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ORIGINAL ARTICLE

Optimization of pH, temperature and Acephate concentration for *In Situ* Acephate mineralization

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ABSTRACT
Pesticides are chemical substances designed to prevent, control, or eliminate pests that threaten agricultural productivity, public health, and stored products. They encompass a wide range of compounds, including insecticides, herbicides, fungicides, and rodenticides, each targeting specific types of pests. While pesticides play a crucial role in ensuring food security and preventing disease transmission, their extensive and indiscriminate use has raised significant environmental and health concerns. Many pesticides are synthetic xenobiotics that are persistent in nature, allowing them to accumulate in soil, water, and living organisms. This persistence can lead to bioaccumulation, disruption of ecological balance, and potential toxic effects on non-target organisms, including humans. Over time, pesticide residues can contaminate food chains, reduce biodiversity, and contribute to the development of resistant pest populations, underscoring the urgent need for safer, sustainable alternatives and effective biodegradation strategies. In the present study, an enrichment culture technique was employed to isolate acephate-degrading bacterial strains from paddy soil samples collected from fields exposed to high levels of acephate application. A total of sixteen pure bacterial isolates were successfully obtained and subjected to screening experiments to assess their growth potential in the presence of increasing concentrations of acephate. From this group, one bacterial strain demonstrating superior performance was selected for in-depth biodegradation studies. Optimization experiments determined that the most favorable conditions for biodegradation were a temperature of 37 °C, pH 8, and an acephate concentration of 1000 ppm. The identified metabolites suggest a multi-step degradation pathway, potentially involving hydrolysis and subsequent mineralization
Keywords: *Acephate, enrichment culture, xenobiotic, mineralization*

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INTRODUCTION

The global human population has been rising steadily, reaching approximately 7 billion at present [9; 14). At the current rate of growth, it is projected to reach around 9.5 billion by 2050, thereby placing tremendous pressure on global food supplies (14). According to FAO [7], food demand is expected to increase by nearly 70% in the coming decades, necessitating intensive cultivation of agricultural crops [9; 25). To meet this challenge, soils are often exploited by extractive cropping practices, which deplete nutrient reserves and lead to nutrient imbalance and soil degradation (28; 13). Soil degradation is broadly defined as the physico-chemical and biological deterioration of soil quality due to anthropogenic activities, resulting in a severe decline in productivity and fertility [5].

Among the dominant forms of soil degradation are erosion and salinity. Erosion, in particular, is accelerated by unsustainable agricultural practices, deforestation, and overgrazing [28]. Globally, these factors have degraded 38% of agricultural land, 21% of permanent pastures, and 18% of forests and woodlands (18; 27). Based on the extent of damage, soils have been classified into lightly (9%), moderately (10%), and strongly (4%) degraded categories [18]. While lightly and moderately degraded soils retain limited agricultural functions and can still support farming with reduced productivity, their

restoration is possible through improved management practices. In contrast, severely degraded soils lose their productivity almost entirely, along with their original biotic functionality [18; 27).

In the case of light to moderate degradation, the removal of organic matter and nutrient-rich top soil results in nutrient depletion, loss of fertility, impaired soil structure, and reduced water-holding capacity. Consequently, agricultural production in such soils is highly dependent on nutrient availability and maintenance of good soil structure to sustain plant growth. Abiotic and biotic factors collectively regulate the activity and diversity of soil microorganisms. The occurrence and abundance of microbes in soil are strongly influenced by vegetation type, soil texture and chemical composition, nutrient availability, pH, moisture, climate, and temperature. These factors not only determine microbial diversity but also govern soil physiology, which may vary seasonally even at the same location. Furthermore, the addition of organic wastes from agricultural fields enriches the soil with nutrients, thereby creating favorable conditions for microbial proliferation [20]. Bacteria, as the most abundant and diverse group of microorganisms [1], play a pivotal role in organic matter decomposition, mineralization of elements, and nutrient cycling [6]. With the advent of high-throughput sequencing technologies, increased focus has been directed toward understanding bacterial community structures across different habitats [30]. Given their critical role in maintaining soil health and enhancing plant growth, detailed knowledge of bacterial communities is essential for advancing sustainable agricultural and ecological practices.

The close association between soil health and microbial activity highlights the significance of bacteria in mitigating the adverse effects of agrochemicals. Since acephate and its toxic metabolite methamidophos persist in agricultural environments, their breakdown relies heavily on the metabolic potential of soil microorganisms. Thus, understanding the interactions between soil bacterial communities and acephate degradation is essential, not only for reducing environmental toxicity but also for maintaining soil fertility and supporting sustainable crop production.

Acephate is a systemic and contact organophosphate insecticide first registered in the United States in 1973. It is widely used to control insect pests in field crops (e.g., tobacco), food crops (e.g., beans, lettuce, and bell peppers), ornamentals, sod, turf, food-handling facilities, as well as residential and commercial environments. Acephate is characterized by its high-water solubility, low soil adsorption, and moderate potential for runoff into surface waters. In the environment, it undergoes degradation within a few days, producing metabolites such as methamidophos, a more toxic organophosphate insecticide. This transformation also occurs in insects, where the toxicity of acephate primarily arises from its metabolic conversion to methamidophos [26]. Considering the persistence and potential toxicity of its degradation products, the role of soil microorganisms, particularly bacteria, becomes highly significant in the detoxification and biodegradation of acephate, thereby mitigating its environmental impact

MATERIAL AND METHODS

Chemical: Analytical grade Acephate (75%)

Isolation of Acephate Degrading Bacteria:

Soil samples were collected from paddy field with prior history of acephate applications and used for the isolation of pesticide degrading bacterial strains. Repeated enrichment culture technique was employed to isolate pesticide degrading bacteria using minimal salt medium following serial dilution method. Sixteen morphologically different bacterial isolates were obtained by plating serial dilutions of the culture on MSM agar plates containing acephate. Four isolates were selected based on maximum growth in MSM Broth by measuring optical density at 600 nm using UV/Visible Spectrophotometer. Degradation of acephate by all the four isolates individually was monitored by Esterase enzyme activity, FTIR and GC – MS analysis. Genomic DNA was extracted from the most efficient pure isolate. Further, molecular identification was carried out by 16S rRNA gene analysis and molecular evolutionary relationship were accomplished using Robust Phylogenetic Tree online tool and the neighbour-joining algorithm. The sequences of the isolate obtained was submitted in GenBank.

Optimisation of growth parameters of bacterial isolate:

Based on Esterase enzyme activity, FTIR and GC – MS results, most efficient isolate was selected for further studies. To increase the degradation ability of the isolate, different physiological parameters like Nitrogen source, NaCl concentration, Acephate concentration, pH and incubation temperature were optimized in Minimal Salt Broth. The un-inoculated sterile minimal salt broth was kept as negative control. All sets were performed in triplicates [22].

Effect of Nitrogen Sources

The effect of various nitrogen sources on degradation study of bacteria was studied by adding organic nitrogen sources at 1% (w/v) level into optimization medium. The organic nitrogen sources such as casein hydrolysate, yeast extract, beef extract and peptone were used. Each flask, containing MSM broth

supplemented with the selected nitrogen source individually, was inoculated with the bacterial culture and incubated at 37 °C for 24hrs. After the incubation period, OD value was taken.

Effect of NaCl Concentration

Different NaCl concentration of 0.05%, 0.10%, 0.15%, 0.20% and 0.25% was added to the MSM broth individually with chosen nitrogen source. Each flask containing different concentration of NaCl was inoculated with the bacterial culture and incubated at 37 °C for 24hrs. After the incubation period, OD value was taken.

Effect of pH

The efficacy of the pH was determined by using alkaline and acidic pH range, MSM broth with its chosen nitrogen source and NaCl concentration, were inoculated with 2% (v/v) of seed culture and incubated at different pH values such as 5.0, 6.0, 7.0, 8.0 and 9.0 for 24hrs isolate. After the incubation period, OD value was taken.

Effect of Incubation Temperature

To determine the effective incubation temperature, MSM broth with its chosen nitrogen source, NaCl and pH were inoculated with 2% (v/v) of seed culture and incubated at different temperatures such as 25 °C, 35 °C, 37 °C, 40 °C and 45°C for 24hrs. After the incubation period, OD value was taken.

Effect of Acephate Concentration

Different concentrations of Acephate were chosen. 50ppm, 100ppm, 200ppm, 300ppm, 400ppm, 500ppm, 600ppm, 700ppm, 800ppm, 900ppm, 1000ppm and 1100ppm was added to the MSM broth individually with its chosen nitrogen source, NaCl concentration and pH. Each flask containing different concentration was inoculated with a bacterial culture and incubated at 37 °C for 24hrs. After the incubation period, OD value was taken at 24 hr.

Optimization of Culture Conditions by Response Surface Methodology (RSM)

The Box–Behnken Design (BBD) of Response Surface Methodology (RSM) with three factors—time (A), pH (B), and substrate concentration (C)—each at three levels (−1, 0, +1), was employed to optimize conditions for maximum acephate degradation. Experiments were designed and analyzed using Design-Expert 10.0.5.2 (State-Ease, Inc., USA), with 17 experimental runs including five replicates at the center point, all conducted in triplicate. The data were fitted to a second-order polynomial equation**:**

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC,$$

where $\boldsymbol{Y}$ represents the predicted response and the β terms denote linear, quadratic, and interaction coefficients. Analysis of Variance (ANOVA) was performed to assess model significance, regression fit, and reliability through $\boldsymbol{F}$ and $\boldsymbol{p}$ values, while three-dimensional response surface plots were generated to visualize the interaction effects and validate the optimization model.

RESULTS AND DISCUSSION

Optimisation of growth parameters of bacterial isolate:

Among four bacterial isolates, PA11 was advanced based on strong esterase activity and supportive FTIR and GC-MS evidence of acephate transformation. It was consistent with reports that organophosphate (OP) degradation is often initiated by (phospho) esterases and related hydrolases in bacteria. Such enzymes underpin OP detoxification and bioremediation frameworks [19].

Among the tested parameters, yeast extract supported the highest bacterial growth (OD_{600} = 0.148; Fig. 1a) due to its rich supply of amino acids, peptides, vitamins, and minerals that promote biomass and enzyme synthesis [8; 16]. The salt tolerance profile (optimum 0.15%; OD_{600} = 0.205; Fig. 1b) suggests that PA11 is adapted to low-salinity environments, consistent with the majority of soil isolates studied by Cycoń et al., [4] as moderate salinity stabilizes cell membranes and osmotic balance, while higher salt levels inhibited growth [17]. Peak growth occurred at pH 8.0 (OD = 0.092; Fig. 1c), indicating that mildly alkaline conditions enhance organophosphate hydrolysis and enzyme activity, consistent with previous reports showing optimal acephate degradation near pH ~8.9 [10; 12; 32]. Temperature optimization revealed maximum growth at 37 °C (OD = 0.109; Fig. 1d), aligning with mesophilic trends (30–37 °C) typically observed for organophosphate degraders [12; 23; 32]. Across acephate concentrations ranging from 50–1100 ppm, the isolate exhibited highest growth at 1000 ppm (OD = 0.208; Fig. 1e), suggesting strong substrate tolerance and efficient metabolic adaptation similar to other reported acephate-degrading consortia [24; 32]. This is comparable to degradation efficiencies reported in strains of *Pseudomonas* and *Burkholderia* that thrive at concentrations up to 1200 ppm [3; 31]. The optimized conditions obtained for PA11 align closely with values reported for other organophosphate-degrading isolates.

Overall, the comparative analysis underscores the potential of PA11 as a robust acephate degrader under agriculturally relevant conditions, and provides a baseline for further RSM-based optimization to enhance biodegradation efficiency [11].

Box- Behnken Design (BBD) of RSM model fitting:

The Box–Behnken Design (BBD) of Response Surface Methodology (RSM) was employed to optimize culture conditions for maximum acephate degradation. Three key factors—pH (A), temperature (B), and acephate concentration (C)—were evaluated at three coded levels (–1, 0, +1) through 17 experimental runs, including six center-point replicates (Tables 2–3). The developed quadratic model expressed the response (Y) as: $Y = 92.55 + 0.1125A + 0.0413B - 0.0263C - 0.1025AB + 0.0125AC - 0.2900BC - 11.49A^2 - 11.26B^2 - 11.23C^2$**.** Statistical analysis confirmed the model's reliability, with a highly significant F-value (8095.34) and $p < 0.0001$, indicating strong model fit and predictive accuracy (Table 4). The lack-of-fit F-value (0.39) and low coefficient of variation (CV = 0.2060%) further validated the precision of the experimental design. The R^2 value of 0.9999 (Table 5) demonstrated that the model explained 99.99% of the response variability, confirming its robustness for predicting acephate degradation under different environmental conditions [12; 24; 32].

Figure 2 (a, b, and c) depicts the combined influence of pH, temperature and substrate concentration on acephate degradation by *Sanguibacter* sp. PA11. The 3D surface plots, represented by a blue-to-red color gradient, show increasing degradation efficiency. Maximum degradation occurred under moderate conditions, particularly at pH 8, 37 °C, and a substrate concentration of 1000 ppm. In contrast, extreme values of these parameters resulted in reduced degradation efficiency. These findings suggest that maintaining balanced environmental conditions promotes optimal enzymatic activity and enhances acephate degradation.

Jabeen *et al.,* (2015) [33] optimized profenofos degradation using a bacterial consortium (PBAC) via Response Surface Methodology (RSM), analyzing the effects of temperature, pH, and pesticide concentration. The consortium, composed of soil bacterial isolates, achieved over 90% degradation efficiency under optimal conditions of 35 °C, pH 7.5 and 200 mg L^{-1} profenofos within 72 hours. Regression modeling confirmed significant effects of all variables, with temperature and pH being the most influential, and kinetic analysis indicated a pseudo-first-order enzyme-catalyzed process. Similarly, John *et al.*, (2016) [34] applied RSM to optimize chlorpyrifos degradation by an assembled bacterial consortium, achieving ~96% degradation at pH 7.0, 35 °C and 200 mg L^{-1} within 72 hours. The validated quadratic model showed excellent fit between predicted and observed results, with degradation following first-order kinetics and involving hydrolase and phosphatase enzymes. Khatoon and Rai (2020) [35] also utilized RSM to optimize atrazine degradation by *Bacillus badius* ABP6, yielding a robust quadratic model (R^2 = 0.9897) and identifying optimal conditions of pH 7.05, 30.4 °C, 145.7 rpm and 200.9 ppm atrazine, resulting in ~89.7% removal efficiency. All studies demonstrated that RSM effectively enhances pesticide biodegradation by defining precise physicochemical parameters for maximal microbial activity.

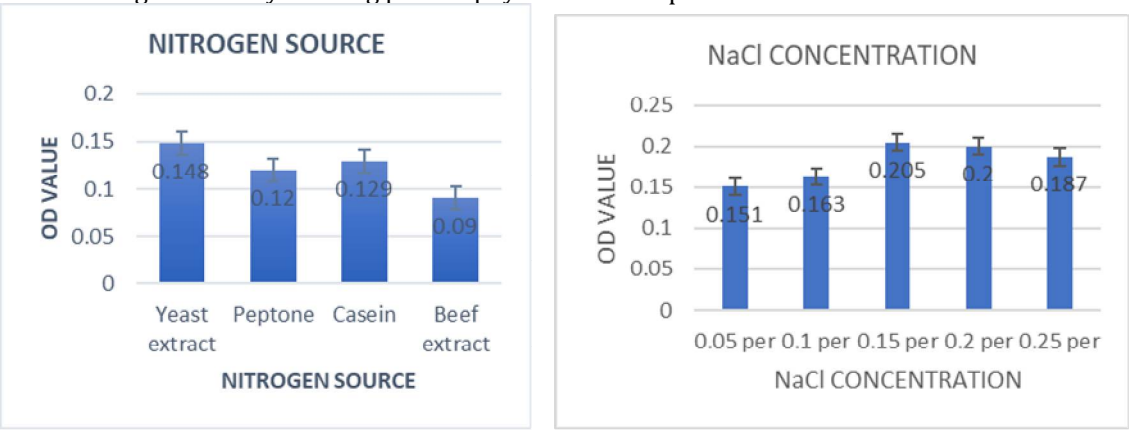

Fig. 1a and 1b: Optimization of selected strain PA11 for Nitrogen source and NaCl Concentration

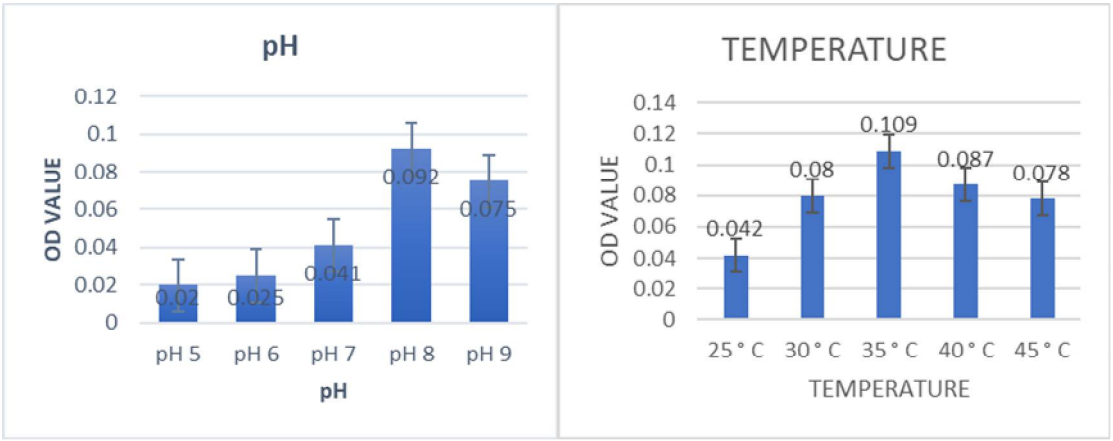

Fig. 1c and 1d: Optimization of selected strain PA11 for pH and Temperature

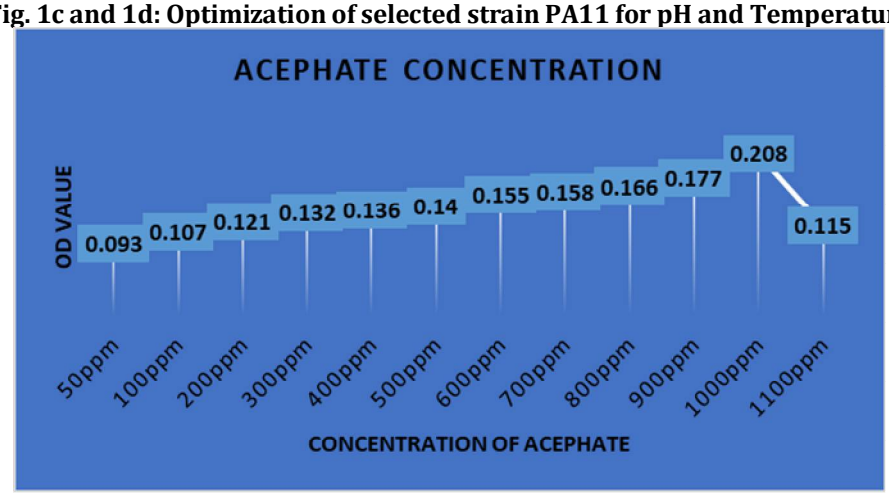

Fig. 1e: Optimization of selected strain PA11 for various Acephate Concentration

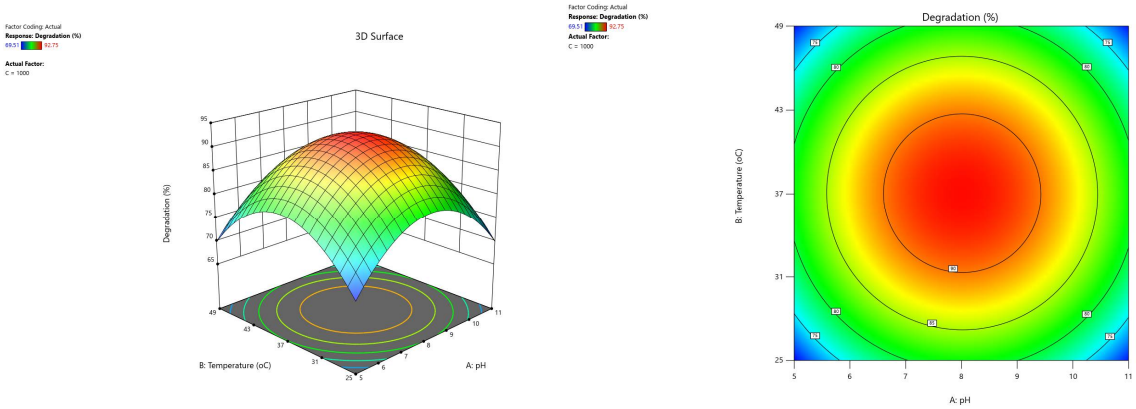

Fig. 2a: 3D surface of Optimized variables pH and Temperature

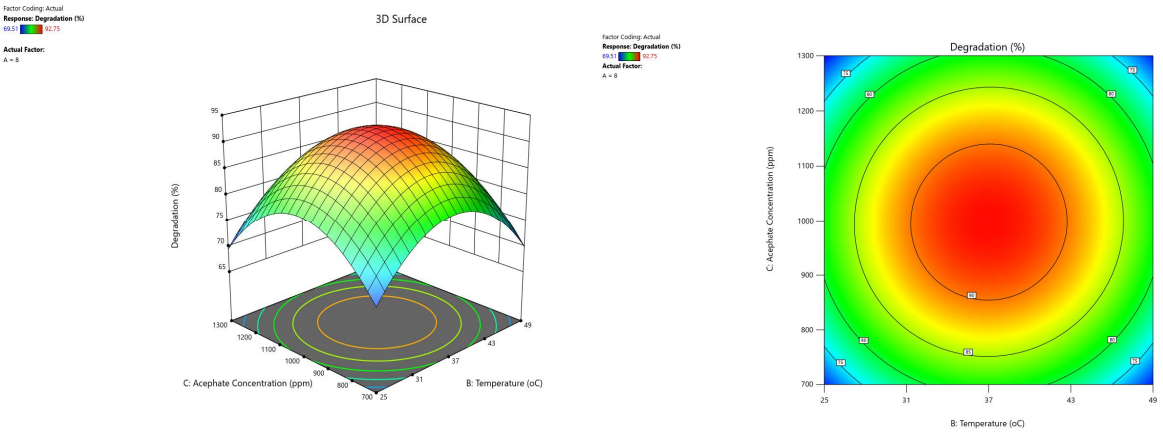

Fig. 2b. 3D surface of Optimized Variables Temperature and concentration

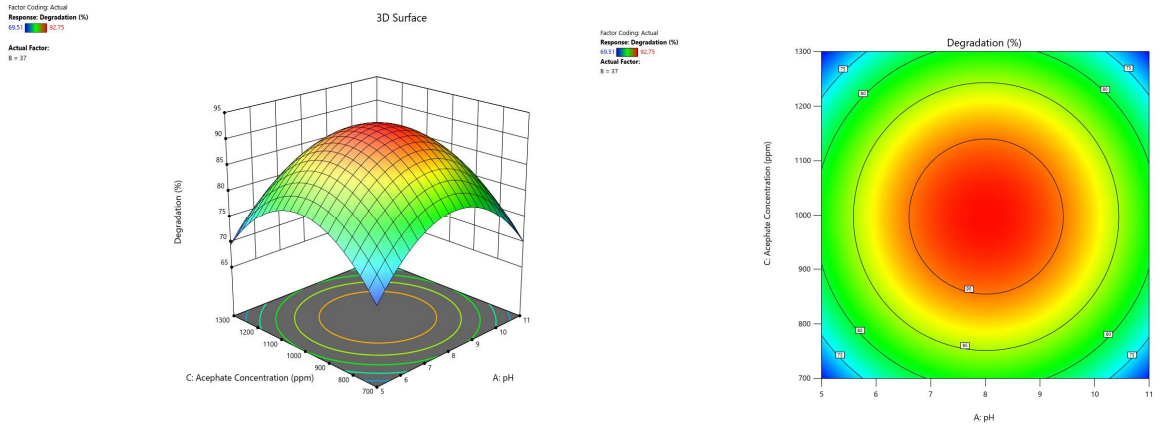

Fig. 2c: 3D surface of Optimized variables pH and concentration

Table. 1. Comparison of Optimized Growth Conditions of PA11 with Reported Studies

Parameter	PA11 (This Study)	Reported Range/Optimum in Literature	References
Nitrogen source	Yeast extract (OD_{600} = 0.148)	Yeast extract enhances microbial growth & pesticide degradation	8; 15
NaCl concentration	0.15% (OD_{600} = 0.205)	Low salinity (~0.1–0.2%) supports growth of organophosphate degraders	21; 4
Acephate concentration	1000 ppm (OD_{600} = 0.208)	Optimum tolerance ~800–1200 ppm for acephate/OP degradation	3; 31
Temperature	37 °C (OD_{600} = 0.109)	30–37 °C for mesophilic pesticide degraders	23; 29
pH	8.0 (OD_{600} = 0.092)	pH 7.5–9.0 favorable for OP hydrolase activity	2, 10

Table 2: Experimental range of variables for the central composite design (CCD)

S. No.	Variable	Coded symbol	Units	Low level (-1)	Mid-level (0)	High Level (+1)
1.	pH	A	-	5	8	11
2.	Temperature	B	^oC	25	37	49
3.	Acephate concentration	C	ppm	700	1000	1300

Table 3: Design of experiment of pH, temperature and substrate concentration on Acephate degradation

run	Factor 1 A: pH	Factor 2 B: Temperature ^{O}C	Factor 3 C: Acephate Conc. ppm	Response 1 Degradation %
1	8	37	1000	92.25
2	11	37	700	70.12
3	8	37	1000	92.55
4	8	49	1300	70.25
5	8	25	1300	69.52
6	11	49	1000	69.87
7	8	37	1000	92.75
8	8	37	1000	92.61
9	11	25	1000	70.06
10	8	37	1000	92.58
11	5	49	1000	69.75
12	8	25	700	70.44
13	5	25	1000	69.53
14	5	37	700	70.02
15	5	37	1300	69.51
16	8	49	700	70.01
17	11	37	1300	69.66

Table 4: Response 1: Degradation

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1812.14	9	201.35	8095.34	< 0.0001	significant
A-pH	0.1012	1	0.1012	4.07	0.0834	
B-Temperature	0.0136	1	0.0136	0.5473	0.4835	
C-Acephate Concentration	0.3403	1	0.3403	13.68	0.0077	
AB	0.0420	1	0.0420	1.69	0.2348	
AC	0.0006	1	0.0006	0.0251	0.8785	
BC	0.3364	1	0.3364	13.53	0.0079	
A^2	555.54	1	555.54	22335.65	< 0.0001	
B^2	533.75	1	533.75	21459.66	< 0.0001	
C^2	531.38	1	531.38	21364.46	< 0.0001	
Residual	0.1741	7	0.0249			
Lack of Fit	0.0396	3	0.0132	0.3929	0.7656	not significant
Pure Error	0.1345	4	0.0336			
Cor Total	1812.31	16				

Table 5: Fit Statistics

Std. Dev.	0.1577		R^2	0.9999
Mean	76.56		Adjusted R^2	0.9998
C.V. %	0.2060		Predicted R^2	0.9995
			Adeq Precision	190.5776

CONCLUSION

Optimization studies demonstrated that the biodegradation potential of bacterial isolate PA11 was strongly influenced by physicochemical and nutritional factors. Among the nitrogen sources tested, yeast extract supported maximum growth (OD 0.148), indicating its suitability in enhancing enzyme production. Salinity tolerance was optimal at 0.15% NaCl (OD 0.205), suggesting the isolate can adapt to mild osmotic stress. Acephate concentration up to 1000 ppm without the addition of any extra carbon source significantly enhanced growth (OD 0.208), confirming its metabolic adaptability and tolerance to high pesticide levels. Temperature optimization revealed that 37 °C (OD 0.109) was the most favourable condition, consistent with the mesophilic nature of the isolate, while alkaline pH 8 (OD 0.092) further supported its degradation activity.

Overall, the results indicate that isolate PA11 exhibits strong potential for acephate degradation under optimized growth conditions, highlighting its suitability as an effective candidate for the bioremediation of pesticide-contaminated environments."

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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