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Original Paper

Optimization of IAA Production by Halotolerant Bacterial and Fungal Consortia

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The current study focused on optimizing the production of indole-3-acetic acid (IAA) by halotolerant bacterial and fungal consortia, which are crucial for promoting plant growth under saline conditions. Initial screening using Plackett-Burman design identified pH, tryptophan concentration, and incubation period as the most significant factors affecting IAA synthesis. These factors were further optimized using Response Surface Methodology (RSM), resulting in maximal IAA yields of up to 125 µg/mL under conditions of pH 6.25–7.0, tryptophan 0.7–1.2 mg/mL, and incubation of 8.5–12 days. The statistical model exhibited strong significance ($p < 0.0001$) with non-significant lack-of-fit, confirming the model's validity. The findings suggest that tailoring these parameters can significantly boost IAA production in halotolerant consortia, enhancing their potential as bioinoculants in saline-stressed soils. This work provides a solid foundation for developing effective microbial formulations to improve plant productivity in challenging environments.

Keywords: *Bacillus*, CCD-RSM, Halotolerant, IAA, Plackett Burman

1 Introduction

One of the most crucial characteristics of plant growth-promoting microorganisms (PGPMs) is their ability to synthesize phytohormones, particularly indole-3-acetic acid (IAA), which is essential for regulating plant development (Husen, 2003). This trait has been widely studied in both symbiotic and non-symbiotic plant growth-promoting rhizobacteria (PGPR) as well as in phosphate-solubilizing fungi. These microbes directly stimulate plant growth through the production of key hormones such as auxins and gibberellins. As demonstrated by Hayat et al. (2010), several bacterial genera produce IAA and indole-3-ethanol, compounds belonging to the auxin class while earlier work by Vessey (2003) confirmed that auxin biosynthesis is not limited to plants but is also a microbial function. In addition to hormone synthesis, PGPMs contribute to improved plant nutrition by solubilizing inorganic phosphate and mineralizing organic phosphorus compounds, thus enhancing phosphorus availability in the rhizosphere (Xie et al., 1996).

However, a major challenge in agricultural application of these bioinoculants is the reduced performance under salinity stress, which is a growing problem in

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many regions (Tufail et al., 2021). Saline soils disrupt microbial processes and reduce nutrient bioavailability, thereby undermining the efficacy of IAA-producing microorganisms. Although many studies have focused on optimizing IAA production under laboratory conditions, they often overlook the practical reality that field-level performance deteriorates under elevated salt concentrations (Abbas et al., 2019). This creates an urgent need for microbial isolates that not only produce high levels of IAA but also tolerate saline conditions.

Conventional methods for improving IAA synthesis typically involve empirical adjustments that fail to account for the complex physicochemical interactions affecting microbial metabolism. In contrast, statistical tools like Plackett–Burman Design (PBD) and Response Surface Methodology (RSM) offer a structured and efficient means to identify and optimize multiple factors simultaneously (Sasirekha & Shivakumar, 2012). These approaches allow for better prediction and enhancement of microbial performance under variable conditions.

Despite their potential, few studies have applied like co-inoculation with *Trichoderma* and *Bacillus*/*Azotobacter* cell suspensions has been shown to synergistically enhance plant-growth-promoting capacity across various crops, largely due to a combined increase in indole-3-acetic acid (IAA) production (Kasa et al., 2015).

To address this research gap, the present study aims to utilize PBD and RSM to optimize IAA production by salt-tolerant bacterial and fungal consortia. The ultimate objective is to develop efficient, salt-resilient bioinoculant formulations that can improve plant growth and nutrient uptake in saline-affected agricultural soils, thereby contributing to sustainable crop production under abiotic stress conditions.

2 Material and Methods

2.1 Assessment of IAA Production

Halotolerant microbes were isolated from Lonar lake (19° 58′ 17.4″ N 76° 30′ 20.1″ E) water and screened at a concentration of 8% NaCl along with its potential for IAA production. For relative quantification, 0.5 mL of broth culture from each isolate was inoculated into LB broth medium supplemented with 8% NaCl for B1 (PV856458.1), B2 (PV857542.1), and B3 (PV857590.1) adjusted to pH 6.5 ±0.2. A similar protocol was followed for screening fungal isolates F3 (PV857704.1), F7 (PV857723.1), F17 (PV857757.1), and F20(PV857762.1) in PDB broth. On a rotary shaker, cultures were incubated for seven days at 30 °C and 150 rpm, with filter-sterilized L-tryptophan (50 µg/mL). L-tryptophan served as the physiological precursor for microbial auxin biosynthesis. Every experiment was run in triplicate alongside controls. Every 24 hours, 1 mL of culture was harvested and centrifuged at 11180 g for 5 min. 4 mL of IAA reagent (1 mL 0.5 M $FeCl_3$, 30 mL conc. H_2SO_4 [sp. gr. 1.84], and 50 mL distilled water) was added to 1 mL supernatant to make a final volume of 5 mL. After incubation at room temperature for 30 min, development of pink coloration was observed (Joshi et al., 2021). Absorbance was measured at 530 nm using a UV spectrophotometer (2700, Thermo Scientific, USA), and an analytical-grade IAA standard curve was used to calculate the IAA concentration.

2.2 Screening for the Variable Conditions for IAA Production by Plackett Burman Method

Plackett-Burman was a two-factorial design that screened n variables in $n + 1$ tests to determine the key physicochemical parameters needed for higher production of IAA (Plackett & Burman, 1946). The microbes selected for consortia study were *Bacillus haynesii* (B1 and B2) and *Bacillus clausii* (B3) while in the fungi it was selected as *Aspergillus versicolor* (F3, F7, and F20) and *Aspergillus proliferans* (F17). The variables selected for the present study were temperature, pH, incubation days, shaking speed, inoculum size, tryptophan, glucose, peptone, and sodium chloride. Every variable was expressed as a numerical component, and they were all analyzed at two widely separated intervals known as -1 (low level) and +1 (high level). The impact of individual parameters on IAA production was determined by the following equation:

$$E = \left(\sum M+ - \sum M-\right)/N$$

where: E – the influence of the parameter under study and $M+$ and $M-$ were responses (IAA production) from trials where the parameter was at its highest and lowest levels respectively; N – represented the overall number of trials

2.3 Optimization of IAA Production by Using Response Surface Methodology

A central composite design (two-level factorial: quarter fraction) with three primary crucial independent variables viz., pH (5.5 to 7.0), incubation period (5 to 12), and tryptophan (0.4 to 1 µg/mL) was selected.

For each run, the same concentration of the media without inoculum was set to zero and coded as blank. By analyzing the experimental data, these eight factors each having five coded levels, 53 experimental runs, and 2.37841 alpha values were able to improve the estimation of the experimental error and provide additional information regarding yields in the experimental region's interior. The experiments were done usign Statease

Table 1 Experimental trial design for IAA production optimization using PBD

Parameters	Units	-1	1
pH		5	7
Temperature	°C	26	37
Tryptophan	µg/mL	0.01	1
Incubation days	Days	2	10
Shaking	RPM	70	120
Glucose	%	0.1	1
Peptone	%	0.1	0.3
NaCl	%	0.05	2
Inoculum size	CFU	100000	1E+007
Dummy1	g/L	0.01	0.02
Dummy2	g/L	0.01	0.02
pH		5	7

The medium composition for the optimization was a basal medium consisting of KH_2PO_4 (2 g/L), Na_2HPO_4 (1 g/L), $MgSO_4 \cdot 7H_2O$ (0.5 g/L), $ZnSO_4 \cdot 7H_2O$ (0.01 g/L), $FeSO_4 \cdot 7H_2O$ (0.005 g/L), and $MnSO_4 \cdot H_2O$ (0.002 g/L), Dummy 1 and Dummy 2 were included solely to complete the statistical design matrix and do not contribute to the interpretation of results

design expert Version 7. The following second-order polynomial regression equation, which takes into account both the individual and cross effects of each variable, was used to fit the experimental data:

$$Y = \beta_0 + \sum_{i=1}^{k} (\beta_i X_i) + \sum_{i=1}^{k} (\beta_{ii} X_i^2) + \sum_{i=1}^{k} \sum_{r>1}^{k} (\beta_{ir} X_i X_r)$$

where: Y – the predicted response; k – the number of factors (independent variables) studied; β_0 – the constant coefficient (intercept); β_i – the linear coefficient; βi_i – the quadratic coefficient, βir is the interaction coefficient; X_i and X_r – the uncoded independent variables

The equation used to construct surfaces for variables was represented by an interaction coefficient, a squared coefficient, a linear coefficient, and a constant

3 Results and Discussion

Microbial consortia particularly the co-inoculation of bacteria and fungi have long been employed for diverse agricultural purposes, including the suppression of plant diseases and the enhancement of growth through induced systemic resistance (Santoyo et al., 2021). However, limited literature exists demonstrating that indole-3-acetic acid (IAA) production can be effectively achieved using combined bacterial-fungal consortia. In the current study, significant IAA production was achieved using different species of *Bacillus* and *Aspergillus consortia* particularly *Bacillus haynesii*, *Bacillus clausii*, *Aspergillus versicolor* and *Aspergillus proliferans*. Out of these on the individual levels, there are reports that *Bacillus haynesii* has shown to produce IAA in the levels of 27–32 µg/mL (Andy et al., 2023), *Bacillus clausii* in the levels of 7 to 10 µg/mL, *Aspergillus versicolor* in the level of 2.49 µg/mL to 44.57 µg/mL (Hussein & Joo, 2015; Pablo et al., 2025).

3.1 *Screening of Variables for IAA Production by Placket Burman Test*

The results from both the data table and the Pareto chart collectively highlighted key factors affecting IAA production by *Bacillus* and *Aspergillus consortia*. The data table shows IAA yields varying between 70–110 µg/mL across different combinations of parameters, revealing

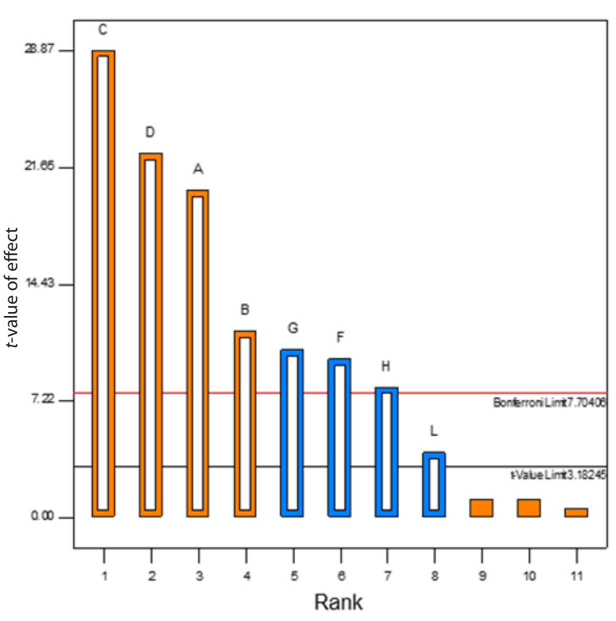

Figure 1 Pareto chart of Plackett Burman

higher production when tryptophan concentration is at 1 mg/mL, pH is maintained around 6–7, and inoculum size is elevated (Table 2). The Pareto chart visually reinforces these observations by displaying tryptophan concentration, pH, and inoculum size as the most significant variables exceeding the significance threshold (Figure 1). Other parameters such as glucose, peptone, NaCl, and incubation period show minimal or negative contributions, indicating they are less critical for maximizing IAA output. This combined analysis identifies the most influential factors, providing a foundation for further optimization using approaches such as response surface methodology. 2 dummy parameters dummy 1 and dummy 2 were added.

The ANOVA table further confirmed these findings: tryptophan (*F* = 833.33, *p* < 0.0001), pH (*F* = 408.33, *p* = 0.0003), and incubation days (*F* = 507, *p* = 0.0002) had highly significant effects on IAA production, with the model itself being significant (*p* = 0.0003) (Table 3). Other variables like glucose, peptone, and NaCl also showed statistically significant effects but with lower *F*-values. The curvature test was significant (*p* = 0.0008), indicating potential model nonlinearity that can be further explored in future optimization studies.

Table 2 Experimental trial design for IAA production optimization using PBD

Run	A: pH	B: Temperature (°C)	C: Tryptophan (mg/mL)	D: Incubation (days)	E: Shaking (rpm)	F: Glucose (%)	G: Peptone (%)	H: NaCl (%)	J: Inoculum size (CFU)	K: Dummy 1	L: Dummy 2	IAA production (μg/mL)
1	7	26	1	10	70	1	0.3	2	100000	0.01	0.01	95
2	7	37	0.01	10	120	1	0.1	0.05	100000	0.02	0.01	97
3	7	26	1	10	120	0.1	0.1	0.05	10000000	0.01	0.02	110
4	6	31.5	0.51	6	95	0.55	0.2	1.02	5050000	0.01	0.01	100
5	7	37	1	2	70	0.1	0.3	0.05	10000000	0.02	0.01	100
6	7	26	0.01	2	120	0.1	0.3	2	100000	0.02	0.02	70
7	5	37	1	2	120	1	0.3	0.05	100000	0.01	0.02	80
8	5	26	0.01	10	70	1	0.3	0.05	10000000	0.02	0.02	70
9	7	37	0.01	2	70	1	0.1	2	10000000	0.01	0.02	76
10	5	37	0.01	10	120	0.1	0.3	2	10000000	0.01	0.01	80
11	5	37	1	10	70	0.1	0.1	2	100000	0.02	0.02	100
12	5	26	0.01	2	70	0.1	0.1	0.05	100000	0.01	0.01	70
13	5	26	1	2	120	1	0.1	2	10000000	0.02	0.01	78

Table 3 ANOVA of Plackett Burman

Source	df	Mean	F value	p-value	Significance
Model	8	271	271	0.0003	significant
A-pH	1	408.33	408.33	0.0003	
B-Temperature	1	133.33	133.33	0.0014	
C-Tryptophan	1	833.33	833.33	< 0.0001	
D-Incubation days	1	507	507	0.0002	
F-Glucose	1	96.33	96.33	0.0022	
G-Peptone	1	108	108	0.0019	
H-NaCl	1	65.33	65.33	0.004	
L-Dummy2	1	16.33	16.33	0.0273	
Curvature	1	194.08	194.08	0.0008	significant
Residual	3	1			
Cor Total	12				

The current study reported that as the salt concentration increased, the IAA production decreased. This was in contrast to the result obtained by Damodaran et al. (2023) who reported increase in IAA production – 58.97 µg/mL with 0% NaCl concentration to 63.30 µg/mL at 7.5% NaCl concentration using halotolerant *Bacillus consortia*.

3.2 Optimization of IAA production by RSM Model

The optimization of IAA production using the RSM model revealed that the highest IAA yields (up to 125 µg/mL) were achieved at pH around 6.25–7.0, tryptophan concentrations of 0.7–1.2 mg/mL, and incubation periods between 8.5 and 12 days (Table 4). Runs with these optimal conditions consistently produced IAA values close to or exceeding 120 µg/mL, which matched well with the point predictions from the model. The model demonstrated strong predictive accuracy, as the experimental results aligned closely with predicted values across various combinations (Figure 2). These findings confirm that the selected ranges of pH, tryptophan concentration, and incubation days significantly influence IAA biosynthesis in halotolerant bacterial consortia, providing an optimized set of conditions for further field trials and application development. The near neutral pH optima and tryptophan dependent control of auxin pathway mirror the prior RSM work in plant beneficial microbes (Lebrazi et al., 2020). Diaporthe terebinthifolii has been reported to be optimized to produce 121.2 µg/mL using 1 mg/mL tryptophan in potato dextrose broth. On the contrary, *Streptomyces fradiae* reached ~82 µg/mL under optimized conditions with 2 g/L tryptophan. Differences in IAA expression trace to:

a) organism/consortium genetics (pathway choice and flux),
b) medium richness and precursor loading,
c) bioprocess variables (oxygen transfer, pH regime),
d) analytical method.

In the current experiment, as seen previously in Plackett Burman test, NaCl reduced the IAA yield, plausible given osmotic energy reallocation and stress response that deprioritizes IAA production. However, there are records of srain dependent exceptions, as in case of Damodaran et al. (2023), who observed enhanced IAA and improved crop performance in sodic soils using halotolerant *Bacillus/Lysinibacillus consortia*, which regulate the IAA production via ACC deaminase and osmoregulatory traits.

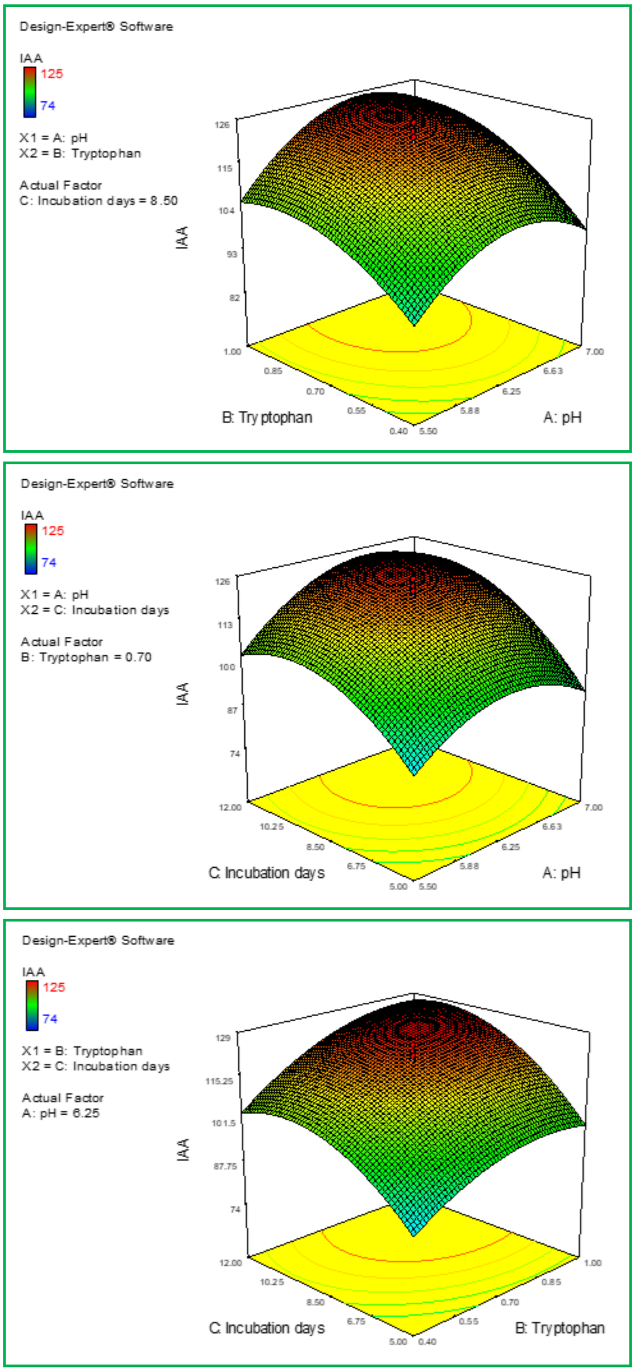

Figure 2 Response Surface Contour plots with respect tryptophan, pH, and incubation days

The ANOVA analysis of the RSM optimization model shows that the model is highly significant ($F = 29.92$, $p < 0.0001$), indicating that the selected factors effectively explain the variability in IAA production (Table 5). Among the factors, pH ($F = 18.01$, $p = 0.0017$), tryptophan ($F = 47.73$, $p < 0.0001$), and incubation days ($F = 69.76$, $p < 0.0001$) had significant positive effects on IAA production. Quadratic terms for pH, tryptophan, and incubation days were also significant, demonstrating the non-linear nature of the system. The lack-of-fit was not significant ($p = 0.0675$), suggesting the model fits well with the experimental data. This statistical validation, combined with the close agreement between experimental and predicted IAA

Table 4 Optimization of IAA by RSM model

Run	A: pH	B: Tryptophan	C: Incubation days	IAA production (µg/mL)	Point prediction
1	5.5	0.4	5	75	75.04
2	4.99	0.7	8.5	82	82.30
3	6.25	0.7	8.5	117	120.87
4	6.25	0.7	8.5	118	120.87
5	5.5	1	5	85	84.99
6	5.5	1	12	100	105.29
7	6.25	0.7	8.5	120	120.87
8	7	0.4	12	97	100.17
9	6.25	0.7	14.39	114	110.48
10	7.51	0.7	8.5	106	101.34
11	7	0.4	5	78	75.87
12	6.25	0.7	8.5	121.5	120.87
13	7	1	5	88	94.32
14	6.25	0.7	2.61	74	72.96
15	5.5	0.4	12	90	86.84
16	6.25	0.7	8.5	123	120.87
17	7	1	12	124	127.12
18	6.25	0.7	8.5	125	120.87
19	6.25	1.2	8.5	125	117.96
20	6.25	0.2	8.5	84	87.22

Table 5 ANOVA analysis of RSM

Source	Sum of squares	df	Mean square	*F*-value	*p*-value Prob > *F*	Significance
Model	6,553.75	9	728.19	29.92	< 0.0001	significant
A-pH	438.24	1	438.24	18.01	0.0017	
B-Tryptophan	1,161.64	1	1,161.64	47.73	< 0.0001	
C-Incubation days	1,697.8	1	1,697.8	69.76	< 0.0001	
AB	36.13	1	36.13	1.48	0.2511	
AC	78.13	1	78.13	3.21	0.1035	
BC	36.13	1	36.13	1.48	0.2511	
A^2	1,527.17	1	1,527.17	62.75	< 0.0001	
B^2	624.31	1	624.31	25.65	0.0005	
C^2	1,527.17	1	1,527.17	62.75	< 0.0001	
Residual	243.39	10	24.34			
Lack of Fit	197.52	5	39.5	4.31	0.0675	not significant
Pure Error	45.88	5	9.18			
Cor Total	6,797.14	19				

Table 6 Model Summary Statistics for Response Surface Regression Analysis

Std. Dev.	Mean	C.V. %	PRESS	R-Squared	Adj R-Squared	Pred R-Squared	Adeq Precision
4.93	102.33	4.82	1,620.82	0.9642	0.932	0.7615	15.511

values (from 72 to 127 µg/mL), confirms the robustness of the model in optimizing IAA production conditions. Overall, the study demonstrates the potential of using RSM to maximize IAA yield from halotolerant consortia by fine-tuning key parameters. The table 6 also shows that the model diagnostics indicated an excellent fit and precision ($R^2 = 0.9642$; adj. $R^2 = 0.932$; CV = 4.82%; SD = 4.93 at mean = 102.33). Adeq Precision was 15.51 (> 4), confirming an adequate signal, and Pred. $R^2 = 0.7615$ with PRESS = 1620.82, supporting acceptable out-of-sample predictability across the experimental range.

From the analysis given, the following quadratic equation was obtained:

$$\text{IAA} = -669.745 + 219.583\ (\text{pH}) + 56.897\ (\text{tryptophan}) + 8.614\ (\text{incubation days}) + 9.444\ (\text{pH} \times \text{tryptophan}) + 1.190\ (\text{pH} \times \text{incubation days}) + 2.024\ (\text{tryptophan} \times \text{incubation days}) - 18.301\ (\text{pH}^2) - 73.132\ (\text{tryptophan}^2) - 0.840\ (\text{incubation days}^2)$$

4 Conclusions

The present study successfully optimized IAA production by halotolerant bacterial and fungal consortia using Plackett–Burman design and Response Surface Methodology. Key factors such as pH, tryptophan concentration, and incubation period significantly influenced IAA yield. The statistical models demonstrated strong predictive accuracy, validating the experimental results and highlighting the non-linear relationships among variables. The optimized conditions produced high IAA levels (up to 125 µg/mL), suggesting practical potential for developing robust bioinoculant formulations. This work lays a solid foundation for further scale-up and field evaluation of halotolerant microbial consortia in saline-stressed agricultural systems.

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Conflict of Interest

The authors declare that they have no conflict of interest regarding the publication of this paper.

Author Contributions

Suvarna Patil: performed the experiments, analyzed data, and wrote the manuscript; Aparna Jobanputra: designed and framed the experimental methodology; Sunil Pathak: assisted in experimental design and provided critical peer review of the manuscript.

AI and AI-Assisted Technologies Use Declaration

AI-based tools were utilized for language refinement and formatting assistance during manuscript preparation. All intellectual content, scientific interpretation, and conclusions are the original work of the authors.

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