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Optimization of Indole-3-Acetic Acid (IAA) Biosynthesis by *Enterobacter Cloacae* 38 in Defatted Soybean Meal Via Response Surface Methodology

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Abstract

Plant growth promoting rhizosphere (PGPR) research has gained significant interest for improving crop productivity and promoting sustainable agriculture. Among various plant hormones, indole-3-acetic acid (IAA) is a key auxin typically produced by PGPR that supports the growth and development of plants. In this study, *Enterobacter cloacae* 38 was identified as an effective IAA-producing bacterium using defatted soybean meal (DSM), a soybean by-product, as an inexpensive substitute for the commonly used Luria–Bertani (LB) medium. This study reports the optimization of indole-3-acetic acid (IAA) biosynthesis by *Enterobacter cloacae* 38 using defatted soybean meal (DSM) as a low-cost alternative medium through response surface methodology (RSM). Unlike conventional soybean-based media for microbial cultivation or biofertilizer production, this study demonstrates DSM as a cost-effective medium for enhancing microbial IAA biosynthesis compared with commercial LB medium. Key culture conditions, including L-tryptophan concentration, pH, and different carbon and nitrogen sources, were screened and optimized to enhance IAA biosynthesis. One-factor-at-a-time (OFAT) analysis showed that *E. cloacae* 38 produced the highest IAA titer of 529.71 µg/mL after 120 h of fermentation. Among the tested parameters, DSM as a nitrogen source and L-tryptophan concentration had the strongest influence on IAA production, whereas carbon sources had no significant effect. Further optimization using response surface methodology (RSM) increased IAA biosynthesis to 627 µg/mL under the optimal conditions of 20 mg/mL DSM, 5 mg/mL L-tryptophan, pH 9, 37 °C, and 120 h incubation. Post-validation experiments confirmed that the DSM-optimized medium produced 25.6% more IAA than commercial LB medium.

Keywords: Defatted soybean meal (DSM), *Enterobacter cloacae*, Indole-3-acetic acid (IAA), Response surface methodology, Optimization.

1. Introduction

Sustainable agricultural production remains a vital challenge due to increasing global food demand and environmental constraints [1]. Overdependence on synthetic pesticides and chemical fertilizers has led to soil degradation and environmental pollution [2]. Therefore, research related to plant growth-promoting rhizobacteria (PGPR) has gained prominence, as these bacteria can improve crop yield, reduce negative environmental impacts, and ultimately help combat global food security issues.

Generally, PGPR are beneficial bacteria that promote the growth and development of plants through several mechanisms, primarily through the production of phytohormones [3]. Among these phytohormones, indole-3-acetic acid (IAA) is the most widely distributed auxin synthesized by plants, various symbiotic and free-living rhizospheric bacteria, including PGPR [4]. Microbially synthesized IAA plays significant roles in plant growth, particularly in cell differentiation, cell division, cell expansion, plant tissue elongation, fruit development, as well as facilitating nutrient and water uptake, which improves the productivity and quality of high-value crops [5,6,7].

Despite its importance, large-scale usage of commercial IAA is typically hindered by its high production cost, instability, and requirement for extreme processing conditions involving highly toxic substances [5]. These limitations highlight the need for alternative, cost-effective, and environmentally friendly strategies for microbial IAA production. In biotechnology processes, production cost is largely influenced by medium composition and its concentration [5]. Carbon and nitrogen sources, in particular, are key growth factors affecting microbial growth and IAA production. Therefore, the use of low-cost and nutrient-rich substrates is essential to improve economic feasibility while maintaining high IAA yield.

Agricultural waste materials have emerged as attractive low-cost substrates owing to their abundance in nature, nutrient composition, and potential to reduce environmental pollution [8]. Various agro-industrial by-products such as molasses, corn steep liquor, and soybean hydrolysates have previously been utilized as low-cost substrates in microbial fermentation for their high nutrient content and economic advantages [8]. Molasses is rich in fermentable sugars but relatively low in nitrogen content, often requiring additional nutrient supplementation [9]. Corn steep liquor contains soluble proteins and vitamins; however, its composition may vary considerably depending on processing conditions, affecting fermentation consistency. Meanwhile, soybean hydrolysates generally provide readily available amino acids but often require enzymatic or chemical pretreatment, increasing processing costs. Among these wastes, defatted soybean meal (DSM), a soybean industry by-product, remains underexplored for microbial IAA production. DSM is relatively known as a high protein substitute meal for animal feed especially in fish, poultry and swine diets, owing to its rich nutritional composition, which includes approximately 50% protein, 25–30% carbohydrate, and other micronutrients [9]. These characteristics suggest that DSM could serve as an effective organic nitrogen source for microbial growth and biofertilizer development. Although numerous studies have reported microbial IAA production using commercial media and selected nutrient supplements, large-scale application remains economically constrained due to the high cost of refined nitrogen sources such as tryptone and yeast extract. In addition, while soybean-derived materials have been explored in several fermentation processes, the potential of defatted soybean meal (DSM) as a low-cost substrate specifically for microbial IAA biosynthesis remains underexplored. Furthermore, limited studies have investigated the optimization of IAA production by *Enterobacter cloacae* using agricultural by-products integrated with statistical optimization approaches such as response surface methodology (RSM). Therefore, this study aimed to address these limitations by evaluating DSM as an economical nitrogen source and optimizing culture conditions to enhance IAA biosynthesis by *Enterobacter cloacae* 38.

Therefore, this study focused on using the agricultural waste DSM as a low-cost nitrogen source to produce IAA from PGPR strains isolated in a previous study. *Enterobacter cloacae* 38 was selected for further optimization as it demonstrated substantially higher IAA production compared to other bacterial isolates screened in this study. The strain produced more than 20-fold higher IAA concentration than *Bacillus paralicheniformis* GRA2 and *Bacillus velezensis* TAP5 under identical cultivation conditions. Previous studies have also reported *Enterobacter* spp. as highly efficient IAA-producing PGPR with strong potential to promote plant growth and nutrient uptake. Despite these promising characteristics, studies focusing on the optimization of IAA biosynthesis by *Enterobacter cloacae* using low-cost agricultural by-products such as defatted soybean meal remain limited. Therefore, *E. cloacae* 38 was considered a suitable candidate for evaluating sustainable and cost-effective microbial IAA production. The bacterial strains have been characterized as potential PGPR capable of producing IAA using LB-tryptophan commercial media [10]. This study has two objectives: to characterize and select the best IAA-producing strain among three bacterial isolates, and to optimize IAA production using DSM as a low-cost medium via one-factor-at-a-time (OFAT) and Central Composite Design of Response Surface Methodology (RSM-CCD).

2. Materials and methods

2.1 Substrate

Defatted soybean meal (DSM) was obtained from D'syira Enterprise in Serdang, Selangor, Malaysia. DSM is a soybean processing by-product rich in organic nutrients, containing approximately 50% protein, 25–30% carbohydrates, minor lipids, minerals, and micronutrients [9]. Prior to use, DSM was sieved to remove large particles and stored in airtight containers at room temperature under dry conditions until further use. The DSM was used directly as an organic nitrogen source without further chemical or enzymatic pretreatment.

2.2 Microorganisms and Culture Maintenance

Enterobacter cloacae 38, *Bacillus paralicheniformis* GRA2, and *Bacillus velezensis* TAP5 isolated from oil palm empty fruit bunch (OPEFB)–manure compost were used in this study [9,10]. Glycerol stocks stored at –80 °C were revived on nutrient agar (Oxoid, UK) and incubated at 37 °C for 24 h. Single colonies were subcultured routinely and maintained on nutrient agar slants at 4 °C for short-term use.

2.3 Screening of IAA-producing Bacterial Isolates

IAA biosynthesis was screened using a colorimetric assay using Salkowski reagent consisting of 0.5 M of FeCl_3 , 95% (w/w) H_2SO_4 , and distilled water in a ratio of 1:30:50 (v/v/v) [11]. Each bacterial isolate (1% v/v) was cultivated in 100 mL of LB broth (BD Difco™, USA) supplemented with 2 g/L L-tryptophan at 37 °C and 150 rpm for 72 h. The samples were then centrifuged at 10,000 rpm for 10 min to obtain cell-free supernatants. One milliliter of supernatant was transferred into 2 mL of Salkowski reagent before being incubated at room temperature in the dark for 20 min. The development of a reddish color from pink qualitatively indicated the presence of IAA. Then, it was confirmed with a quantitative method by measuring the absorbance at 530 nm using a UV–Vis spectrophotometer (Uviline 9400, Secomam, France). The IAA titer was determined using an IAA standard curve ranging from 0 to 120 $\mu\text{g/mL}$. To validate the specificity of IAA detection obtained from the Salkowski assay, HPLC analysis was performed using authentic IAA standard as reference. The HPLC procedure was performed using MeOH-1% aqueous acetic acid (30:70) as the mobile phase, a C18 column (15×4.6 mm, 5 μm), and a 0.8 mL min⁻¹ flow rate. UV detection was monitored at a wavelength of 254 nm.

2.4 Time-Course Profiling of IAA Production

The isolate exhibiting the highest IAA concentration was selected for time-course analysis. Cultures were grown in LB broth added with 2 g/L L-tryptophan at 37 °C and 150 rpm for up to 168 h. Sampling was performed every 24 h to determine cell growth (OD_{600}), IAA concentration, and pH. The fixed cultivation conditions used in this study (37 °C and 150 rpm) were selected based on preliminary cultivation observations on *Enterobacter* spp. for efficient bacterial growth and IAA biosynthesis. The incubation period for optimization experiments was determined from the time-course profiling study, which demonstrated maximum IAA production at 120 h.

2.5 Screening of Culture Conditions and Medium Components

Preliminary screening of key factors influencing IAA production was conducted using a one-factor-at-a-time (OFAT) technique to identify the major medium components influencing IAA biosynthesis prior to statistical optimization. This approach enabled rapid evaluation of individual variables and reduction of experimental complexity before applying the CCD-RSM model. Since only a limited number of culture parameters were investigated during the preliminary stage, OFAT was considered sufficient for selecting the most influential variables for further interaction and optimization studies.

The effects of L-tryptophan concentration, carbon source, and nitrogen source were evaluated independently under constant incubation conditions (37 °C, 150 rpm, 72 h) [12]. All OFAT experiments were conducted at an initial pH of 7.0. During the screening of carbon sources, each tested sugar was added at 1% (w/v). During the screening of nitrogen sources, each tested nitrogen compound was also added at 1% (w/v). These fixed concentrations were used to enable a direct and consistent comparison among different substrates prior to subsequent statistical optimization.

2.5.1 Effect of L-Tryptophan Concentration

The impact of L-tryptophan concentration on IAA biosynthesis was examined by supplementing LB broth with 0, 1, 2, 5, 7, or 9 g/L L-tryptophan. Cultures were incubated for 72 h, and IAA concentration was determined.

2.5.2 Effect of Carbon Sources

To evaluate the impact of carbon sources on IAA biosynthesis, glucose, galactose, sucrose, fructose, lactose, and dextrose were tested individually by supplementing the carbon component in LB medium. Media were supplemented with NaCl and 2 g/L L-tryptophan to maintain osmotic balance and precursor availability, respectively [13]. Cultures were incubated for 72 h prior to IAA analysis. Positive control comprised cell in LB medium without additional carbon supplementation, while negative control contained the same medium supplemented with carbon sources but no cell.

2.5.3 Effect of Nitrogen Sources

The effect of different nitrogen sources on IAA biosynthesis was determined by replacing the nitrogen components of LB broth (tryptone and yeast extract) with ammonium sulfate, sodium nitrate, tryptone, peptone, yeast extract, skim milk powder, or DSM. All media were supplemented with NaCl and 2 g/L L-tryptophan and incubated for 72 h before analysis. Positive control consisted of cell in LB medium without substituted nitrogen sources, while negative control contained the same medium with replaced nitrogen sources but no cell.

2.6 Optimization of IAA Biosynthesis Using Response Surface Methodology

Based on preliminary results, DSM concentration, L-tryptophan concentration, and initial pH were selected as independent parameters for IAA biosynthesis optimization via central composite design (CCD) under RSM. A total of 20 experimental runs, with six center points, were designed by Design-Expert® software (version 11, Stat-Ease Inc., Minneapolis, MN, USA) [9]. Every variable was analyzed at five levels coded as $-\alpha$, -1 , 0 , $+1$, $+\alpha$, where α represents the axial distance required to ensure rotatability of the design. All experiments were conducted at 37 °C, 150 rpm, and an incubation period of 120 h. A second-order polynomial function, including linear, quadratic, and interaction effects, was assumed for the system's behavior, as depicted in Equation 1.

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_{ij} \quad (\text{Equation 1})$$

where (Y) represents the predicted IAA concentration, (β_0) is the intercept coefficient, (β_i) represents the linear coefficients, (β_{ii}) represents the quadratic coefficients, (β_{ij}) represents the interaction coefficients, and (X_i) and (X_{ij}) are the coded independent variables [9]. Analysis of variance (ANOVA) was conducted to evaluate the statistical significance and adequacy of the model and its individual terms. To visualize the interaction effects between the parameters and to identify the optimal fermentation conditions for maximum IAA production, response surface plots were generated.

2.7 Model Validation

To validate the optimized conditions predicted by RSM, IAA production by selected strain was evaluated under the optimized DSM-based medium and compared with commercial LB medium. Sampling was performed every 24 h up to 120 h, and IAA concentration was quantified. For model validation, all cultivation conditions including inoculum size, incubation temperature (37°C), agitation speed (150 rpm), fermentation duration, and sampling intervals were maintained identical for DSM-based and LB-based media. The culture medium composition was the only variable altered to evaluate the effect of DSM on IAA biosynthesis.

2.8 Statistical Analysis

All experiments were conducted in triplicate, and data are presented as mean $\pm$ standard deviation (SD). One-way analysis of variance (ANOVA) was performed using SPSS software (version 25.0, IBM, USA), followed by Tukey's post hoc tests for multiple means comparisons. Statistical significant was set at $p < 0.05$.

3. Results and discussion

3.1 Screening of bacterial isolates for IAA production

Three bacterial isolates (*Enterobacter cloacae* 38, *Bacillus paralicheniformis* GRA2, and *Bacillus velezensis* TAP5) were screened for their capacity to synthesize indole-3-acetic acid (IAA) via Salkowski colorimetric assay after 72 h of incubation in LB medium supplemented with L-tryptophan [14-15]. All isolates showed positive color development (from pink to red), indicating the capacity to synthesize IAA; however, substantial quantitative differences were observed among the strains. *Enterobacter cloacae* 38 produced significantly higher IAA concentration ($326.04 \pm 14.36 \mu\text{g/mL}$) ($p < 0.05$) compared to *Bacillus paralicheniformis* GRA2 and *Bacillus velezensis* TAP5, with an IAA yield exceeding 20-fold relative to the Bacillus isolates (Table 1). Similar observations have been reported in previous studies where *Enterobacter* spp. demonstrated substantially greater IAA biosynthesis capacity than several other PGPR genera due to efficient tryptophan utilization and auxin biosynthetic activity [16-18]. The higher IAA production exhibited by *Enterobacter cloacae* 38 may be associated with the efficient utilization of tryptophan-dependent IAA biosynthetic pathways commonly reported in *Enterobacter* spp., including the indole-3-pyruvate (IPyA) pathway [17-19]. Previous studies have shown that *Enterobacter* species possess strong metabolic adaptability and efficient amino acid assimilation systems that enhance precursor conversion toward auxin biosynthesis [18,19]. In addition, regulatory mechanisms associated with nitrogen metabolism and secondary metabolite production may further contribute to higher IAA accumulation than the tested Bacillus strains under similar cultivation conditions. Based on its significantly higher IAA yield, *E. cloacae* 38 was selected for subsequent experiments.

Table 1 IAA concentration of different IAA-producing strains after 72 hours incubation in LB medium supplemented with 2g/L L-tryptophan.

Sample	IAA concentration ($\mu\text{g/mL}$)
Control	0.00 ± 0.00
E38	326.04 ± 14.36
GRA2	13.80 ± 4.42
TAP5	15.89 ± 5.89

3.2 Time-Course Profiling of IAA Production by *Enterobacter cloacae* 38

The profiling pattern of cell growth, IAA production, and pH variation by *E. cloacae* 38 was monitored over 168 h. Based on Figure 1, IAA production progressively increased with incubation time and reached a maximum concentration ($529.71 \pm 13.77 \mu\text{g/mL}$) at 120 h. The gradual accumulation of IAA alongside increasing bacterial growth suggests that IAA biosynthesis was associated with active cell metabolism and continued during prolonged cultivation. After 120 h, a reduction in IAA concentration was observed, potentially due to enzymatic degradation of IAA, oxidative decomposition, reassimilation by bacterial cells, or reduced stability under prolonged incubation conditions [17–19]. Previous studies have reported that certain microorganisms are capable of utilizing IAA as a secondary carbon source or metabolizing IAA through catabolic pathways when nutrients become limited during extended cultivation periods. In addition, changes in medium pH and accumulation of metabolic by-products may further influence IAA stability and bioavailability. Similar declining trends in microbial IAA concentration after prolonged fermentation have also been reported in *Enterobacter* spp. and other PGPR cultures [6,16,18]. The culture pH steadily increased from near neutral to alkaline throughout incubation and achieved the highest pH at 168 hours (8.67 ± 0.01). The gradual increase in culture pH observed during incubation may be associated with bacterial amino acid metabolism and the release of alkaline metabolites such as ammonium ions during nitrogen assimilation processes [19,21]. Similar pH elevation trends during microbial fermentation have been previously reported in *Enterobacter* and other bacterial cultures cultivated in protein-rich media. However, further metabolic analysis is warranted to confirm the exact mechanisms contributing to pH variation in the present study.

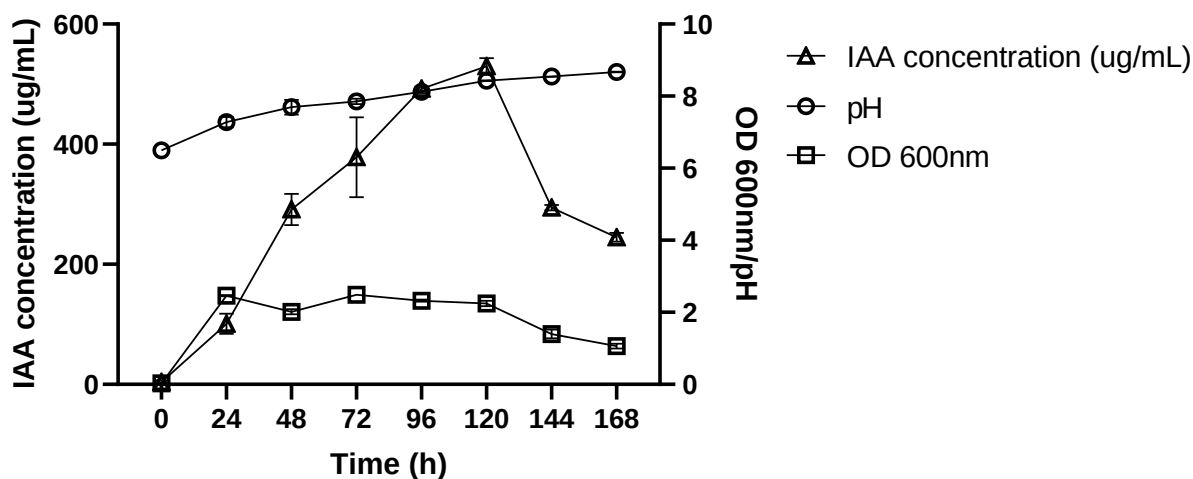


Figure 1 IAA production profiling of *Enterobacter cloacae* 38 throughout 168-hour incubation in LB medium supplemented with 2g/L. Each data point represents the mean $\pm$ SD of three replicates.

3.3 Effect of Medium Components on IAA Biosynthesis

3.3.1 Effect of L-Tryptophan Concentration

L-tryptophan concentration had a significant impact on IAA production by *E. cloacae* 38 (Figure 2A). The concentration of IAA was directly proportional to the increase in L-tryptophan concentration, in which 9 g/L L-tryptophan achieved the highest IAA concentration of $734.78 \pm 26.64 \mu\text{g/mL}$. This indicates the tryptophan-dependent nature of IAA biosynthesis in *E. cloacae*, in which L-tryptophan acts as the major precursor for auxin synthesis [17,19]. However, the increase in IAA concentration beyond 2 g/L was non-significant, suggesting substrate saturation or feedback regulation of the IAA biosynthetic pathway, indicating that excessive precursor supplementation may not be economically advantageous.

3.3.2 Effect of Carbon Sources

Different carbon source supplementations produced no significant improvement in IAA biosynthesis compared with positive control or among the carbon sources tested, despite glucose yielding the highest measured IAA concentration ($20.44 \pm 9.76 \mu\text{g/mL}$) and dextrose showing no detectable IAA production ($0 \mu\text{g/mL}$) (Figure 2B). This indicates that carbon availability was not a limiting factor for IAA production under the tested conditions. Although certain carbon sources such as glucose showed relatively higher measured IAA concentrations compared to other treatments, statistical analysis revealed no significant differences ($p > 0.05$) among the tested carbon sources and the positive control. The observed numerical variations may reflect normal biological variability rather than a substantial effect of carbon source supplementation on IAA biosynthesis under the tested conditions.

The limited impact of carbon sources suggests that carbon substrates were primarily utilized for cellular maintenance and energy generation rather than secondary metabolite production, especially in the absence of an optimal nitrogen source. Similar results have been reported where nitrogen availability exerted a greater regulatory effect on IAA biosynthesis than carbon source selection [20-21].

3.3.3 Effect of Nitrogen Sources

In contrast to carbon sources, nitrogen sources markedly influenced IAA production (Figure 2C). DSM exhibited the highest IAA concentration ($313.93 \pm 6.81 \mu\text{g/mL}$), followed closely by yeast extract ($305.729 \pm 8.84 \mu\text{g/mL}$) and peptone ($296.875 \pm 0.74 \mu\text{g/mL}$). The superior performance of DSM in supporting IAA biosynthesis may be attributed to its rich organic nutrient composition, particularly its high protein and peptide content, which can enhance bacterial growth and precursor availability for tryptophan-dependent auxin biosynthesis pathways [9,17–19]. DSM contains abundant amino acids, carbohydrates, vitamins, and micronutrients that may support cellular metabolism and enzymatic activities involved in the conversion of L-tryptophan into IAA intermediates such as indole-3-pyruvate (IPyA). In addition, organic nitrogen sources are generally more readily assimilated than inorganic nitrogen sources, allowing improved metabolic flux toward secondary metabolite production, including auxin biosynthesis [20,21]. The presence of peptides and slowly metabolized nutrients in DSM may also provide sustained nutrient release during prolonged cultivation, contributing to enhanced IAA accumulation compared to conventional LB medium. Inorganic nitrogen sources such as ammonium sulfate and sodium nitrate resulted in minimal IAA production, potentially due to limited assimilation efficiency and lack of growth-associated cofactors/micronutrients required for IAA synthesis. These results support the selection of DSM as a promising low-cost nitrogen source for further optimization.

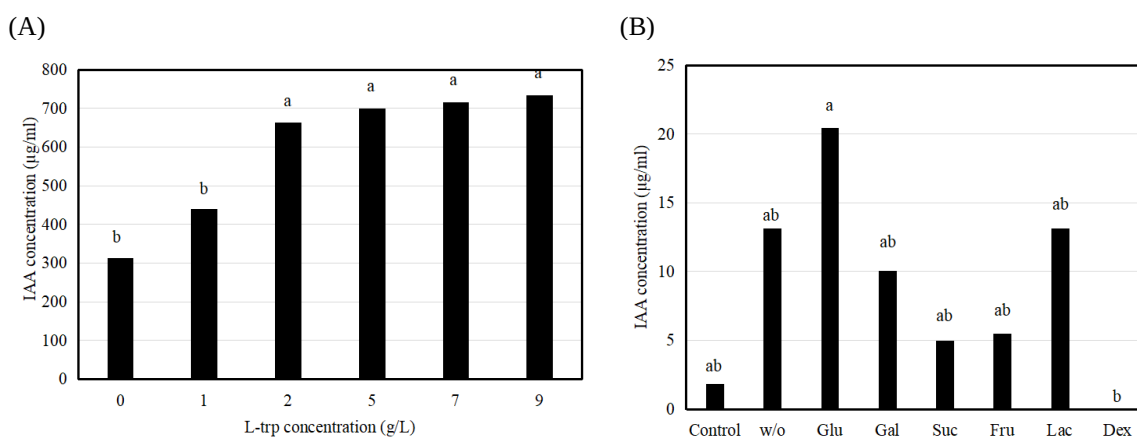


Figure 2 IAA concentration of *Enterobacter cloacae* 38 under different culture conditions: (A) various L-tryptophan concentrations supplemented in LB medium after 72 h incubation; (B) various carbon sources supplemented with NaCl and 2 g/L L-tryptophan after 72 h incubation; and (C) various nitrogen sources supplemented with NaCl and 2 g/L L-tryptophan after 72 h incubation. Each data point represents mean $\pm$ SD of three replicates. Different lowercase letters indicate statistically significant differences at $p < 0.05$.

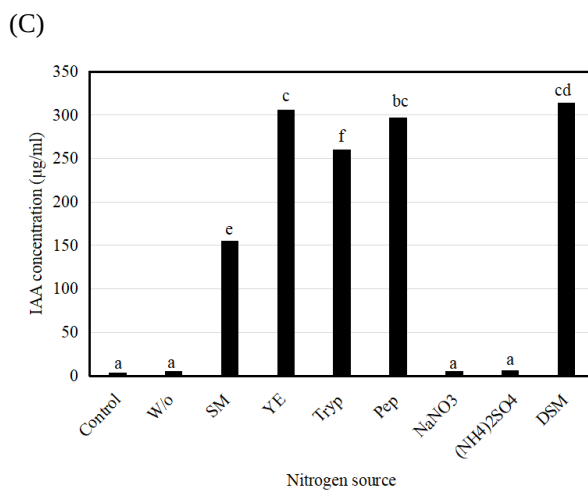


Figure 2 IAA concentration of *Enterobacter cloacae* 38 under different culture conditions: (A) various L-tryptophan concentrations supplemented in LB medium after 72 h incubation; (B) various carbon sources supplemented with NaCl and 2 g/L L-tryptophan after 72 h incubation; and (C) various nitrogen sources supplemented with NaCl and 2 g/L L-tryptophan after 72 h incubation. Each data point represents mean $\pm$ SD of three replicates. Different lowercase letters indicate statistically significant differences at $p < 0.05$. (Cont.)

3.4 Optimization of IAA Biosynthesis Using Response Surface Methodology

According to preliminary results, DSM concentration, L-tryptophan concentration, and initial pH were selected for optimization using central composite design (CCD). The experimental results showed wide variation in IAA biosynthesis, ranging from 9.64 to 543.62 $\mu\text{g/mL}$, indicating strong interactive effects among the selected parameters (Table 2). To identify the optimum level of each parameter to produce maximum IAA titer, a second-order polynomial model was designed. A second-order polynomial (Equation 2) was developed to predict IAA concentration.

$$Y = 434.51 + 91.88A + 96.24B + 42.12C + 98.61AB + 57.46AC + 4.17BC - 126.37A^2 - 31.88B^2 - 39.93C^2 \quad (\text{Equation 2})$$

The regression analysis of the second-order polynomial model demonstrated a significant fit to the experimental data ($p < 0.001$) with an R^2 value of 0.9172 (91.72%) (Table 3) [4-5,22]. An R^2_{Adj} value of 0.8427 indicates a good model fit with low predication error, confirming the model's significance [22]. The lack of fit yielded an F value of 4.91 and a p value of 0.0528, indicating that the model was not significantly related to pure error [22]. Initial pH and DSM concentration were identified as the most influential factors affecting IAA production due to high F-values comparatively to L-tryptophan. In addition, evaluating the mutual interaction between the parameters is crucial according to the p values. Based on Table 3, all the independent parameters except L-tryptophan concentration showed a significant impact on IAA production, while, in terms of interaction between variables, only AB and A2 had a significant effect on IAA production. These interactions are typically illustrated using graphical representations derived from the regression equations, including 3D response surface curves. Figure 3 illustrates that increasing pH up to 9 and DSM concentration up to 20 mg/mL significantly increased IAA production; however, DSM concentration, with a higher F value (22.76), had a greater impact on IAA production than pH (20.75). The significant interaction between pH and DSM concentration suggests that nitrogen availability is strongly pH-dependent in regulating IAA biosynthesis. The model predicted a maximum IAA production of 626.86 $\mu\text{g/mL}$ at pH 8.95, 20 mg/mL DSM, and 5 mg/mL L-tryptophan.

The significant interaction observed between DSM concentration and initial pH indicates that nutrient utilization and IAA biosynthesis by *E. cloacae* 38 were strongly influenced by the surrounding physicochemical environment. Initial pH may affect nutrient solubility, membrane transport activity, enzyme stability, and the metabolic conversion efficiency of L-tryptophan into IAA intermediates [17–19]. At alkaline pH conditions, improved availability and assimilation of organic nitrogen compounds from DSM may have enhanced cellular metabolism and auxin biosynthetic activity. In addition, DSM concentration likely influenced the balance between nutrient availability and metabolic regulation, where insufficient DSM may limit precursor supply, while excessive nutrient concentration could alter metabolic flux and reduce biosynthetic efficiency. The interaction between DSM and L-tryptophan further indicates that optimal precursor utilization depends on sufficient organic nutrient availability to support enzymatic conversion toward IAA production.

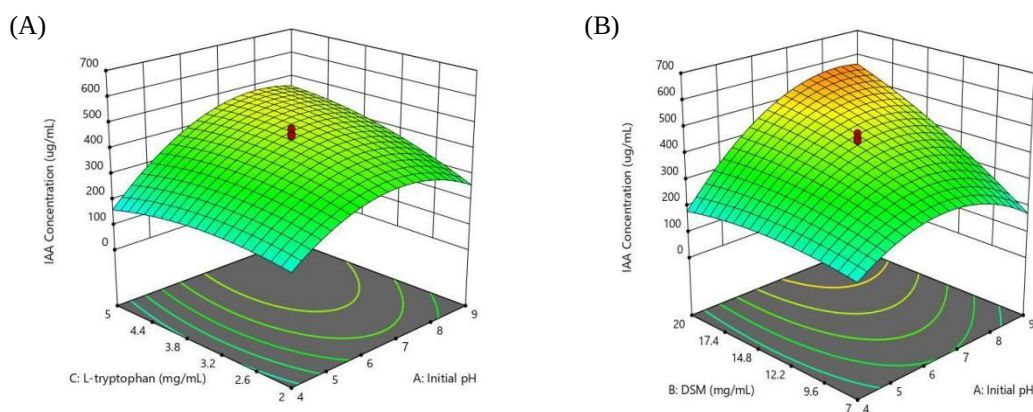
Table 2 Experimental design and RSM-CCD results.

Run	A: Initial pH	B: DSM (mg/mL)	C: L-tryptophan (mg/mL)	IAA Concentration (µg/mL)	
				Observed	Predicted
1	6.5	13.5	3.5	332.16	386.59
2	6.5	13.5	6.02269	378.91	430.69
3	6.5	13.5	3.5	310.42	372.5
4	4	7	2	112.37	122.84
5	9	7	2	152.34	134.81
6	6.5	24.4317	3.5	411.98	578.15
7	10.7045	13.5	3.5	144.01	172.81
8	9	20	2	331.77	386.13
9	4	7	5	128.78	142.53
10	6.5	13.5	3.5	399.22	479.06
11	9	20	5	543.62	652.34
12	6.5	13.5	3.5	376.82	452.19
13	2.29552	13.5	3.5	9.64	6.76
14	4	20	2	222.79	140.14
15	9	7	5	186.59	223.91
16	6.5	13.5	3.5	377.08	452.5
17	6.5	13.5	0.977311	252.21	237.86
18	4	20	5	13.41	16.09
19	6.5	13.5	3.5	383.20	459.84
20	6.5	2.56835	3.5	113.28	135.94

Table 3 A full second-order polynomial model's regression analysis for IAA concentration optimization of *E. cloacae* 38.

Source	Sum of Squares	Df	Mean Square	F-value	p-value	Remarks
Model	615600	9	68397.86	12.31	0.0003	Significant
A- Initial pH	115300	1	115300	20.75	0.0010	
B- DSM	126500	1	126500	22.76	0.0008	
C- L-tryptophan	24230.52	1	24230.52	4.36	0.0633	
AB	77793.43	1	77793.43	14.00	0.0038	
AC	26412.06	1	26412.06	4.75	0.0542	
BC	139.19	1	139.19	0.0251	0.8774	
A ²	230100	1	230100	41.42	< 0.0001	
B ²	14644.89	1	14644.89	2.64	0.1355	
C ²	22975.66	1	22975.66	4.14	0.0694	
Residual	55562.56	10	5556.26			Not significant
Lack of Fit	46161.66	5	9232.33	4.91	0.0528	
Pure Error	9400.90	5	1880.18			
Cor Total	671100	19				

Fit statistics: $R^2 = 0.9172$; Adjusted $R^2 = 0.8427$; Predicted $R^2 = 0.4273$; C.V.% = 24.91; df: degree of freedom

**Figure 3** Response surface curve for the interactions among initial pH (A), DSM (B), and L-tryptophan (C) that towards the IAA production by *E. cloacae* 38.

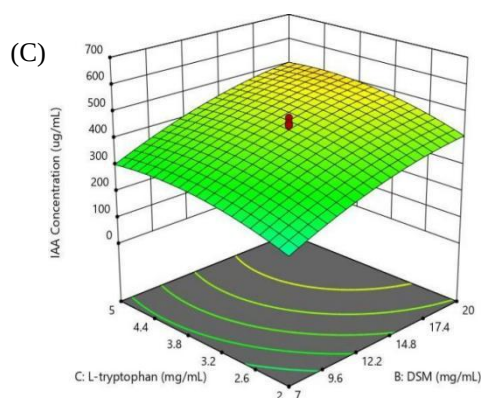


Figure 3 Response surface curve for the interactions among initial pH (A), DSM (B), and L-tryptophan (C) that towards the IAA production by *E. cloacae* 38. (Cont.)

3.5 Model Validation and Comparison with Commercial Medium

Experimental validation under optimized conditions yielded an IAA concentration of 650.52 ± 26.96 µg/mL, closely matching the model prediction with 96.2% accuracy. This confirms the robustness and reliability of the RSM model despite inherent biological variability. When compared with commercial LB medium (518.06 ± 15.12 µg/mL), the optimized DSM medium produced 25.6% higher IAA (650.52 ± 26.96 µg/mL) after 120 h (Figure 4). According to these results, it can be concluded that combining DSM with the precursor L-tryptophan (5 mg/mL) at alkaline pH can effectively substitute for expensive refined media while enhancing auxin production.

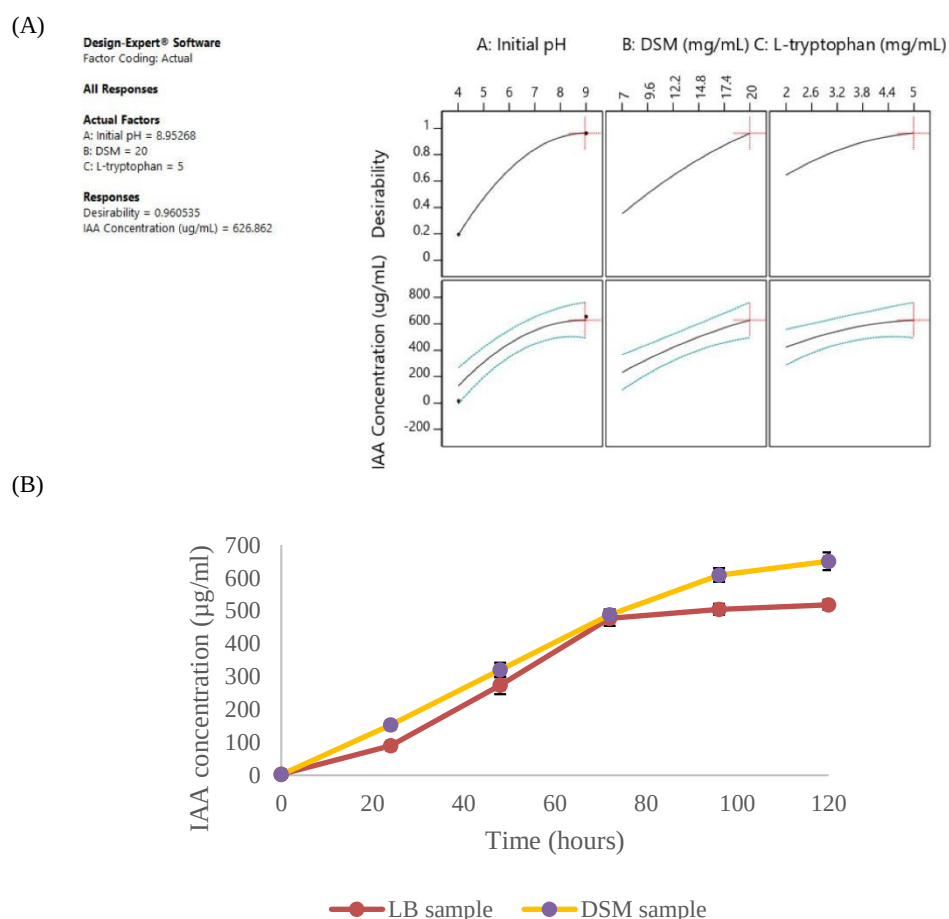


Figure 4 Validation of the optimization model: (A) desirability-based prediction; (B) time profiling of *Enterobacter cloacae* 38 IAA production in LB supplemented media and optimized DSM media throughout 120 hours incubation. Each data point represents the average mean $\pm$ SD of three replicates.

3.6 Economic and Practical Implications

The DSM-based medium with additional L-tryptophan was calculated to be 99.35% cheaper than commercial LB medium with additional L-tryptophan (Table 4). This higher cost arises because commercial LB medium contains expensive tryptone and yeast extract as part of its composition [23]. However, in this optimized DSM medium, those expensive nitrogen sources have been replaced with cheap DSM. This substantial cost reduction highlights the feasibility of DSM as a substrate for large-scale and sustainable IAA production. Although minor preprocessing may be required to improve nutrient bioavailability, the overall economic and environmental benefits strongly favor DSM as an industrial fermentation substrate. While the present findings demonstrate the feasibility of utilizing DSM as a low-cost substrate for enhanced microbial IAA biosynthesis, further studies involving bioreactor-scale cultivation, process stability, downstream processing, and comprehensive economic assessment are required before industrial-scale application can be established.

Table 4 Percentage cost difference between DSM–tryptophan optimized medium and commercial LB–tryptophan medium using feed-grade L-tryptophan (per liter volume).

Media	Base media cost (per liter)	Addition of 1g/L L-trp (feed grade)	Total cost (per L)
LB broth- tryptophan	1.95 USD	0.01 USD	1.96 USD
DSM-tryptophan	0.0025 USD	0.01USD	0.13USD

4. Conclusion

In conclusion, all objectives have been successfully achieved, demonstrating the potential of *Enterobacter cloacae* 38 as an efficient IAA-producer using a low-cost defatted soybean meal (DSM)-based medium. Optimization using Central Composite Design (CCD) revealed the maximum IAA biosynthesis of 650.52 ± 26.96 µg/mL under optimal conditions comprising 20 mg/mL of DSM supplemented with 5 mg/mL of L-tryptophan, initial pH 9, temperature 37 °C, and a fermentation period of 120 h. This result suggests that *E. cloacae* 38 can achieve a high IAA yield when cultivated in optimized DSM medium supplemented with L-tryptophan as an effective and economical nitrogen source. Comparative evaluation using conventional LB broth further validated the superiority of the optimized DSM medium, which resulted in a 25.6% higher IAA production at 120 h. This study demonstrates the potential of *Enterobacter cloacae* 38 as an efficient microbial producer of indole-3-acetic acid (IAA) using defatted soybean meal (DSM) as a sustainable low-cost substrate. Beyond achieving enhanced IAA production through RSM-based optimization, this study provides new insight into the feasibility of replacing expensive commercial media with nutrient-rich agricultural by-products for microbial auxin biosynthesis. The findings contribute to the development of economically viable and environmentally sustainable strategies for PGPR-based biofertilizer production and highlight the broader potential of agro-industrial waste valorization in microbial biotechnology.

These findings highlight the potential of DSM as an economical substrate for laboratory-scale microbial IAA production and provide a foundation for future scale-up and process development studies involving PGPR-based biofertilizer applications. The successful application of defatted soybean meal (DSM) in enhancing IAA biosynthesis demonstrates the potential of agricultural by-products as economical fermentation substrates for PGPR-based metabolite production. Future studies may further investigate other nutrient-rich agro-industrial residues with comparable composition to improve process sustainability and evaluate their suitability for large-scale microbial IAA production.

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6. Authors contributions

Razak, AA.: Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft; Roslan, MAM.: Conceptualization, Formal analysis, Methodology, Software, Supervision, Validation, Writing - review and editing; Halim, M.: Supervision, Validation, Writing – review & editing; Pannerchelvan, S.: Validation, Writing - review and editing; Rahman, NA.: Conceptualization, Resources, Funding acquisition, Project administration, Supervision, Validation, Writing – review & editing.

7. Conflicts of interest

The authors declare that they have no conflict of interest.

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