., Cherdyntseva, T. A., Botina, S. G., and Netrusov, A. I.:** Bacteria associated with orchid roots and microbial production of auxin, Microbiol. Res., **162**, 69–76 (2007).
28. **Glickmann, E. and Dessaux, Y.:** A critical examination of the specificity of the Salkowski reagent for indolic compounds produced by phytopathogenic bacteria, Appl. Environ. Microbiol., **61**, 793–796 (1995).
29. **Orozco-Sánchez, F., Sepúlveda-Jiménez, G., Trejo-Tapia, G., Zamilpa, A., and Rodríguez-Monroy, M.:** Oxygen limitations to grow *Azadirachta indica* cell culture in shake flasks, Rev. Mex. Ing. Química, **10**, 343–352 (2011).
30. **de Carvalho-Neto, D. P., de Melo-Pereira, G. V., Finco, A. M. O., Letti, L. A. J., da Silva, B. J. G., Vandenberghe, L. P. S., and Soccol, C. R.:** Efficient coffee beans mucilage layer removal using lactic acid fermentation in a stirred-tank bioreactor: kinetic, metabolic and sensorial studies, Food Biosci., **26**, 80–87 (2018).
31. **Potumarthi, R. Ch S. and Jetty, A.:** Alkaline protease production by submerged fermentation in stirred tank reactor using *Bacillus licheniformis* NCIM-2042: effect of aeration and agitation regimes, Biochem. Eng. J., **34**, 185–192 (2007).
32. **Bruno, L., Pacenza, M., Forgione, I., Lamerton, L. R., Greco, M., Chiappetta, A., and Bitonti, M. B.:** In *Arabidopsis thaliana* cadmium impact on the growth of primary root by altering SCR expression and auxin-cytokinin cross-talk, Front. Plant Sci., **8**, 1323 (2017).
33. **Murashige, T. and Skoog, F.:** A revised medium for rapid growth and bio assays with tobacco tissue cultures, Physiol. Plant, **15**, 473–497 (1962).
34. **Pérez-Hernández, J., Nicasio-Torres, M. D. P., Sarmiento-López, L. G., and Rodríguez-Monroy, M.:** Production of anti-inflammatory compounds in *Sphaeralcea angustifolia* cell suspension cultivated in stirred tank bioreactor, Eng. Life Sci., **19**, 196–205 (2019).
35. **Singh, V. K., Singh, A. K., Singh, P. P., and Kumar, A.:** Interaction of plant growth promoting bacteria with tomato under abiotic stress: a review, Agric. Ecosyst. Environ., **267**, 129–140 (2018).
36. **Wu, R., Chen, G., Pan, S., Zeng, J., and Liang, Z.:** Cost-effective fibrinolytic enzyme production by *Bacillus subtilis* WR350 using medium supplemented with corn steep powder and sucrose, Sci. Rep., **9**, 6824 (2019).
37. **Gotor-Vila, A., Usall, J., Torres, R., Abadias, M., and Teixidó, N.:** Formulation of the biocontrol agent *Bacillus amyloliquefaciens* CPA-8 using different approaches: liquid, freeze-drying and fluid-bed spray-drying, BioControl, **62**, 545–555 (2017).
38. **Gunjal, A., Kapadnis, B., and Pawar, N.:** Agroindustry by-products as a carrier resource for plant-growth-promoting rhizobacterium, *Bacillus subtilis*, J. Mater. Cycles Waste Manag., **14**, 274–280 (2012).
39. **Koga, J.:** Structure and function of indolepyruvate decarboxylase, a key enzyme in indole-3-acetic acid biosynthesis, Biochim. Biophys. Acta, **1249**, 1–13 (1995).
40. **Sivasakthi, S., Usharani, G., and Saranraj, P.:** Biocontrol potentiality of plant growth promoting bacteria (PGPR) - *Pseudomonas fluorescens* and *Bacillus subtilis*: a review, Afr. J. Agric. Res., **916**, 1265–1277 (2014).
41. **Duca, D., Lorv, J., Patten, C. L., Rose, D., and Glick, B. R.:** Indole-3-acetic acid in plant-microbe interactions, Int. J. Gen. Mol. Microbiol., **106**, 85–125 (2014).
42. **Prabakaran, G. and Balaraman, K.:** Development of a cost-effective medium for the large scale production of *Bacillus thuringiensis* var *israelensis*, Biol. Control, **36**, 288–292 (2006).
43. **Malik, D. K. and Sindhu, S. S.:** Production of indole acetic acid by *Pseudomonas* sp.: effect of coinoculation with *Mesorhizobium* sp. *Cicer* on nodulation and plant growth of chickpea (*Cicer arietinum*), Physiol. Mol. Biol. Plants, **17**, 25–32 (2011).
44. **Pirog, T. P., Iutynska, G. O., Leonova, N. O., Beregova, K.,A., and Shevchuk, T. A.:** Microbial synthesis of phytohormones, Biotechnol. Acta, **11**, 5–24 (2018).
45. **Kizhakedathil, M. P. J. and Devi, C.,S.:** Rhizoshpheric bacteria isolated from the agricultural fields of Kolathur, Tamilnadu promotes plant growth in mustard plants, Biocatal. Agric. Biotechnol., **16**, 293–302 (2018).
46. **Donoso, R., Leiva-Novoa, P., Zúñiga, A., Timmermann, T., Recabarren-Gajardo, G., and González, B.:** Biochemical and genetic bases of indole-3-acetic acid (auxin phytohormone) degradation by the plant-growth-promoting rhizobacterium *Paraburkholderia phytofirmans* PsJN, Appl. Environ. Microbiol., **83**, e01991-16 (2016).
47. **Laird, T. S., Flores, N., and Leveau, J. H. J.:** Bacterial catabolism of indole-3-acetic acid, Appl. Microbiol. Biotechnol., **104**, 9535–9550 (2020).
48. **Greenhut, I. V., Slezak, B. L., and Leveau, J. H. J.:** *iac* gene expression in the indole-3-acetic acid-degrading soil bacterium *Enterobacter soli* LF7, Appl. Environ. Microbiol., **84**, e01057-18 (2018).
49. **Scott, J. C., Greenhut, I. V., and Leveau, J. H. J.:** Functional characterization of the bacterial iac genes for degradation of the plant hormone indole-3-acetic acid, J. Chem. Ecol., **39**, 942–951 (2013).

50. **Moradkhani, H., Izadkhah, M. S., Anarjan, N., and Abdi, A.:** Oxygen mass transfer and shear stress effects on *Pseudomonas putida* BCRC 14365 growth to improve bioreactor design and performance, Environ. Sci. Pollut. Res., **24**, 22427–22441 (2017).
51. **Martinez, I., Santos, V. E., Gomez, E., and Garcia-Ochoa, F.:** Biodesulfurization of dibenzothiophene by resting cells of *Pseudomonas putida* CECT5279: Influence of the oxygen transfer rate in the scale-up from shaken flask to stirred tank reactor, J. Chem. Technol. Biotechnol., **91**, 184–189 (2016).
52. **Anderlei, T., Zang, W., Papaspyrou, M., and Büchs, J.:** Online respiration activity measurement (OTR, CTR, RQ) in shake flasks, Biochem. Eng. J., **17**, 187–194 (2004).
53. **Escobar, S., Rodriguez, A., Gomez, E., Alcon, A., Santos, V. E., and Garcia-Ochoa, F.:** Influence of oxygen transfer on *Pseudomonas putida* effects on growth rate and biodesulfurization capacity, Bioprocess Biosyst. Eng., **39**, 545–554 (2016).
54. **da Silva, T. A. F., de Freitas, L. S., da Silva, L. V. J. B., Duarte Neto, J. M. W., da Silva, G. R., Maranhão, L. M. de A. C., de Lacerda, C. A., Oliveira, J. de P., Bezerra, R. P., and Porto, A. L. F.:** Optimization of a culture medium based on forage palm for 8-endotoxin production. Biocatal. Agric. Biotechnol., **27**, 101664 (2020).
55. **Palmerín-Carreño, D. M., Castillo-Araiza, C. O., Rutiaga-Quiñones, O. M., Verde-Calvo, J. R., and Huerta-Ochoa, S.:** Kinetic, oxygen mass transfer and hydrodynamic studies in a three-phase stirred tank bioreactor for the bioconversion of (+)-valencene on *Yarrowia lipolytica* 2.2ab, Biochem. Eng. J., **113**, 37–46 (2016).
56. **Amer, M., Feng, Y., and Ramsey, J. D.:** Using CFD simulations and statistical analysis to correlate oxygen mass transfer coefficient to both geometrical parameters and operating conditions in a stirred-tank bioreactor, Biotechnol. Prog., **35**, e2785 (2019).
57. **Salazar-Magallon, J. A., Murillo-Alonso, K. T., Garcia, L. L., Pena-Chora, G., and Hernandez-Velazquez, V. M.:** Scale-up from a shake flask to a bioreactor, based on oxygen transfer for the production of spore-crystal complexes from *Bacillus thuringiensis*, J. Pure Appl. Microbiol., **10**, 871–878 (2016).
58. **Contesto, C., Milesi, S., Mantelin, S., Zancarini, A., Desbrosses, G., Varoquaux, F., Bellini, C., Kowalczyk, M., and Touraine, B.:** The auxin-signaling pathway is required for the lateral root response of Arabidopsis to the rhizobacterium *Phyllobacterium brassicacearum*, Planta, **232**, 1455–1470 (2010).
59. **López-Valdez, F., Fernández-Luqueño, F., Ceballos-Ramírez, J. M., Marsch, R., Olalde-Portugal, V., and Dendooven, L.:** A strain of *Bacillus subtilis* stimulates sunflower growth (*Helianthus annuus* L.) temporarily, Sci. Hortic., **128**, 499–505 (2011).