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Enhanced Production of Indole-3-Acetic Acid by *Bacillus* Strains Using Immobilized Cell Systems on AMBERJET® 4200 for Potential Application as Agricultural Bioinoculants

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Abstract: Plant growth-promoting bacteria (PGPB) play a pivotal role in sustainable agriculture through the production of phytohormones such as indole-3-acetic acid (IAA). Among them, *Bacillus* spp. Are particularly noted for their metabolic versatility and environmental resilience. This study aimed to compare IAA production by free-living versus immobilized *Bacillus* strains (RC9 and RC15), isolated from coffee rhizospheres, using AMBERJET® 4200 Cl^- as a porous solid support. The strains were cultured in a minimal medium supplemented with tryptophan, and IAA synthesis was quantified via colorimetric (Salkowski reagent) and thin-layer chromatography (TLC) methods. Immobilized cells demonstrated significantly higher IAA yields and specific productivity compared to their free-cell counterparts ($p < 0.05$), even when cell density was suboptimal levels. Notably, the RC9 immobilized derivative outperformed all experimental set ups, suggesting enhanced bioconversion efficiency and possible activation of endogenous biosynthetic pathways. These findings underscore the potential of immobilization technology to optimize microbial auxin production and contribute to the development of bioinoculants for sustainable crop management.

Key words: indole-3-acetic acid (IAA), plant growth-promoting bacteria (PGPB), cell immobilization, bioinoculants.

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The immobilization of cell or enzyme involves physically confining or localizing cells and/or enzymes in a specific region of space, retaining their catalytic properties and activities (Woodward, 1985). Cell immobilization is a process of undeniable importance in biotechnology. One of its best-known aspects is the use of relatively expensive catalysts such as enzymes in various industrial processes that require reuse of the catalyst to make the process economically viable (Guisan, 2006; Mateo *et al*., 2007). Reverse immobilization methods allow cells to be detached under non-extreme conditions (Weber and Thomas, 2008). The use of reversible methods for cell immobilization is highly attractive, primarily for economic reasons, as the support can be regenerated and replenished with new cells when functional activity declines. Some of the most widely used reversible immobilization methods involve electrostatic interactions between the support and the cell (Ji *et al*., 2019; Zhang *et al*., 2019). Plant growth-promoting bacteria (PGPR) synthesize 3-indoleacetic acid (IAA). This important auxin, secreted by bacteria, contributes to the endogenous pool of plant hormones, mimicking the effect of exogenous IAA application (Glick *et al*., 1999). In this sense, bacterial IAA stimulates the development of the root system and the overall growth of the host plant. Likewise, the resulting increase in the production of plant metabolites, used by the bacteria for their own growth, has been shown a reciprocal benefit in the plant-bacteria relationship (Patten and Glick, 2002). The promotion of root growth is one of the pivotal factors by which the beneficial effects of different PGPRs are evaluated (Tilak *et al*., 2006). In this term, bacterial production of IAA and the high sensitivity of roots to this hormone would be fundamental in the response to inoculation (Turan *et al*., 2006). Bacteria that secrete low levels of IAA would stimulate root elongation, while highly auxin-producing bacteria would promote the formation of lateral roots or the development of absorbent hairs (Rocha *et al*., 2019). Bacteria possess a wide plasticity and variety in their metabolism, manifested in a great diversity of metabolic pathways that generate many intermediates for the anabolism of many metabolites (Taiz and Zeiger, 2010). The shikimic acid and chorismic acid pathways generate precursors of various aromatic metabolic intermediates such as indole rings that can be used in the synthesis of aromatic compounds such as 4-aminobenzoic acid, gallic acid, L-tryptophan or IAA (Khan *et al*., 2016; Normanly, 2010; Uribe *et al*., 2020) an economically important frankincense-producing tree found in the desert woodlands of Oman, is least known for its endophytic fungal diversity and the potential of these fungi to produce extracellular enzymes and auxins. We isolated various fungal endophytes belonging to Eurotiales (11.8%).

Material and Methods

Strains

The following strains were used: RC-9 and RC-15, rhizospheric coffee isolates belonging to the genus *Bacillus* sp., from the Department of Microbiology and Virology, Faculty of Biology, University of Havana.

Immobilization of live microbial cells on the AMBERJET® 4200 Cl− porous solid support

Immobilization of the strains RC-9 and RC-15 by ionic adsorption on AMBERJET® 4200 Cl^- porous solid support (Rohm and Haas Company, 2025) was performed according to Del Monte-Martínez *et al*., 2021. Immobilization was controlled by the parameter differential immobilization grade (cells) (diff. IGCell; expressed on cell number/support g) according to Del Monte-Martínez *et al*., 2021. The experimental parameter practical maximum quantity of cells to immobilize (pMQCell) was calculated from the obtained diff. IGCell as previously described (Rohm and Haas Company, 2025).

IAA Production

IAA production from cell suspensions and their corresponding immobilized derivatives was carried out using the following culture medium: 10.0 g sucrose, 2.5 g KH_2PO_4, 1.0 g $(NH4)_2HPO_4$, 0.20 g $MgSO_47H_2O$, 0.01 g $FeSO_47H_2O$, 0.007 g $MnSO_4H_2O$, supplemented with 20 µg/mL Trp. The final bioconversion assay volume was 5 mL. For the immobilized derivatives, 1 g of support was used with a cell load corresponding to pMQCell. The IAA was then sieved using a constant temperature cabinet (Stuart, UK) for 24 hours at 30°C. After the assay, samples were collected for product quantification. For strain comparison assays, to maintain the same number of cells in cell

suspension and immobilized derivatives, the amount corresponding to the pMQCell of strain RC-15 reported by Del Monte-Martínez *et al*., 2021 was used. For the immobilized derivative of strain RC-9, an IGCell diff of 1.78 (*108 Cells/g of Support) was used, corresponding to 26% of its pMQCell.

IAA quantification

IAA production was determined following the methodology of (Glickmann and Dessaux, 1995). 500 µL of the supernatant was added and mixed with 500 µL of the Salkowski reagent (12.0 g of anhydrous (FeCl3) in 1000 mL of 7.9 mol/L (H2SO4). This mixture was incubated in the dark for 30 minutes, and the reaction was carried out by exposing the sample to light. The absorbance of the samples was measured at a wavelength of 530 nm in a UV-Visible spectrophotometer (Genesys 200, USA). The assays were performed in triplicate. Solutions of tryptophan and IAA at 100 µg/mL were used as controls.

Determination of IAA production by Thin Layer Chromatography (TLC)

5x10 cm silica gel TLC plates (Merck, Germany) were used for chromatography. Applications were made at 1 cm from the base (of the plate) with a 0.5 mm distance between each layer. 5 µL of the sample was added for each application. The mobile phase used was isopropanol: ammonia: water (10:1:1) (Sánchez *et al*., 2019). The plates were developed with UV light at 258 nm. The plates were developed with ultraviolet light at 258 nm in a Development Cabinet (Liuyi, China). The chromatographic plates were photographed with a Cannon Camera (Canon Inc., Japan), and the images were processed using ImageJ version 2.0 software.

Determination of IAA Bioconversion Percentage: The bioconversion percentage was calculated according to equation (1)

Bioconversion (%) = (Conc.IAA)/(Conc.Trp)*100

where, Conc.IAA is the concentration of IAA produced; ConcTrp is the initial concentration of Trp

Determination of Specific Productivity for IAA Production

The specific productivity for IAA production was determined according to equation (2)

ProdSp = (Conc.IAA)/(Conc.Cell)

where, ProdSp is the specific productivity for IAA production; Conc. IAA is the concentration of IAA produced; Conc . Cell is the concentration of cells used

Results and Discussion

AIA is one of the auxins of greatest interest due to its positive effect on the promotion of plant growth; its production is considered crucial characteristics present in PGPB (Idris *et al*., 2007). Bacterial derivatives immobilized in the production of plant growth stimulating phytohormones are aimed at contributing to an ecological and sustainable agriculture, as well as to the sustainability of Plant Biotechnology companies (Nonhebel, 2015; Rajneesh *et al*., 2017). In this work, the detection and semi-quantification of IAA was performed by the colorimetric method using Salkowski's reagent and by thin layer chromatography (TLC)

Production of IAA by suspension cells and immobilized cells

The production of AIA by cells in suspension and immobilized was monitored using a colorimetric method that employs Salkowski's reagent as a developer (Glickmann and Dessaux, 1995). This reagent allows the detection of AIA and other indole compounds (Trujillo *et al*., 2007). These other indole compounds have auxin-like activity, which supports the relevance of using this method (Valencia, 2020). The application of this method as a routine test for bioconversion processes in IAA production is supported by: i) its ease of handling, ii) the rapid obtaining of results, and iii) its economic feasibility. For all these reasons, it is one of the most widely used methods for the detection and semi-quantification of AIA production (Torres-Rubio *et al*., 2000).

In a comparative study on IAA production, the number of cells applied in the bioconversion process was maintained as a control parameter for both experiments with suspended cells and immobilized cells (Fig. 1 A and B). Similar biomass concentrations of the strains under study (cell suspensions and immobilized derivatives) were used, with a total number of cells of approximately 1.75 * 108. This study

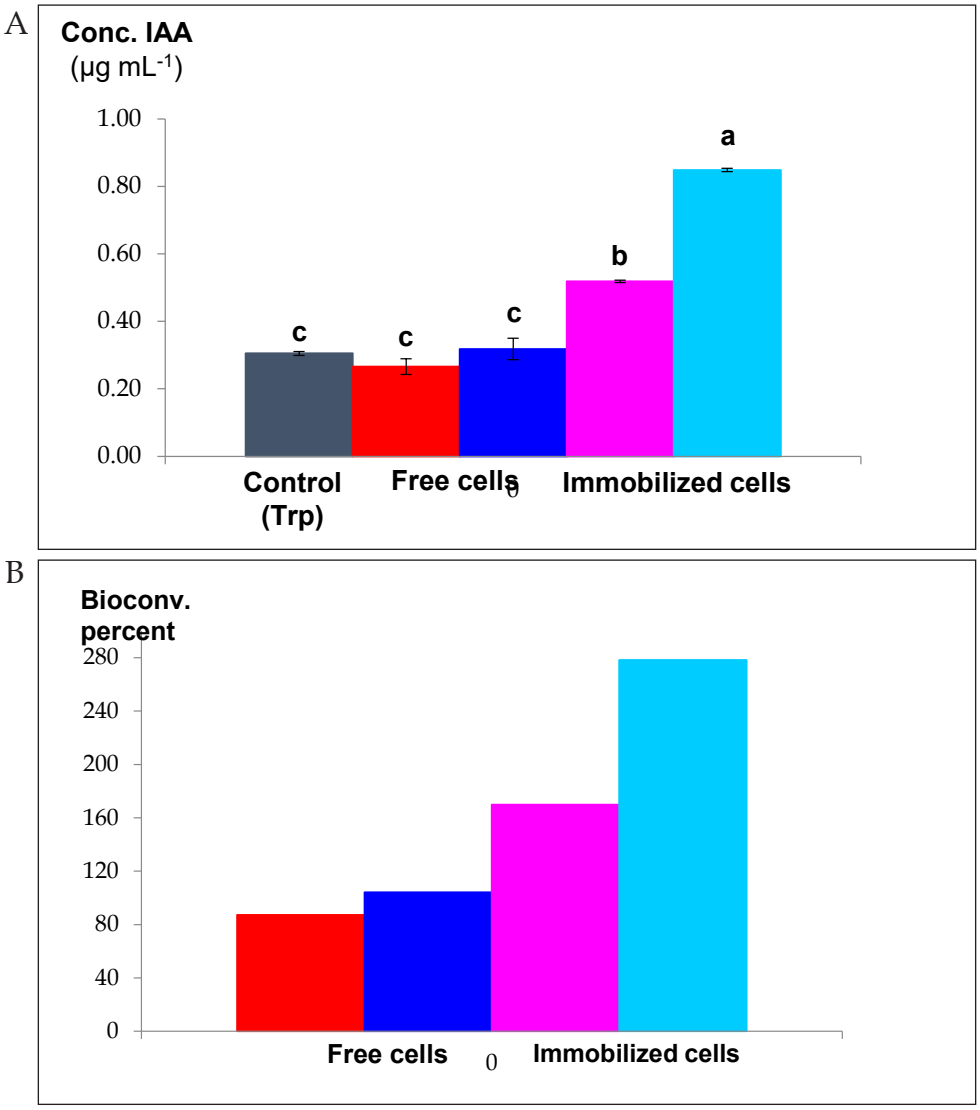

Fig. 1. A: IAA production (µg/mL) in the Trp/IAA bioconversion process. B: Percentage of bioconversion in the Trp/IAA process. Error bars represent the standard deviation from the mean (n=3). Different letters indicate statistically significant differences between Trp/IAA bioconversion values for p<0.05, according to Tukey's mean comparison test. Legends: Control Trp precursor of the bioconversion process. Cell suspension of RC15 strain. Cell suspension of RC9 strain. Immobilized derivative of strain RC15. Immobilized derivative of RC9 strain.

allowed us to compare indole production values between strains and to understand the behavior between cell suspensions and their respective immobilized derivatives. Indole production was positive for both strains analyzed and for the immobilized derivatives, reaching values ranging from 0.26 to 0.88 µg/mL of indoles, according to the indole quantification standard curve obtained with AIA as the standard, with a slope value of 0.0171 and an intercept of 0.0414, with an R2 = 0.9941. The analysis of indole production by the cell suspensions behaved similarly for both strains and behaved equimolarly (with no statistically significant differences for a $p < 0.05$) with the amount of Trp added as an inducer in the culture medium. Indole production by the immobilized derivatives was significantly higher than that of their respective parent cell suspensions (with statistically significant differences for $p<0.05$). For both derivatives, indole production levels exceeded the concentration of Trp used as an inducer in the bioconversion medium. This behavior suggests that, in some way, the effect produced by cell immobilization triggers the activation of indole synthesis through endogenous pathways, even when Trp has been depleted from the bioconversion medium.

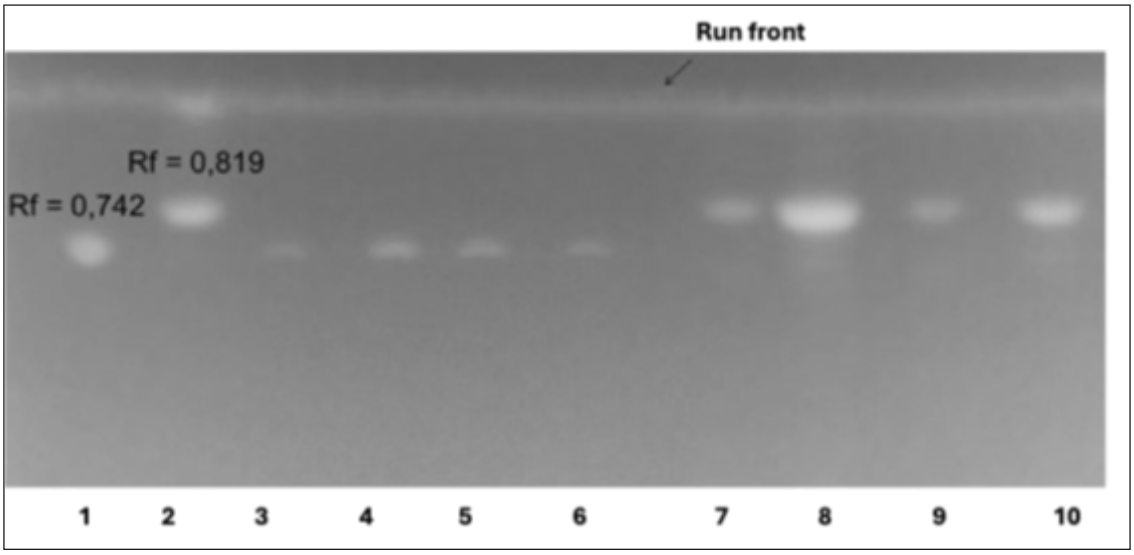

Fig. 2. Thin layer chromatography photograph of the samples (5 µL) of supernatants from the bioconversion tests for each of the strains under study and their corresponding immobilized derivatives, at the beginning and end of the process (24 hours), 25°C. Lanes: 1 and 2, Trp and IAA patterns. Lanes: 3 to 6, initial samples from the bioconversion process for cell suspensions and their respective immobilized derivatives of strains RC 9 (3 and 4) and RC 15 (5 and 6). Lanes: 7 to 10, final samples from the bioconversion process for cell suspensions and their respective immobilized derivatives from strains RC 9 (7 and 8) and RC 15 (9 and 10).

This novo synthesis behavior, independent of tryptophan, has been reported in the literature for the production of AIA and other indole-based growth promoters in different bacteria and fungi (Normanly, 2010; Taiz and Zeiger, 2010).

The analysis of the bioconversion percentage in the IAA production process (Fig. 1B) showed a marked difference between the cell suspension systems and the immobilized derivatives produced from their corresponding strains. The immobilized derivative RC 15-AMBERJET® 4200 Cl^- showed a productivity 1.9 times higher than the cell suspension RC 15. The behavior of the immobilized derivative RC 9-AMBERJET® 4200 Cl^-, in a comparison similar to the previous one, showed a productivity of 2.6 times higher. The behavior between both cell suspensions was similar. In the comparison between the two immobilized derivatives, RC 9-AMBERJET® 4200 Cl^- was 0.875 times higher than RC 15-AMBERJET® 4200 Cl^-. This last result is even more important because we are comparing an immobilized derivative with its optimal immobilized enzyme load (RC 15-AMBERJET® 4200 Cl^-) and another immobilized derivative that is only 26% of its total capacity. Therefore, the productivity obtained by RC 9-AMBERJET® 4200 Cl^- should be highlighted.

The greater bioconversion obtained by the immobilized derivatives compared to their parent cell suspensions can also be explained by the advantages of this process and not necessarily by the reuse of the biocatalyst. The immobilization process allows the generation of a heterogeneous material in which the conditions of agitation, nutrient transfer, and oxygen transfer are favored, showing superior efficiency to homogeneous systems such as cell suspensions (Abdel-Naby *et al*., 2011). This biocatalyst with cells immobilized by electrostatic adsorption minimizes the diffusion restrictions present in other immobilized systems as a result of cell immobilization on the surface of the support (Woodward, 1985). These findings are consistent with previous studies reporting high IAA production by *Bacillus* spp. (Reetha *et al*., 2014; Suliasih and Widawati, 2020), who demonstrate the ability of Bacilli strains to produce this phytohormone. The RC-9 strain was a better producer than RC15, a result that is consistent with the findings of (Bello, 2014; Tejera-Hernández *et al*., 2011) in previous studies.

It should be noted that, to rule out the presence of other non-phytohormonal compounds in the total indole production, a TLC chromatographic test was performed as a process control (Fig. 2). IAA and Trp (100

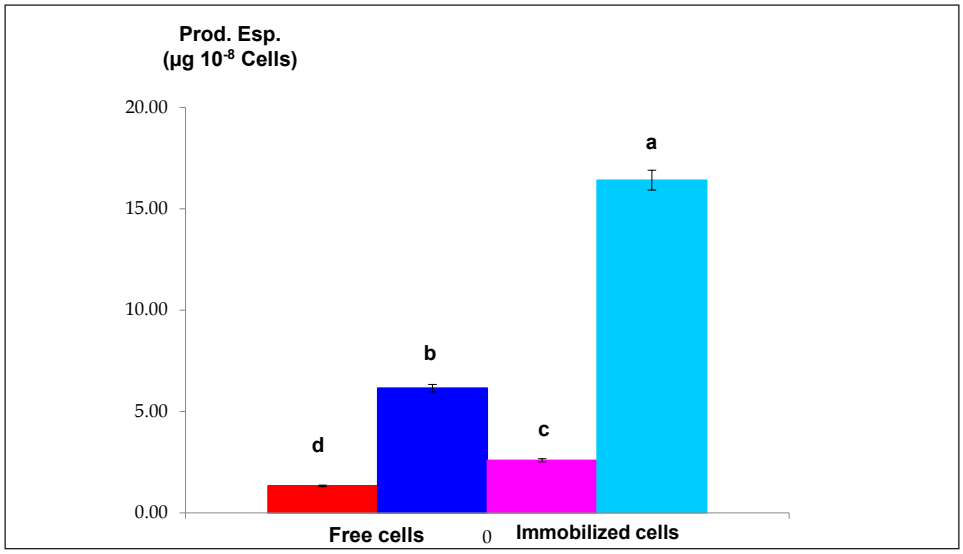

Fig. 3. Specific productivity (Esp.Prod) behavior for all samples studied in the Trp/IAA bioconversion process. Error bars represent the standard deviation from the mean (n=3). Different letters indicate statistically significant differences between Trp/IAA bioconversion values for p<0.05, according to Tukey's mean comparison test. Legends: Cell suspension of strain RC 15. Cell suspension of strain RC 9. Immobilized derivative of strain RC 15. Immobilized derivative of strain RC 9.

µg in each case) of analytical quality were used as standards. The mobile phase used allowed both compounds to be separated (Rf-IAA: 0.819 and Rf-Trp: 0.742) and therefore: i) discriminate in each of the samples from the biotransformation, ii) the presence or absence of each of these compounds, iii) the correlation between the intensity of the band in the chromatography with the levels of total indole production, and iv) the purity of the product of the bioconversion process for each strain under study and its respective immobilized derivatives.

The specific productivity (Prod. Esp.) (Fig. 3) analyzed for the results of the bioconversion process in obtaining IAA showed a similar behavior to the analysis performed on the bioconversion percentages. The main difference in this analysis is based on the comparison under optimized conditions for each immobilized derivative; in both cases, the same amount of g of biocatalyst was used. In all cases, statistically significant differences were obtained for the samples tested.

In the comparison of productivity between free cell in the behavior of strain RC 9, it was 4.62 times higher than RC 15. The most outstanding result is the higher productivity found for the optimized derivative RC 9-AMBERJET® 4200 Cl^- (5.37 times higher) compared to that obtained for RC 15-AMBERJET® 4200 Cl^-.

The promising results obtained in this study highlight the potential of immobilized *Bacillus* strains as effective bio-inputs for sustainable crop production. The enhanced IAA synthesis observed in immobilized systems suggests that these biocatalysts could be formulated into bioinoculants or biostimulants capable of promoting root development, nutrient uptake, and plant vigor under field conditions. The use of solid supports such as AMBERJET® 4200 Cl^- not only improves microbial efficiency but also offers practical advantages for formulation stability, shelf life, and gradual release of active metabolites in the rhizosphere.

Limitations and future work

Although the immobilization strategy using AMBERJET® 4200 Cl^- significantly enhanced IAA production by *Bacillus* strains, this study was conducted under controlled laboratory conditions using synthetic minimal media. As such, the actual performance and stability of the immobilized derivatives under real-world soil or rhizosphere conditions remain to be validated. Furthermore, the potential interaction of these immobilized biocatalysts with native microbial communities and plant systems has not yet been explored. Future studies should focus on evaluating the agronomic effectiveness of these immobilized strains through greenhouse and field trials, assessing their influence on plant growth,

yield components, soil microbial dynamics, and bioinoculant formulation stability.

Conclusions

This study demonstrated the ability of *Bacillus* strains RC9 and RC15, along with their immobilized derivatives, to produce indole derivates particularly IAA. Immobilization on AMBERJET® 4200 Cl^- significantly enhanced IAA production, with the RC9 immobilized derivative exhibiting the highest specific productivity. These results indicate that cell immobilization promotes microbial auxin biosynthesis and holds promise for developing effective bioinoculants. Future studies should evaluate the performance of these immobilized strains in greenhouse and field trials to validate their efficacy in promoting plant growth under practical agricultural conditions.

Author Contributions

FCA: Conceptualization, Data curation, Formal analysis, Methodology, Writing– original draft.

MPA: Investigation.

JGB: Investigation, Methodology.

MRB: Methodology, Supervision.

AdMM: Project administration, Software, Resources, Supervision, Writing– review & editing.

ROH: Project administration, Software, Resources, Supervision, Writing – review & editing.

Conflicts of Interest

There are no conflicts to declare.

Data Availability Statement

A data availability statement (DAS) is required to be submitted alongside all articles. Please read our full guidance on data availability statements for more details and examples of suitable statements you can use.

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