

Communication

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Communication

A Minimal Synthetic IAA Pathway in *Escherichia coli* Using Avocado Seed Hydrolysate: A Sustainable and Didactic Platform for Synthetic Biology

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Abstract

Indole-3-acetic acid (IAA) is the main natural auxin and a key regulator of plant growth. However, most commercial auxins are synthetically produced from non-renewable resources. Here, we present a minimal synthetic biology platform for microbial IAA production that also serves as a teaching model for genetic circuit design and bioprocess development. We developed codon-optimized versions of the *iaaM* and *iaaH* genes, which encode tryptophan 2-monooxygenase and indole-3-acetamide hydrolase, and assembled them into a compact expression cassette in *Escherichia coli* TOP10. Correct expression of both enzymes was confirmed by SDS-PAGE. The engineered strain was cultivated in a low-cost medium made from avocado seed hydrolysate, an agro-industrial waste, supplemented with tryptophan as a precursor. IAA levels of about 300 µg/mL were measured after 48 hours using the Salkowski assay and HPLC, with the medium costing five times less locally than traditional LB. The supernatants containing biosynthetic IAA induced strong root formation in tobacco leaf explants, confirming biological activity. Since this workflow follows the Design–Build–Test–Learn (DBTL) cycle: Design (pathway selection and codon optimization), Build (plasmid assembly), Test (protein expression, metabolite quantification, plant bioassays), and Learn (medium and process optimization), it provides a sustainable production method and an accessible educational platform for synthetic biology.

Keywords: indole-3-acetic acid; auxin; *Escherichia coli*; synthetic biology; genetic circuit; avocado seed hydrolysate; circular bioeconomy; plant growth regulator

1. Introduction

The rapid increase in the global population and the intensification of agriculture have raised serious concerns about food security and environmental sustainability [1]. Achieving a stable food supply without worsening soil degradation, biodiversity loss, and greenhouse gas emissions requires more sustainable methods for producing agricultural inputs. In this context, phytohormones such as auxins play a key role, as they regulate important aspects of plant growth, development, and stress responses, and are widely used as plant growth regulators in agriculture and horticulture [2].

Synthetic biology provides a complementary and increasingly effective method for microbial production of valuable agricultural compounds [3]. Applying rational design principles to engineer metabolic circuits allows for the development of sustainable, scalable, and traceable processes to produce molecules that improve crop productivity, even in challenging conditions like drought or nutrient shortages [4].

Indole-3-acetic acid (IAA) is the main natural auxin in plants and a key regulator of cell division and elongation, apical dominance, organ formation, gravitropism, and nutrient uptake [5]. The

worldwide market for plant growth regulators, including synthetic auxin analogues, has grown steadily in recent years due to increasing demand for tools to improve crop performance [6]. However, the industrial production of IAA and its analogues primarily depends on chemical processes from non-renewable feedstocks, which produce hazardous by-products and face regulatory and environmental limitations [7]. Regulatory restrictions on chemically produced phytohormones in several regions have heightened interest in microbial production as a sustainable alternative [8]. In the context of tightening regulations on synthetic auxins and the growing market for biobased plant growth regulators, microbially produced IAA from renewable substrates represents an attractive alternative that is compatible with emerging sustainable-use policies.

Indeed, microorganisms are attractive hosts for phytohormone production because of their genetic tractability, rapid growth, and suitability for large-scale cultivation. The indole-3-acetamide (IAM) pathway—comprising two enzymatic steps catalyzed by tryptophan 2-monooxygenase (*IaaM*) and indole-3-acetamide hydrolase (*IaaH*)—is the most common Trp-dependent route for IAA biosynthesis in plant-associated bacteria [5] and has been successfully recreated in heterologous hosts. Notably, Wu et al. (2021) achieved IAA titers of up to 7.10 g/L in *E. coli* MG1655 through whole-cell catalysis of the IAM pathway using 10 g/L of external L-tryptophan as substrate [9]; the indole-3-pyruvic acid (IPyA) pathway in *E. coli* DH5 α expressing *ipdC*, *aspC*, and *iad1* produced approximately 1.1 g/L IAA with 2 g/L Trp in LB medium after 48 hours [10]; and de novo IAA biosynthesis from glucose without adding external Trp reached about 0.7 g/L by overexpressing both the Trp biosynthetic pathway and downstream IAA-forming enzymes [11]. A summary of key studies is shown in Table 1. The didactic accessibility column qualitatively scores how easily each platform can be implemented in standard teaching laboratories, based on circuit complexity, strain requirements, and media components. Although these platforms can reach high titers, they generally require significant metabolic engineering, high Trp doses, specialized expression strains, or complex media—limitations that greatly hinder their use in teaching laboratories or resource-limited settings. Therefore, a simple, modular design that maintains the essential enzymatic pathway of auxin biosynthesis while being accessible for teaching and prototype development is needed. Incorporating agro-industrial residues as low-cost culture substrates also supports a circular bioeconomy approach [12].

Beyond its utility as a minimal auxin module, an IAM-based two-gene cassette also provides a convenient readout to probe L-tryptophan overproduction in engineered strains, since IAA titers can directly reflect flux through the Trp pool in chassis optimized for aromatic amino acid biosynthesis [13]. In this sense, the IAM construct can serve as a simple reporter module to evaluate L-tryptophan overproduction strategies in *E. coli*, complementing recent efforts that apply metabolic engineering and fed-batch process optimization to achieve high L-Trp titers.

Table 1. Key studies on IAA production in engineered *Escherichia coli*. IAM, indole-3-acetamide pathway; IPyA, indole-3-pyruvic acid pathway; de novo, pathway from glucose without added tryptophan. Yield shown as μg IAA per mg of L-Trp supplied (when applicable). ND, not determined; —, not applicable.

Reference	Strain	Pathway	Key genes	Medium	Trp added (g/L)	IAA titer Δ (mg/mL)	Yield ($\mu\text{g}/\text{mg}$ Trp)	Didactic accessibility
[10]	DH5 α	IPyA	<i>ipdC</i> , <i>aspC</i> , <i>iad1</i>	LB + 2 g/L Trp	2.0	~1.1 mg/mL	~550	Low (3-gene, specialized promoters)
[9]	MG1655 MIA-6	IAM	<i>iaaM</i> , <i>iaaH</i> (heterologous)	Whole cell + 10 g/L Trp	10.0	7.10 mg/mL	~710	Low (extensive rewiring, metabolic engineering)
[14]	Lemo21(D E3)	IAM	<i>iaaM</i> , <i>iaaH</i> (plant microbiome)	M9 + no Trp added	0	~1.2 $\mu\text{g}/\text{mL}$	—	Moderate (2-gene, SUMO-fusion, biocatalytic focus)
[11]	BL21(DE 3)	IPyA	<i>ipdC</i> , <i>trpABCDE</i> , <i>aldH</i>	Glucose (de novo)	0	~0.7 mg/mL	—	Low (full Trp pathway + de novo)

[15]	<i>DH5α</i>	IAM	<i>iaaM, iaaH</i> (BioBrick)	LB + Trp	ND	~55 μM (~9.6 μg/mL)	ND	Moderate (BioBrick format, LB-based)
[16]	<i>E. coli</i> Nissle 1917	IPyA	<i>aspC, ipdC, iad1</i> + RiboJ	LB + IPTG	ND	μg/mL range	ND	Moderate (3-gene, specialized chassis, biosensor integration)
[17]	Cell-free (chemo (TMO enzyme) enzymatic)	IAM	TMO (<i>iaaM</i> homolog) + acid hydrolysis	Continuous flow, L-Trp feed	ND	11.16 mg/mL/day	48.50 %	Low (requires purified enzyme, flow reactor)
This work	<i>TOP10</i>	IAM	<i>iaaMs, iaaHs</i> (codon-optimized)	ASH + M9 salts + 0.5 g/L Trp	0.5	~303 μg/mL	~60.6	High (2-gene, standard strain, low-cost medium, full DBTL)

^Δ Some titer units are modified from the original authors and reported as mg/mL; it is numerically equivalent to g/L.

We present a straightforward synthetic pathway for producing IAA in *E. coli* using an inexpensive culture medium made from avocado seed waste [18]. We designed and assembled improved versions of the *iaaM* and *iaaH* genes, which encode enzymes required for IAA production, and combined them into a compact package compatible with a common vector. The resulting construct allows IAA synthesis from tryptophan with just two enzymatic steps, and its expression can be easily confirmed by standard laboratory methods. Avocado seed hydrolysate supports the engineered strain's growth and IAA production while reducing the cost of the growth medium by more than five times compared to regular LB, linking synthetic biology with circular bioeconomy concepts.

Along with measuring IAA through colorimetric and chromatographic methods, we demonstrate its effectiveness in a tobacco leaf rooting test, where it consistently promotes root growth at levels higher than those in commercial IAA. The entire process follows the Design–Build–Test–Learn (DBTL) cycle used in synthetic biology: the Design phase involves selecting pathways and optimizing codons; the Build phase includes cloning and assembling the pIAAMHs expression vector; the Test phase comprises analyzing protein expression, measuring metabolites, and validating function in plant tissue; and the Learn phase focuses on understanding how the medium influences the process, the indolic compounds produced, and any challenges that need to be addressed in future designs. Each stage relies solely on widely available strains, vectors, and analytical methods, making the platform directly applicable to teaching labs with limited infrastructure. This work offers a sustainable method for producing microbial IAA and a teaching framework based on the DBTL cycle that can be adapted to various educational and research settings [19].

Figure 1 illustrates the conceptual workflow (left) that outlines the engineering approach within the synthetic biology framework. On the right is the experimental approach used in this study to replicate the conceptual model. Since each step in this workflow relies on standard molecular biology and analytical techniques, the system is particularly well-suited as a hands-on training platform for synthetic biology.

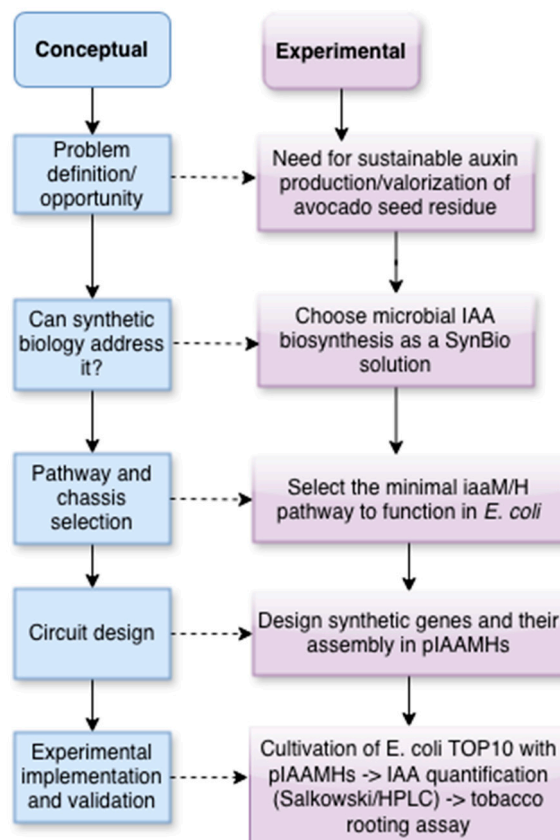


Figure 1. Conceptual and experimental workflow for engineering IAA-producing *E. coli* from avocado seed waste. The diagram maps each stage of the study onto the **Design–Build–Test–Learn (DBTL)** engineering cycle. Left panel (Conceptual layer): the four DBTL phases and their logical dependencies. Right panel (Experimental layer): the related lab activities—**Design**, selecting pathways using computer models and optimizing codons; **Build**, creating and assembling pIAAMHs; **Test**, analyzing protein expression, measuring metabolites, and conducting plant tests; **Learn**, determining media choices and process issues that will guide the next round. Vertical arrows connect each DBTL phase to its experimental implementation, and horizontal arrows indicate the linear progression from problem definition to plant-based validation.

2. Results

2.1. Assembly of the Genetic Circuit for IAA Synthesis in *E. coli*

2.1.1. Design to Complete the Metabolic Pathway for IAA Synthesis from Tryptophan Precursor in *E. coli*

The genetic circuit for IAA production was designed to consider the various pathways in organisms that use tryptophan as a common precursor for IAA synthesis. We chose the pathway with the fewest enzymatic steps to simplify construction. The selected method involves two key enzymes: tryptophan 2-monooxygenase (encoded by *iaaM*) and indole-3-acetamide hydrolase (encoded by *iaaH*). This way, the regulatory circuit was arranged to reflect the natural order of enzymatic reactions, with *iaaM* preceding *iaaH*. The nucleotide sequences of these genes were based on those of *Pseudomonas savastanoi* [19].

2.1.2. Construction of the Plasmid pIAAMHs

Each gene was modified for proper function in *E. coli* and included the standard Shine-Dalgarno sequence from the BioBricks collection, located 6 base pairs before the start codon (ATG), along with a double stop codon. The synthetic genes were inserted into the pUC57 vector at the EcoRV restriction

site, which has blunt ends. Primers with NdeI and EcoRI restriction sites were designed for the *iaaMs* gene, and primers with EcoRI and XbaI sites were made for the *iaaHs* gene (the 's' stands for synthetic) to distinguish it from the original DNA. The primers were designed to ensure correct insertion into the pCOLD™ I expression vector. Using these primers, the genes were amplified from pUC57 with a high-fidelity DNA polymerase. After verifying the sizes of the amplified products by gel electrophoresis, the bands were excised and purified using the Zymoclean Gel Recovery Kit, then inserted into the open pJET1.2 cloning vector at a 3:1 vector-to-insert ratio. The pJET1.2 vector is commercially available and open (linear) with blunt ends at the center of its multiple cloning sites, making it suitable for cloning PCR products and further manipulation. Next, the pJET1.2 vectors containing the respective genes were cut with the corresponding restriction enzymes, and each gene was cloned into the pCOLD I expression vector—first separately to confirm proper protein expression (see below), and then together using the EcoRI restriction site as a common intermediate to ligate both genes. This placed the two genes between the promoter and the transcriptional terminator of pCOLD I. The final construct was verified by DNA sequencing. The resulting genetic circuit plasmid, shown in Figure 2, was named pIAAMHs (“s” for synthetic genes). It was introduced into the *E. coli* TOP10 strain for subsequent experiments.

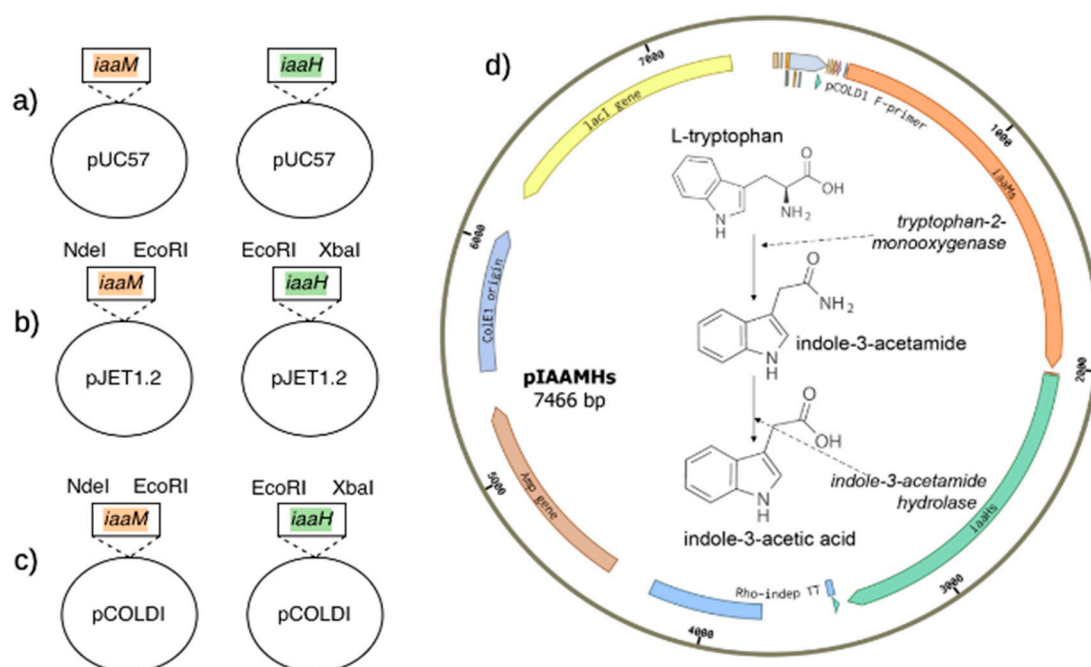


Figure 2. Construction of the genetic circuit to produce IAA from tryptophan in *E. coli*. First, the synthesized genes were received in the pUC57 vector (a). Then, genes were amplified with newly designed primers, and the PCR products were cloned into pJET1.2 (b). After restriction enzyme digestion, the genes were individually cloned into the pCOLD I vector (c). Finally, the gene circuit was assembled in pCOLD I to create the pIAAMHs vector (d). Data and the final plasmid figure were generated in Benchling software [21].

2.2. Verification of Correct Protein Expression for IAA Synthesis in *E. coli*

Before the final assembly of the genetic circuit in pIAAMHs, we performed a thorough assessment to ensure each synthetic gene functions properly. Our main goal was to verify the expression of each protein individually. This step was crucial to confirm that the genetic constructs were properly designed and inserted into the pCOLD I plasmid, so that their transcription is driven by the plasmid promoter and their translation occurs within the correct open reading frame, using the designed translation initiation sequence based on the Shine-Dalgarno. TOP10 *E. coli* cultures, each containing pCOLD I plasmids with the *iaaM* and *iaaH* synthetic genes, were induced and left overnight to produce the proteins. The cellular pellets were separated, heat-disrupted in Laemmli

buffer, clarified, and the supernatant was run on a polyacrylamide gel, as shown in Figure 3. The results confirm that both proteins were expressed at the expected sizes, providing important evidence of successful protein production.

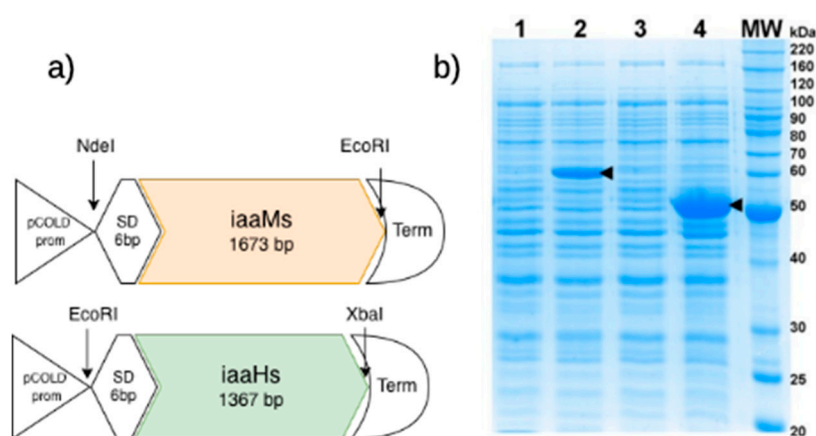


Figure 3. Confirmation of protein expression for IAA synthesis. Individual genetic constructs with genes for *iaaM* (monooxygenase) and *iaaH* (hydrolase) proteins in *E. coli* (a). Induced (lanes 2 for IAAM and 4 for IAAH) and uninduced (lanes 1 and 3) cultures (b). Arrows indicate the recombinant proteins (enzymes) compared to the MW markers.

2.3. IAA Production in *Escherichia coli*

IAA production was assessed using the pIAAMHs construct in ASH medium (avocado seed hydrolysate). This medium was employed to evaluate IAA production in a low-cost culture medium and to integrate avocado agroindustrial waste into a circular economy. An *E. coli* TOP10 strain carrying the empty pCOLD I vector, which lacks the genes necessary for IAA synthesis, served as a negative control. Cultures were grown in 125 mL baffled Erlenmeyer flasks with a working volume of 25 mL. The cultivation conditions included constant agitation at 150 rpm, 0.5 g/L of L-tryptophan as an IAA precursor, and 25 μ L of IPTG (0.1 mM final concentration). The inducing conditions are detailed in the Methods section.

IAA synthesis commenced 4 hours after induction in the ASH medium (not shown) and peaked at 48 hours. IAA production was tracked using the Salkowski assay and thin-layer chromatography (TLC). Figure 4 presents a typical standard curve prepared with sigma IAA and the Salkowski reagent. The figure also illustrates the visual results of the Salkowski reaction with water, medium, non-induced, and induced samples at 48 hours of culture. In Figure 4c, a TLC plate is shown with the non-induced (ni) and induced (in) pIAAMHs, the IAA standard (st), and the mixture of st + in. The medium appears to reduce IAA migration in the samples; the same effect is observed when the commercial standard IAA is mixed with *E. coli*-produced IAA, as in the in + st sample, which runs like the supernatant of the induced culture (in). The Salkowski reaction results indicate that the TOP10 *E. coli* strain harboring the induced pIAAMHs plasmid produces 302.629 ± 3.044 μ g/mL of IAA 48 hours after induction.

To quantify IAA production more accurately, we used an HPLC system (Figure 5). 1 mL of each culture was centrifuged, the supernatant was filtered through a 0.22 μ m filter, and 1 μ L was injected into the HPLC system. Controls included the ASH medium, the TOP10 strain alone, and the strain with the uninduced pIAAMHs plasmid. Only the induced construct (Figure 5e) produced measurable IAA. A calibration curve (not shown) indicated that the TOP10 *E. coli* strain carrying the induced pIAAMHs plasmid produces 313.471 ± 4.064 μ g/mL of IAA at 48 hours after induction. This production is comparable to that calculated using the Salkowski method.

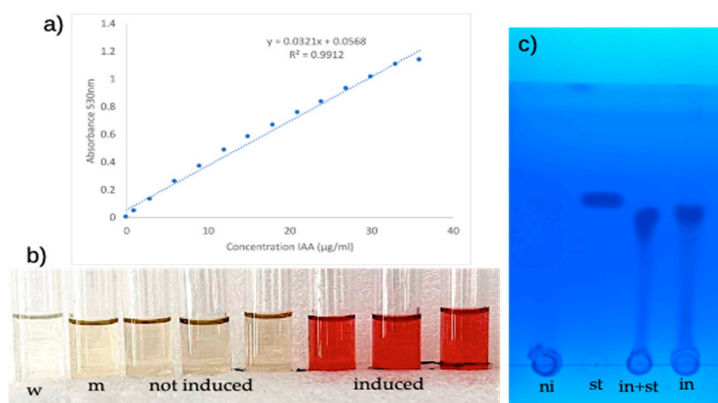


Figure 4. Identification of IAA production in *E. coli*. a) Salkowski standard curve; b) measuring IAA production using the Salkowski method; w, water control; m, medium control; not induced and induced cultures of TOP10 pIAAMHs at 48 hours (repeated three times). TLC of samples was revealed with a UV 355 nm lamp (ni, not induced; st, sigma IAA standard; in, induced; in+st, the mixture of standard plus induced samples).

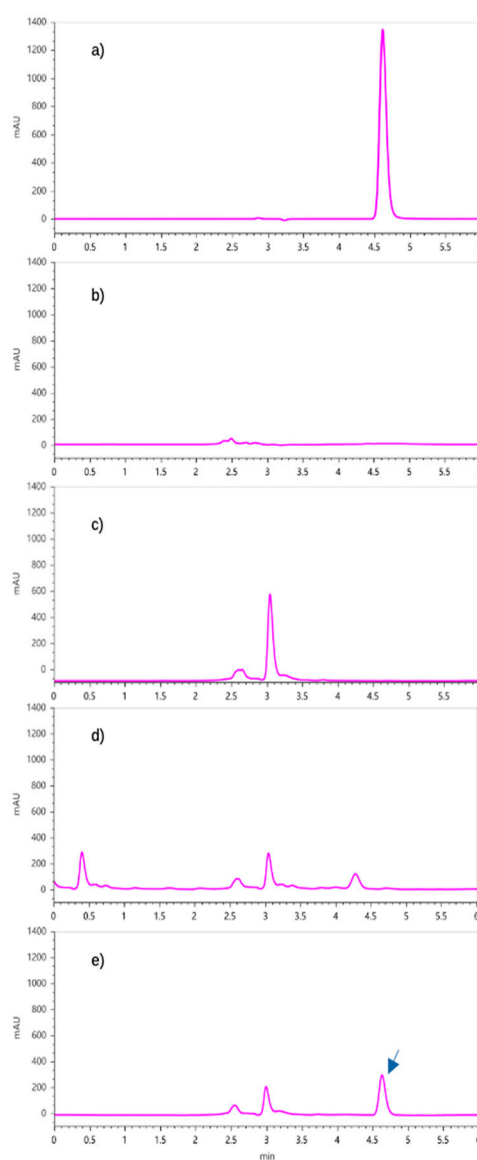


Figure 5. HPLC analysis of IAA production in *E. coli*. a) Standard IAA; b) ASH medium; c) *E. coli* TOP10 culture in ASH; d) TOP10 with pIAAMHs at 48 hours, not induced; e) TOP10 with pIAAMHs at 48 hours, induced. The IAA peak, indicated by an arrow in e), is comparable to the standard in a).

Finally, to test the biological activity of the synthesized IAA, we used tobacco leaf fragments. For this experiment, we used mid-aged leaves, cut them into pieces about 0.25×0.5 cm, and placed the abaxial side on Petri dishes containing solid MS medium with IAA, as shown in Figure 6. After 28 days, IAA treatment resulted in the emergence of roots from the leaf fragments. This effect was observed in approximately 50% of leaf fragments exposed to synthetic IAA, but in 100% of leaf fragments exposed to the same amount of IAA produced by *E. coli*. In four replicated plates (n=40), the Kruskal-Wallis + pair comparisons analysis indicates that the three treatments are different.

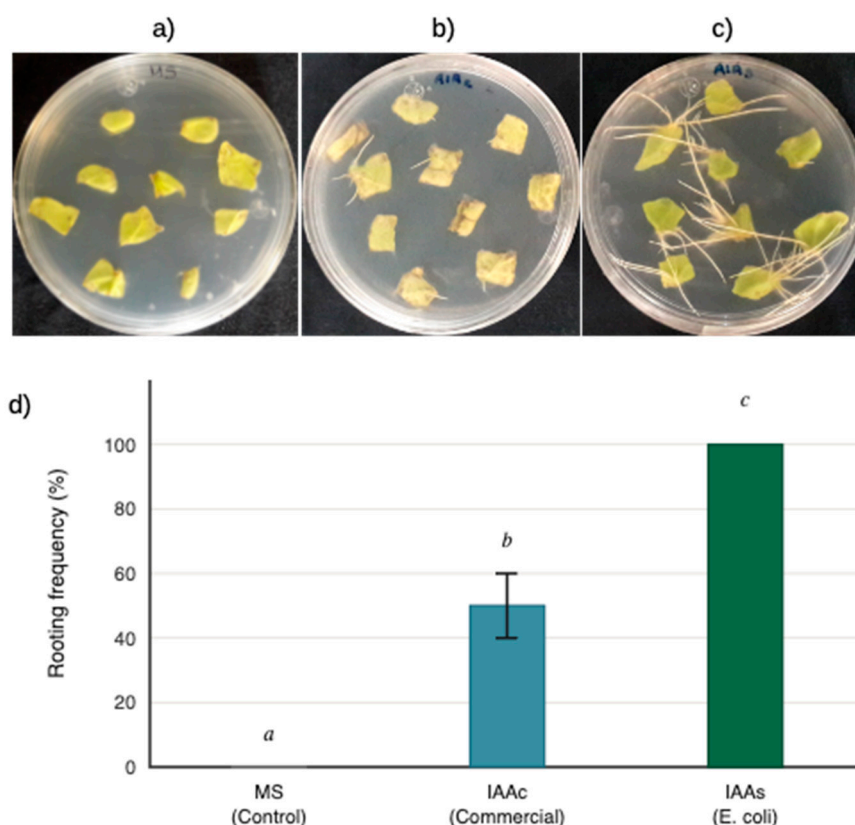


Figure 6. Biological activity of the IAA produced in *E. coli*. **a)** Tobacco leaf fragments in MS medium; **b)** Tobacco leaf fragments in MS medium with 7.5 μM of standard IAA; **c)** Tobacco leaf fragments in MS medium with 7.5 μM of synthesized IAA in *E. coli*. Photographs are taken 28 days after incubation begins. **d)** The same results are shown in a graph based on experiments with quadruplicate samples (n=40).

3. Discussion

This work demonstrates that a functional two-step IAA biosynthetic pathway can be successfully implemented in a standard *E. coli* cloning strain using a compact synthetic construct encoding *iaaM* and *iaaH*. Codon optimization for the *E. coli* K-12 codon usage and the inclusion of a consensus Shine-Dalgarno sequence resulted in a compact expression module with strong protein production under pCOLD I control, as shown by SDS-PAGE at the expected molecular weights for IaaM (~62 kDa) and IaaH (~45 kDa). The IAA titer of approximately 303 $\mu\text{g}/\text{mL}$ obtained in ASH medium with 0.5 g/L external L-tryptophan corresponds to a molar yield of about 60.6 μg IAA per mg of L-Trp supplied (Table 1). Although this titer is significantly lower than those reported in high-yield platforms that use extensive metabolic rewiring, standard LB medium, or high-density Trp loading (Table 1), it is achieved with a two-gene minimal construct in a non-optimized cloning strain and still lies above the range of physiologically active auxin concentrations typically effective at nanomolar to low-micromolar levels in planta and in agronomic applications [22], meaning that

the culture supernatant could be diluted approximately 100–200-fold to reach agronomically active concentrations (1–3 μM), based on measured titer values [5,23].

Beyond its use as a minimal auxin module, the pIAAMHs construct also provides a convenient readout of intracellular L-tryptophan availability, and we have already used this genetic system as a probe in computational and experimental studies to understand and optimize bacterial tryptophan production. Thus, the pIAAMHs cassette not only enables sustainable IAA production but also serves as a practical reporter module for evaluating L-tryptophan overproduction strategies in *E. coli*, as illustrated by our recent work on algorithm-guided optimization of Trp-producing strains [24].

The genetic circuit is based on BioBrick-compatible part design principles, and the pCOLD I expression vector was chosen as the final chassis due to its proven structural stability over a long operational history, in our hands for over ten years. It is also worth noting that a recent reconstruction of the same IAM pathway using SUMO-tagged IaaM and IaaH in *E. coli* Lemo21(DE3) produced only ~1.2 mg/L IAA from endogenous tryptophan without exogenous Trp supplementation [14], underscoring that tryptophan availability is the primary bottleneck in minimal IAM systems and that the ~303 $\mu\text{g}/\text{mL}$ titer obtained here with 0.5 g/L L-Trp in ASH represents a practically relevant production level for a two-gene, single-vector construct. A known limitation of this vector is its requirement for cold-shock induction (temperature downshift combined with IPTG), which limits direct scalability in continuous fermentation systems. This issue is discussed in the DBTL learning phase below.

Cultivating the pIAAMHs construct in ASH medium demonstrated that agro-industrial residues can support both bacterial growth and IAA production at measurable and biologically relevant levels (~303 $\mu\text{g}/\text{mL}$ Salkowski; 313 $\mu\text{g}/\text{mL}$ HPLC at 48 hours). Analysis of cell-associated IAA after biomass sonication confirmed that most IAA is secreted into the culture supernatant, which makes downstream recovery easier. The ASH-based medium is estimated to be 5 times cheaper than traditional LB and is locally available, offering a significant economic benefit for pilot-scale or industrial production. This also supports the circular bioeconomy approach by using avocado seed waste as a fermentation substrate in several studies, as we have done [18,25–28]. However, the requirement for additional L-tryptophan supplementation partially offsets this advantage, as discussed below in the limitations section. The variable composition of ASH—especially its complex sugar and nitrogen content—creates a chromatographic background that complicates baseline separation during HPLC analysis (Figures 5b-d), as discussed further in the Limitations section. This variability presents a challenge for the Learn phase of the DBTL cycle, as it requires students to assess substrate quality, optimize chromatographic methods, and understand the impact on process reproducibility. On the other hand, the ASH medium, although complex by its vegetal origin it is not as rich in amino acids as LB, so IAA production and quantification are cleaner in ASH and are observed either with Salkowski reactions (Figure 4), as in the HPLC profiles (Figure 5), where the strain with the construction but not induced does not produce IAA at a detectable level.

The biosynthetic IAA produced by the pIAAMHs construct in ASH medium showed strong biological activity in the tobacco leaf explant rooting test. Importantly, supernatant from non-induced pIAAMHs cultures did not promote root growth (0% rooting, $n = 40$), confirming that the auxin-like activity depends strictly on circuit induction and IAA production in these conditions. At a concentration of 7.5 μM , the biosynthetic IAA caused root formation in 100% of leaf fragments, with at least one root, compared to about 50% with an equivalent amount of commercial IAA (Kruskal-Wallis, $p < 0.05$, $n = 40$, four replicate plates). The higher rooting success of the biosynthetic mixture versus the pure standard at the same IAA level suggests that other auxin-related compounds may be present in the crude supernatant. HPLC analysis (Figures 5c–e) detected a compound at ~4.3 min in non-induced cultures, which disappears with circuit induction alongside IAA build-up at 4.7 min (Figure 5e). This likely represents a metabolic precursor or shunt product of the IAM pathway—probably indole-3-acetamide (IAM), the direct product of IaaM before hydrolysis by IaaH. Identifying this compound using LC-MS/MS is necessary, as it may contribute to the increased plant response and be relevant to the development of crude IAA-based biological inputs for agricultural

use. Notably, Menon et al. (2022) detected both IAM and IAA by LC-MS in *E. coli* cultures co-expressing *iaaM* and *iaaH*, confirming that incomplete hydrolysis by IaaH is a consistent feature of this pathway in heterologous hosts [14]. This precedent strengthens the interpretation that the 4.3 min peak likely corresponds to IAM and supports the use of LC-MS/MS as the definitive analytical tool.

Beyond its technical outcomes, the system presented here serves as a complete and pedagogically coherent training platform that maps directly onto the DBTL engineering cycle of synthetic biology [29,30]. This alignment is especially valuable because, as recent studies of synthetic biology education have shown, executing a full DBTL cycle within a single course allows students to achieve higher cognitive levels of application, analysis, and synthesis—far beyond what passive instruction alone can provide [31]. The platform is intentionally designed to be adaptable across different academic levels: undergraduate students in introductory biotechnology or molecular biology courses can engage with the Build and Test phases (cloning, SDS-PAGE, Salkowski assay), while graduate students and advanced researchers can expand their work into the Learn phase by optimizing induction conditions, substrate makeup, or genetic circuit design. A structured overview of the proposed learning modules for both levels is provided in Table 2. Using only widely available biological components—such as standard cloning strains (TOP10), a commercially available expression vector (pCOLD I), colorimetric and chromatographic assays (Salkowski, TLC, HPLC), and tobacco explants as a scalable plant model—ensures that the protocol can be easily transferred to teaching labs in institutions with limited infrastructure. Additionally, including avocado seed hydrolysate in the culture medium introduces a practical circular bioeconomy element that can serve as a discussion point for sustainable biotechnology in coursework. This platform could therefore act as a model for research-based courses that connect rational genetic circuit design with the sustainable biosynthesis of important plant hormones. The broad applicability of synthetic biology circuits for phytohormone biosynthesis is further illustrated by the recent engineering of *E. coli* Nissle 1917 to produce IAA in response to inflammatory signals via an inducible IPyA pathway with RiboJ-insulated genetic circuits [16], demonstrating that the IAM and IPyA circuit architectures explored in Table 1 extend across chassis and application domains. Comparing these circuit designs — IAM versus IPyA, inducible versus constitutive, agricultural versus biomedical context — constitutes a natural extension of Module M1 for advanced students.

Several limitations of the current work also identify productive paths for future development within the DBTL framework. First, IAA accumulation in ASH medium requires supplementation with 0.5 g/L external L-tryptophan; by contrast, LB alone supports ~80 µg/mL IAA without added Trp (data not shown), while ASH supplemented with 0.5 g/L Trp achieves ~303–313 µg/mL and valorizes avocado seed waste within a circular bioeconomy framework. This reliance makes ASH less cost-effective than LB when tryptophan costs are factored in; future design iterations should consider overexpressing tryptophan biosynthesis, as in the study by Guo *et al.* [11], or using alternative Trp-rich agro-industrial substrates to enable autonomous precursor production; the multiplex metabolic engineering strategies recently reviewed by Nsanzabera and Liu (2025) provide a practical framework for prioritizing which precursor supply interventions are most impactful in *E. coli* IAA platforms [32]. Second, the cold-shock induction mechanism of pCOLD I limits scalability: while suitable for educational purposes and effective at improving protein solubility at 16°C, this system would need to be replaced with a constitutive or heat-insensitive promoter in larger-scale applications. Replacing pCOLD I with an equivalent constitutive or IPTG-inducible promoter at 37°C is a natural next step in the design process; the continuous-flow chemo-enzymatic approach demonstrated by Mapinta et al. (2025), which achieved gram-scale IAA synthesis using purified TMO (functionally equivalent to IaaM) under mild conditions, illustrates an alternative scalability strategy that bypasses induction constraints entirely and could serve as an advanced comparison case in module M6 [17]. Third, IAA was measured at only one time point (48 hours post-induction); a kinetic profile of production would enhance understanding of productivity dynamics and help determine optimal harvest timing. Fourth, the chromatographic peaks at 4.3 minutes (non-induced) and their

disappearance after induction have not been verified by mass spectrometry; LC-MS/MS analysis would clarify whether these peaks correspond to indole-3-acetamide (IAM), indole-3-acetaldehyde, or other intermediates in the pathway and would strengthen the interpretation that the crude preparation has superior plant bioactivity. Further experimentation can include testing skimmed milk [33] or lactose [34] as replacements for the IPTG in induction. From an educational standpoint, framing each of these limitations as explicit iterative design challenges—with specific experimental modules assignable to student teams—would significantly increase the platform's educational value, particularly by enabling multiple cycles of hypothesis-driven circuit redesign within the same analytical framework.

Table 2. Proposed pedagogical modules for the pIAAMHs DBTL platform, designed for scalable use across undergraduate (UG) and postgraduate (PG) levels. DBTL, Design–Build–Test–Learn cycle; RBS, ribosome-binding site; IAM, indole-3-acetamide; LC-MS, liquid chromatography–mass spectrometry.

Module	DBTL phase	Key activities	Learning objectives	Level	Estimated sessions
M1: Circuit design and codon optimization	Design	Pathway selection (IAM vs. IPyA); sequence retrieval from NCBI; codon optimization using online tools (e.g., Benchling, IDT); Shine-Dalgarno design; primer design with restriction sites	Apply rational design principles to a heterologous metabolic pathway; understand codon usage bias and its effect on translation; design oligonucleotides for directional cloning	UG/PG	2–3 (dry lab)
M2: Modular cloning and plasmid assembly	Build	PCR amplification of synthetic genes; agarose gel verification; restriction digestion and ligation; transformation into TOP10; colony selection and Sanger sequencing verification	Execute a complete multi-step cloning workflow; interpret gel electrophoresis results; apply troubleshooting logic to cloning failures; understand vector-insert compatibility	UG/PG	4–6 (wet lab)
M3: Protein expression verification	Test	Bacterial growth and IPTG/cold-shock induction; cell lysis in Laemmli buffer; SDS-PAGE and Coomassie staining; molecular weight estimation from gel	Verify recombinant protein production; interpret SDS-PAGE results; compare induced vs. uninduced expression profiles; understand induction conditions and their limitations	UG/PG	2–3 (wet lab)
M4: IAA production and quantification	Test	Shake-flask cultivation in ASH medium; Salkowski colorimetric assay and standard curve; TLC with R _f determination; HPLC fluorescence quantification	Apply colorimetric and chromatographic methods for metabolite quantification; calculate production titers and conversion yields; compare analytical methods for accuracy and sensitivity	UG/PG	3–4 (wet lab)
M5: Biological validation	Test	Tobacco leaf explant preparation and aseptic inoculation; MS medium preparation with IAA dilution series; incubation and rooting scoring; statistical analysis (Kruskal–Wallis)	Design and execute a plant bioassay with appropriate controls; apply non-parametric statistics to bioassay data; interpret auxin dose-response in tissue culture	UG/PG	4–5 (wet lab + observation period)
M6: Circuit redesign — promoter/RBS variants	Learn → Design	Comparative promoter analysis (constitutive vs. cold-shock); design of alternative RBS sequences; cloning of variant constructs; repeat M3–M4 with new design	Formulate and test design hypotheses; understand how regulatory elements affect expression; compare performance of multiple circuit variants; practice iterative engineering logic	PG	4–6 (dry + wet lab)
M7: Substrate optimization and metabolome analysis	Learn → Design	Comparison of ASH batches and alternative agro-industrial substrates; time-course IAA quantification; LC-MS/MS identification of co-produced indolic compounds	Understand the effect of medium composition on microbial metabolism; apply mass spectrometry for compound identification; discuss circular bioeconomy implications	PG	4–5 (wet lab + instrument access)

The modular design of pIAAMHs is ideal for iterative redesign. Promoter variants, alternative ribosome-binding site sequences, orthologous *iaaM/iaaH* genes from other phytopathogenic or PGPR bacteria, or different chassis organisms can each be tested as separate DBTL cycles using the same analytical workflow. This modular approach—where student teams independently modify one circuit parameter and compare results with the reference pIAAMHs system—serves as the primary

way to incorporate this platform into advanced synthetic biology or biotechnology courses at both the undergraduate and graduate levels. Thus, while our two-gene system is not designed to compete with state-of-the-art multiplexed IAA platforms in terms of volumetric productivity, it occupies a distinct niche as a didactically accessible, waste-based prototype that can serve both as a teaching tool and as a screening cassette for tryptophan-overproducing strains.

4. Materials and Methods

4.1. Bacterial Strains and Media

For DNA cloning, plasmid transformation, gene expression, and indole acetic acid (IAA) production, we consistently used *Escherichia coli* TOP10 (Thermo Scientific, Waltham, MA, USA). The strain was maintained and cultured in Lysogeny Broth (LB) medium, with carbenicillin (Cb) added at 100 µg/mL when necessary. All molecular biology techniques, including PCR, ligation, vector cloning, and other relevant protocols, were carried out according to the protocols outlined in Current Molecular Biology Protocols [35]. The production medium was based on avocado seed hydrolysate, as described in a previously published work [18]. This sustainable approach aims to utilize natural resources efficiently to produce IAA, aligning with the broader goals of environmentally friendly and economically viable processes.

4.2. Gene Synthesis

The synthetic gene sequences used in this study were purchased from Gene Script Biotech Co. (NJ, USA). Each of the two genes was designed to include a Shine-Dalgarno sequence to promote effective translation initiation, and their codon usage was tailored for the *Escherichia coli* K12 strain. Two nonsense stop codons were placed at the ends of each gene to ensure proper termination of translation. The supplementary materials provide detailed information on the specific gene sequences used in this study for reference. These design features were implemented to maximize translational efficiency and metabolic compatibility with the *E. coli* K-12 expression machinery. Table 3 lists the primers used to clone the synthetic genes.

Table 3. Primers used in this study to clone the synthetic *iaaM* and *iaaH* genes into the pCOLD I expression vector.

Primer name	5'-3' DNA sequences *	descriptions
<i>iaaMrbsF</i>	TCT <u>CATATG</u> CGTCGAGATATA AAGGAGATATA CATG	Forward primer with the NdeI restriction site
<i>iaaMR</i>	<u>GGAATTCGATGGT</u> TAATAGCGATACGATACG AT	Reverse primer with EcoRI restriction site
<i>iaaHrbsF</i>	CCG <u>GAAATTCGATA</u> CCCAATA AAGGAGATATA CATG	Forward primer with the EcoRI restriction site
<i>iaaHR</i>	TGCGTCTAGATATTGGGTCTTAGGTGGTATGC GG	Reverse primer with XbaI for restriction site

*Underlined are the enzymes' restriction sites, in bold is the RBS (ribosome binding site or Shine-Dalgarno sequence), and the beginning protein coding sequences are in italics. .

4.3. Bacterial Culture for Protein Expression

Before preparing the final plasmid to produce indole acetic acid (IAA), it was verified that each protein was properly expressed from the pCOLD I expression vector (Takara Bio Inc., Kusatsu, JP). A single colony, selected for its resistance to carbenicillin and with confirmed genetic circuitry for each gene, was grown overnight in 5 mL of LB medium in 50 mL conical tubes at 37 °C with continuous stirring (150 rpm) until the OD reached 0.6-0.7 at 595 nm. Afterwards, the cultures were

cooled on ice for 20 minutes, induced with 100 μ M isopropyl β -D-1-thiogalactopyranoside (IPTG), and incubated at 16 $^{\circ}$ C for 2 hours, then shifted to 37 $^{\circ}$ C for a total of 48 hours. The induction process followed the instructions in the pCOLD I manual [36] to ensure effective expression of the target genes and successful IAA production.

4.4. Protein Electrophoresis

E. coli colonies were grown overnight at 37 $^{\circ}$ C with agitation at 150 rpm in 50 mL conical tubes containing 5 mL of LB medium supplemented with carbenicillin. To prepare samples for protein electrophoresis, 1 mL from both induced and uninduced cultures was collected. The cells were centrifuged at 10000 rpm for 1 min, and the pellets were resuspended in 100 μ L of 2X Laemmli buffer. The suspension was then heated at 95 $^{\circ}$ C for 10 minutes to denature the proteins. Afterward, the samples were quickly cooled on ice for 5 minutes, centrifuged at 10,000 rpm for 1 minute, and 10 μ L of each supernatant was loaded onto a 10% SDS-PAGE gel. The gels were run until the dye front of the marker reached the end, then stained with Coomassie Brilliant Blue (Bio-Rad, Hercules, CA, USA) and destained to visualize the separated proteins.

4.5. Bacterial Culture for IAA Production

E. coli colonies were pre-cultured overnight at 37 $^{\circ}$ C and 150 rpm in 50 mL conical tubes with 5 mL of LB medium containing carbenicillin. This culture was then used to inoculate 25 mL of fresh ASH medium (10% v/v) in 125 mL shake flasks. The ASH medium was prepared from a concentrated solution to achieve a final reduced-sugar concentration of 16 g/L, as measured with a Brix meter (Sper Scientific 300058 refractometer, Shenzhen, China). Additionally, the ASH solution was supplemented with M9 salts: Na₂HPO₄·7H₂O (7 g/L), KH₂PO₄ (3 g/L), NaCl (0.5 g/L), and NH₄Cl (1 g/L). Cultures were grown in an orbital shaker at 37 $^{\circ}$ C and 150 rpm until reaching an OD of approximately 0.6, then induced with 100 μ M IPTG and supplemented with 0.5 g/L tryptophan as an IAA biosynthetic precursor. The cultures were then incubated at 16 $^{\circ}$ C for 2 hours, shifted to 37 $^{\circ}$ C, and continued for an additional 46 hours (total of 48 hours post-induction) before biomass collection.

4.6. Qualitative Identification of Produced IAA

The qualitative determination of IAA from *E. coli* cell-free cultures was performed using thin-layer chromatography. 50 mL of supernatant was adjusted to pH 3 with 10N HCl. After extracting the sample with twice its volume of ethyl acetate, vigorously mixing, and separating the layers in a separatory funnel, the organic phase was concentrated using a rotavapor. The concentrated extract was then resuspended in 1 mL of ethanol and filtered through a 0.25 μ m filter. The silica gel layer was eluted with a mobile phase composed of 60% ethyl acetate and 40% chloroform (v/v). Chromatograms were visualized under UV light at 355 nm, and the retention factor (R_f) of IAA was determined relative to a synthetic IAA standard (Sigma Co., Burlington, MA, USA).

4.7. HPLC Quantification of IAA

The system includes a quaternary liquid chromatograph (Wayeal LC3210, Anhui Wanyi Science and Technology Co., CN) equipped with a fluorescence detector and an autosampler. Chromatographic separation was conducted at 40 $^{\circ}$ C in a temperature-controlled chamber on a C18 column (4.6 \times 250 mm, 5 μ m, 120 \AA). The mobile phase consisted of 80:20 acetonitrile to water at a flow rate of 1 mL/min, with an injection volume of 1 μ L. The detector's excitation and emission wavelengths were set at 280 nm and 350 nm, respectively.

4.8. In Vitro Rooting Activity of IAA

Supernatants from bacterial cultures were diluted to achieve different levels of indole acetic acid (IAA), as shown in Figure 6. These diluted supernatants were mixed with warm Murashige and Skoog (MS) medium, then poured into Petri dishes to solidify and create a suitable environment for

testing IAA activity. To assess the effect of IAA on plant growth, small squares (approximately 0.25×0.5 cm) of tobacco leaves from one-month-old plants (*Nicotiana tabacum* cv. Xanthi) were carefully excised and aseptically placed onto the prepared Petri dishes. The dishes were incubated in a growth chamber under a 16/8 light/dark photoperiod, with 54 $\mu\text{mol m}^{-2} \text{s}^{-1}$ provided by Sylvania fluorescent white lamps (28°C, RH 35%). This setup allowed observation of potential physiological responses and growth patterns in tobacco leaves exposed to different IAA concentrations in the supernatant. The results of these plant bioassays were vital in determining the effectiveness and influence of the produced IAA on plant growth and development.

5. Conclusions

This study establishes a minimal two-gene synthetic pathway for IAA biosynthesis in *E. coli* TOP10 cultivated in avocado seed hydrolysate, integrating auxin production with agro-industrial waste valorization within a circular bioeconomy framework. The biosynthetic IAA reached a titer of approximately 303 $\mu\text{g/mL}$ at 48 hours after induction, with a molar yield of about 60.6 $\mu\text{g/mg}$ L-Trp, and maintained full biological activity, as shown by its superior root-promoting effect on tobacco leaf explants compared to a commercial IAA standard at the same molar concentration. The root-inducing activity depended strictly on circuit induction, confirmed by the lack of rooting in non-induced culture supernatants, validating the specificity of the genetic construct. The entire workflow—from in silico gene design and codon optimization through modular cloning, protein expression verification, metabolite measurement, and plant bioassay—represents a self-contained implementation of the Design–Build–Test–Learn (DBTL) engineering cycle and offers a structured, accessible training platform for synthetic biology applicable at both undergraduate and postgraduate levels. The platform's main limitations—such as reliance on external Trp, the cold-shock induction requirement of pCOLD I, and the variability of ASH composition—are identified as explicit challenges for future iterative cycles, emphasizing the iterative and self-correcting nature of DBTL-based engineering education. Therefore, this work provides both a sustainable microbial production prototype for a key agricultural plant hormone and a scalable pedagogical framework for training the next generation of synthetic biologists.

Supplementary Materials: The following supporting information can be downloaded at [**Author Contributions:** Conceptualization, A.L.H.-O. and A.M.-A.; methodology, A.L.H.-O. and L.C.A.-R.; software, A.M.-A.; validation, A.L.H.-O.; formal analysis, A.L.H.-O. and L.C.A.-R.; investigation, A.L.H.-O. and A.M.-A.; resources, A.L.H.-O. and A.M.-A.; writing—original draft preparation, A.M.-A.; writing—review and editing, A.L.H.-O. and A.M.-A.; visualization, A.M.-A.; supervision, A.L.H.-O.; project administration, A.L.H.-O. and A.M.-A.; funding acquisition, A.M.-A. All authors have read and agreed to the published version of the manuscript.](https://gitlab.com/amalib/iaa-dbt1: pIAAMHs plasmid, <i>iaaM</i>, and <i>iaaH</i> DNA synthetic sequences in .gb format.</p></div><div data-bbox=)

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