

Scale-Up Production of and Identification of Indole Acetic Acid using Waste Glycerol as a Carbon Source in 2-Litre Fermenter

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Abstract

The microbial production of Indole-3-acetic acid (IAA) develops a sustainable and low-cost methods to other traditional methods. This study investigates the scalability of IAA production using four isolated microbial strains *Enterobacter asburiae*, *Bacillus licheniformis*, *Achromobacter mucicolens*, and *Pseudomonas fulva* in a 2-litre fermenter with an optimized Yeast Extract Glycerol (YEG) medium, where crude glycerol was used as a carbon source. The results of microbial fermentation, with *Enterobacter asburiae* achieving yields of up to 3477 µg/ml through the indole-3-pyruvate pathway. Critical fermentation parameters such as pH, temperature, and agitation speed were maintained, contributing to consistent IAA production levels. The use of crude glycerol not only enhanced yields but also provided a low-cost substrate, aligning with sustainability goals in agriculture by reducing reliance on synthetic chemicals. Extraction and quantification methods such as High-Performance Liquid Chromatography (HPLC) and Fourier Transform Infrared Spectroscopy (FTIR), DSC and XRD ensured presence of IAA levels in samples. This study highlights the potential for scaling up microbial IAA production through optimized fermentation processes, with significant implications for biotechnological applications in sustainable agriculture and future research directions focusing on further optimization and genetic enhancements.

Key words: Scale up studies, Indole-3-acetic acid, FT-IR, HPLC, XRD, DSC, Endophytes, *Triticum aestivum*.

Introduction

Indole-3-acetic acid (IAA), a major phytohormone plays an important role in maintaining numerous functional processes in plants, including cell division, root elongation and healthy growth of the plants. Its significance in agricultural practices has spurred extensive research into efficient and scalable methods for its production. Recent advancements in biotechnology, particularly in the realm of fermentation technology, offer promising avenues for enhancing IAA production (1;2).

Microbes present in the root area of different plants produces Indole acetic acid as a byproduct, as a secondary metabolite (3). IAA helps roots grow longer and develop more secondary roots and branches, which improves nutrient absorption. (4;5). The bacterium *Niallia circulans* has shown potential biofertilizer due to its ability to enhance plant health by the production of growth hormone Auxin. Use of an economical growth medium as an alternative to the standard Luria Broth (LB) for cultivating *N. circulans* and enhancing IAA production and the study's findings indicated that the newly developed PYM medium could be effectively utilized for large-scale production of *N. circulans* in bioreactors (6).

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Microbial IAA production could be a green alternative to chemical synthesis, which is not only costly but unstable, and uses toxic materials (7). While microbial IAA has been studied for years, large-scale bioreactor production remains unclear. Typically, small batch tests optimize parameters before scaling up. However, these may not transfer directly to large-scale fermentation. Thus, optimization study is needed for large scale IAA production. *R. fluvialis* DMKU-CP293 strain was used for the production in fermenter using OFAT and RSM methods. They tested the yeast IAA's ability to suppress the weeds (8;9).

In the present study we have selected four potent novel endophytes isolated from wheat (*Triticum aestivum*) for large scale production of IAA (10), based on the optimization study all four strains had shown efficient producer of IAA (11). The study focused on scaling up the production of IAA utilizing a 2-litre fermenter, aiming to bridge the gap between laboratory-scale synthesis and industrial application. Innovative and cost-effective strategies employed to maximize IAA yield while ensuring operational efficiency and economic viability. Furthermore, we explore the broader implications of such advancements in the context of sustainable agriculture and biotechnological innovation.

Materials and Methods

Microorganism selection for IAA production

Four isolated and identified (our previous study) endophytes *Enterobacter asburiae* (S2), *Bacillus licheniformis* (S4), *Achromobacter mucicolens* (S5) and *Pseudomonas fulva* (S8) were screened and selected because they gave maximum result in our optimization study (10,11).

Preparation of fermentation media

In our optimization study, YES (Yeast Extract Sucrose) fermentation medium gave maximum IAA production after 48 hr of incubation in shake flask fermentation (11). For Scale

up studies, in YES medium, Sucrose was replaced with crude glycerol as carbon source. Hence, YEG (Yeast Extract Glucose) medium was prepared. Where 0.1% Yeast extract, 1 % Crude glycerol, 0.05% Magnesium sulphate 0.05 % Magnesium sulphate 0.01%, Sodium chloride and 0.2 % L- tryptophan as a substrate was used (9;12).

Preparation of crude glycerol

Glycerol in the medium was the rich source of carbon in the medium. For 10 ml of glycerol, 100 ml used mustard oil and methoxide (Methanol + Potassium hydroxide) was added in the oil and kept overnight for rotatory shaker and separate 10 ml glycerol using separating funnel (13,14).

Production of IAA in shake flask fermentation

100 ml of YEG media for each four strain was prepared, seed cultures of each strain were inoculated and incubated at 100 rpm at 37°C for 48 hr. After 48 hr of growth, culture was recovered for IAA quantification and compared with standard graph. Each treatment was performed in triplicates.

Scale-up production of IAA in a 2-litre fermenter

Initially, Seed cultures were inoculated in 200 ml Nutrient broth media at 100 rpm and 37°C for 24 hr. Optical Density were measured at 600 nm after 24 hr of growth. (15). Two litres of YEG fermentation medium were prepared in fermenter vessel and autoclave the whole unit, when it cools down 10% seed culture were added in the vessel under sterile condition, other fermentation parameters were maintained such as agitation speed were 200 rpm and incubation temperature was 37°C kept constant and pH were monitored during the batch production of IAA. After 48 hr of batch production in fermenter 5 ml of media for quantification was collected and OD were taken at 530 nm to checked the concentration (16).

Extraction and purification of IAA

After batch cultivation in 2-litre fermenter YEG media was centrifuged and cells were separated and 1 N HCl in the supernatant and pH was adjusted to 2.5 to acidify the solution. Add the equal volume of ethyl acetate in the supernatant and repeat this step two times and collected the ethyl acetate layer (17) and evaporated at 40°C using a rotatory evaporator and crystalline powder was obtained and moisture for removed and store for further analysis. (18;19).

Characterization of indole acetic acid

Various analytical technique like HPLC, DSC, FTIR and XRD were used to characterize the Indole acetic acid produced by endophytes.

Characterization of indole acetic acid by high performance liquid chromatography (HPLC)

The IAA samples were analyzed on an HPLC system (Agilent Technologies, Germany). Identification of IAA was performed on C18 column at 25°C temperature (20). Methanol and 1.5 % acetic acid (70:30 v/v) as a mobile phase was used and flow rate was 2 ml/min with 20 ml injection volume (21). To observe the good intensity of the peak two wavelength 254 nm and 280 nm was set to monitored the indole acetic acid. All the samples were compared with graph of standard IAA (22).

Characterization of indole acetic acid by FT-IR

To identify the functional groups in the samples FTIR analysis was done (23;24). The IAA powdered form of sample and standard were analysed and compared with IAA standard. Wavelength range 400–4000 cm^{-1} was selected for the analysis of functional groups. (25;26).

Characterization of indole acetic acid by X-ray diffraction (XRD)

XRD analysis is a technique used to

identify the crystalline structure of a compound by measuring the diffraction pattern of X-rays passed through the sample. An advanced X-ray diffractometer (XRD 6000, Shimadzu, Japan) was used for investigate the patterns of the IAA samples (27). For analysis angle range of 10° to 80° with Copper K-alpha radiation was set with voltage of 45Kv and current was 100mA. The step scan mode with a step counting time of 0.02 seconds was used for the XRD analysis. The scanning rate was set at 0.02°/min to ensure a thorough and precise analysis of the diffraction patterns of the samples (28). All the samples were analysed and result were compared with standard IAA result.

Characterization of indole acetic acid by differential scanning calorimetry (DSC)

DSC is the thermal analytical technique to analyse the physical properties of any sample/compound changes with different temperature against time. DSC analysis was performed using HITACHI system (DSC7000X, JAPAN). Nitrogen gas was used to maintain the temperature of the system and flow rate was 65 ml/min. 5 mg powdered sample of IAA and standard IAA were placed in aluminium pan and temperature was set from 10°C to 350°C with heating rate was about 25°C/min. IAA sample results were compared with standard IAA. (27,29).

Result and Discussion

Microorganism selection for IAA production

Four isolated and identified endophytes *Enterobacter asburiae*, *Bacillus licheniformis*, *Achromobacter mucicolens* and *Pseudomonas fulva* were screened and selected because they gave maximum result in YES media (Yeast Extract Sucrose) with optimized condition in our previous study.

Preparation of crude glycerol

Crude Glycerol was prepared using mustard oil. In mustard oil methoxide was added and kept overnight for rotatory shaker. Two layers were formed Biodiesel was Glycerol was

settled at the lower part in separating funnel. Approximately 10 ml glycerol was obtained.



Fig. 1: Glycerol and Biodiesel layers

Production of IAA in shake flask fermentation

100 ml of Yeast Extract Glycerol (YEG) media for four strain was prepared separately; strains were inoculated in the respective flask and incubated at 37° C for 48 hr. After 48 hr of growth, culture media was centrifuged & supernatant was used for primary quantification of IAA using spectrophotometer and compared with standard graph. Each treatment was performed in triplicates and the result presented in the table are mean and standard deviation of the triplicate.

Table 1: IAA Concentration in shake flask

Isolated Endophytes	IAA Concentration in YEG ($\mu\text{g}/\text{ml}$)
<i>Enterobacter asburiae</i> (S2)	243.4 \pm 3.5
<i>Bacillus licheniformis</i> (S4)	115.53 \pm 6.8
<i>Achromobacter mucicolens</i> (S5)	248.66 \pm 2.3
<i>Pseudomonas fulva</i> (S8)	84.66 \pm 4.2

Preparation of fermentation media in 2-litre laboratory fermenter

2 litre YEG fermentation medium was prepared in fermenter vessel and autoclaved the whole unit and 10% respective endophytic strains was inoculated when the OD₆₀₀ reached around 4.0×10^9 cells/ml endophytic strains was added in the fermentation vessel under sterile conditions.

Scale-up production of IAA in a 2 L fermenter

All the fermentation parameters were maintained and after 48 hr of batch production in fermenter, 5 ml of media was collected and OD was taken by spectrophotometer at 530 nm and IAA concentration was measured and compared with standard. All the strains showed 5-6 times increase their productivity. *Achromobacter mucicolens* (S5) gave maximum production of 1665.45 $\mu\text{g}/\text{ml}$ followed by *Enterobacter asburiae* (S2) which gave 1213.2 $\mu\text{g}/\text{ml}$. For statistical validation of result obtained, two batches of each strain were studied and considered as duplicate.

Table 2: IAA concentration in 2-litre fermenter

Isolated Endophytes	IAA Concentration in YEG ($\mu\text{g}/\text{ml}$)
<i>Enterobacter asburiae</i> (S2)	1213.2 \pm 31.53
<i>Bacillus licheniformis</i> (S4)	683.8 \pm 38.89
<i>Achromobacter mucicolens</i> (S5)	1665.45 \pm 61.73
<i>Pseudomonas fulva</i> (S8)	569.55 \pm 30.90

Extraction and purification of IAA

After batch cultivation in 2 L fermenter YEG media was centrifuged and the supernatant containing IAA was acidified. The ethyl acetate fraction was evaporated and IAA crystalline powder was settled at bottom of the flask. After removing moisture approximately 2-3 gm of pinkish white crystalline powder was obtained in all the strains.

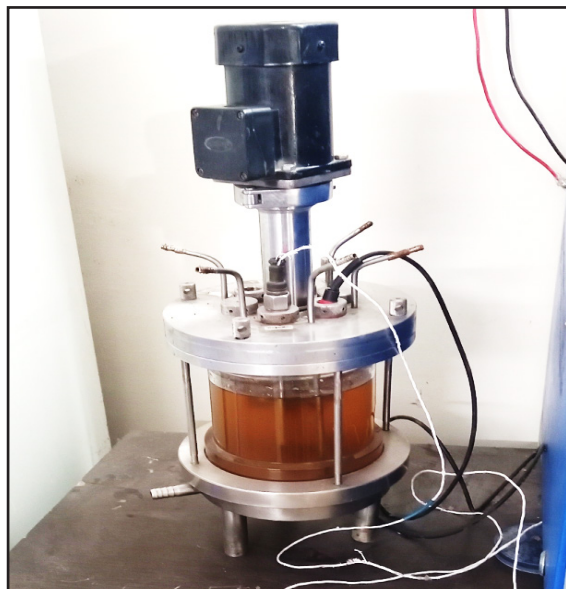


Fig. 2: Production of IAA in 2l fermenter by *Achromobacter mucicolens* (S5)



Fig. 3: IAA crystalline Powder produced by *Achromobacter mucicolens* (S5)

Characterization of Indole acetic acid

Characterization of Indole acetic acid by high performance liquid chromatography (HPLC)

The IAA standard and extracted samples were analyzed by HPLC system (Agilent Technologies, Germany). To observe the good intensity of the peak two wavelength 254 nm and 280 nm was set to monitored the indole acetic acid. Both the wavelengths showed IAA peak in all the samples but at 254 nm all peaks are sharp and present around retention time

3.36 min in standard as well as in sample also and it gave clear indication of the presence of IAA. All the samples were compared with standard IAA graph.

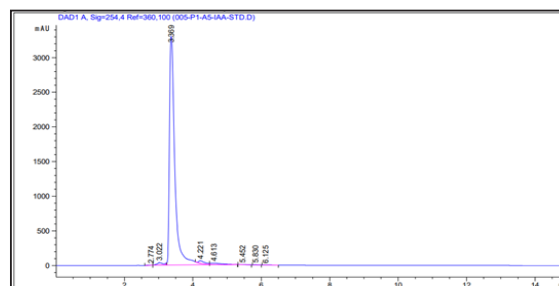


Fig.4: HPLC chromatogram of Standard IAA

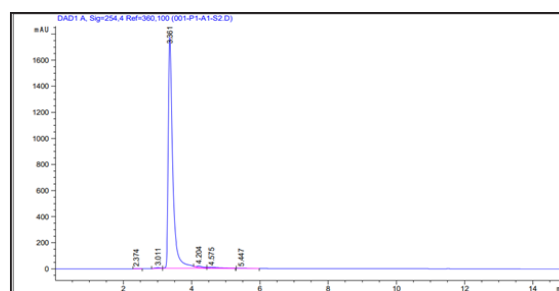


Fig.5: HPLC chromatogram of IAA by S2

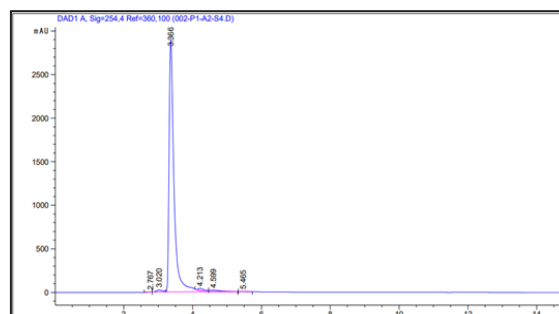


Fig. 6: HPLC chromatogram of IAA by S4

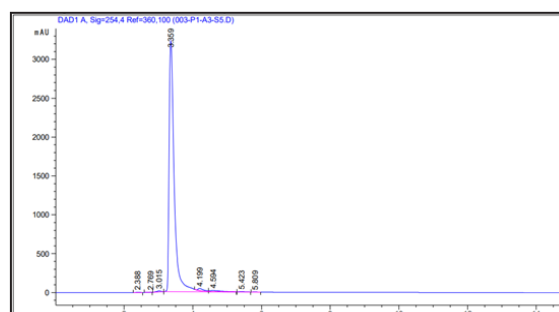


Figure 7: HPLC chromatogram of IAA by S5

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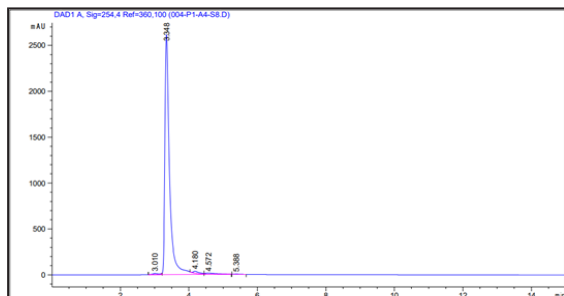


Fig 8: HPLC chromatogram of IAA by S8

Characterization of indole acetic acid by FT-IR

Extracted powder IAA produced by different strains was used to identify the functional groups present and compared with IAA standard powder, 400–4000 cm^{-1} was wavelength was selected. In figure transmittance vs absorbance graph of all the samples with standard were plotted in ORIGIN software. One mg of the extracted IAA was finely powdered and KBR pellet was prepared & used for analysis. Using Origin software, combined graph of standard IAA and samples was plotted. Peaks corresponding to C=C groups, N-H groups, C=O moiety and C-OH group were showed intense peak in the sample and standard (Figure:9). The N-H stretching of Indole ring was observed in the range of 3380 to 3390 cm^{-1} in all the samples and standard. Indole ring C-H bond was appeared around 1100 cm^{-1} and Alkyl (-CH₂) ring was present around 1450 cm^{-1} in all the samples and standard, the peak appeared around 1330 cm^{-1} is of C-OH moiety. FT-IR analysis showed strong indication of the presence of IAA in all the samples.

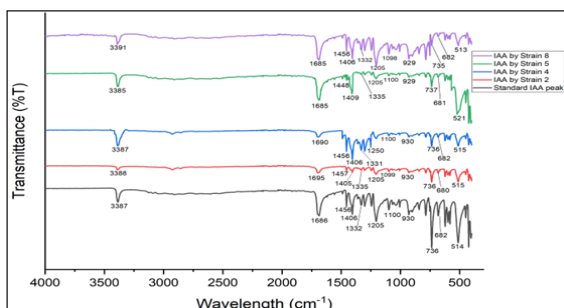


Fig. 9: Combined FT-IR graph of IAA Standard and samples

Characterization of indole acetic acid by XRD

The XRD patterns were recorded, XRD pattern for standard IAA shows sharp, well-defined peaks at specific angles, indicating the crystalline nature of the compound. The sharp peak at 15.48° theta reflects the crystal structure of the IAA molecule, where the spacing between atomic planes (lattice spacing) is specific to IAA. This peak serves as a reference point to compare the patterns of IAA produced by strains S2, S4, S5 and S8. In samples S2, S4, S5, and S8, peaks were observed at similar angles or close to 15.48°, indicating that these samples also contain a crystalline phase that matches the crystal structure of IAA.

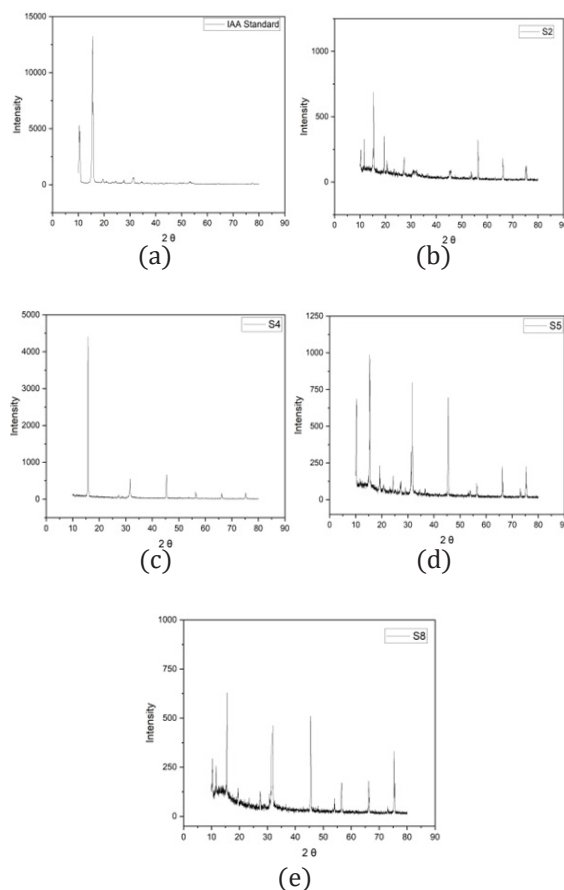


Fig.10: XRD graph (a) IAA Standard (b) IAA by S2 (c) IAA by S4 (d) IAA by S5 (e) IAA by S8

Characterization of indole acetic acid by differential scanning calorimetry (DSC)

DSC analysis was also performed to confirm the presence of Indole-3-Acetic Acid (IAA) in the samples S2, S4, S5, and S8 by comparing their thermal profiles with that of standard IAA. The thermal behaviour of the samples showed a prominent endothermic peak at around 169°C and the melting point of pure IAA ranging 168-169°C. This observation indicated the IAA in all the tested samples. In addition to the characteristic IAA peak, small endothermic peaks were also observed around 100°C, 210°C and 280°C across all the samples.

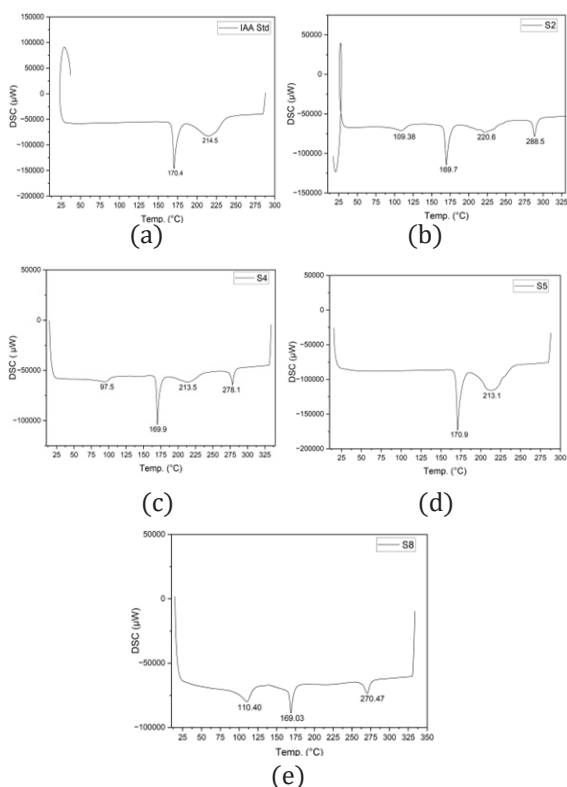


Fig. 11: DSC graph (a) IAA Standard (b) IAA by S2 (c) IAA by S4 (d) IAA by S5 (e) IAA by S8

Discussion

The production of Indole-3-acetic acid (IAA) by bacteria is increasingly recognized as a low-cost alternative method as compared to

chemical production. This study aims to scale up IAA production using four microbial strains *Enterobacter asburiae*, *Bacillus licheniformis*, *Achromobacter mucicolens*, and *Pseudomonas fulva* in a 2-litre fermenter with an optimized Yeast Extract Glycerol (YEG) medium, utilizing crude glycerol obtained from used mustard oil.

Initially production of IAA was checked in shake flask fermentation by all the selected endophytes and maximum production 243.4 µg/ml and 248.66 µg/ml by *Enterobacter asburiae* (S2) and *Achromobacter mucicolens* (S5) respectively. In scale up study amount of IAA was quantified is 1213.2 µg/ml by *Enterobacter asburiae* (S2) and 1665.45 µg/ml by *Achromobacter mucicolens* (S5) and around 10-fold increase in the yield was observed and it is quite high than the (10.7 µg mL⁻¹ IAA) by Sarmiento et al.

This study successfully maintained critical fermentation parameters such as pH, temperature, and agitation speed, leading to consistent IAA production levels. The HPLC results confirm the microbial production for IAA, aligning with recent advancements in biotechnological approaches. For instance, *Enterobacter* strains have been reported to achieve IAA yields of up to 3477 µg/ml through the indole-3-pyruvate pathway, which utilizes tryptophan as a precursor for biosynthesis. In comparison, *Rhizobium* sp. produced 90.21 µg/ml under optimized conditions, showcasing the variability in IAA production across different strains (30;31).

The extraction method involved ethyl acetate followed by High-Performance Liquid Chromatography (HPLC) and Fourier Transform Infrared Spectroscopy (FTIR) analysis, standard techniques for accurately quantifying IAA levels. These methods ensure reliable measurement and characterization of IAA produced during fermentation.

Conclusion

This study highlights the feasibility of scaling up microbial IAA production using

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cost-effective and sustainable fermentation processes. The use of crude glycerol as a carbon source, combined with optimized fermentation conditions, resulted in high yields of IAA comparable to those reported in previous studies. These findings have significant implications for the biotechnological production of phytohormones and their application in sustainable agriculture. Future research could explore further optimization of fermentation parameters and investigate the potential of genetic modifications to further enhance IAA yields.

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