Environment and Ecology 43 (3) : 725—735, July—September 2025
Article DOI: https://doi.org/10.60151/envec/FNSP2704
ISSN: 0970-0420 (Print), ISSN: 3049-3625 (Online)

Optimization of Substrates and Incubation Time for Dye Decolorization using Response Surface Methodology in *Bacillus subtilis* (MTCC-2414) and *Pseudomonas putida* (MTCC-2445)

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Received 20 May 2025, Accepted 13 July 2025, Published on 7 August 2025

ABSTRACT

The rising industrial use of dyes has resulted in un-treated effluents being discharged into water bodies, causing toxic, carcinogenic effects on humans and disrupting aquatic ecosystems. Traditional physico-chemical used for dye removal methods face limitations such as toxic by-product formation, sludge accumulation, and incomplete degradation. Microbial bioremediation offers a sustainable alternative for textile dye decolorization. This study explored the use of a microbial consortium comprising *Bacillus subtilis* (MTCC-2414) and *Pseudomonas putida* (MTCC-2445), optimizing substrate type and concentration to enhance decolorization efficiency. Response Surface Methodology (RSM) was used to identify critical parameters influencing degradation. Statistical analyses including ANOVA, Tukey's HSD, and Principal Component Analysis (PCA), along with contour plots, helped evaluate substrate effects and visualize interactions. Among tested substrates, glucose showed the highest initial decolorization efficiency, though prolonged incubation improved results with all substrates, including sugarcane and rice bran. The findings confirm the effectiveness of *Bacillus subtilis* and *Pseudomonas putida* as bioremediation agents for treating dye-contaminated wastewater. Overall, the study supports microbial bioremediation as an eco-friendly, efficient, and sustainable approach for industrial dye pollution, offering a promising alternative to conventional methods.

Keywords Textile dye degradation, Response surface methodology, Substrate standardization, *Bacillus subtilis* (MTCC-2414), *Pseudomonas putida* (MTCC-2445).

INTRODUCTION

In recent years, the widespread use of dyes in industries such as textiles, printing, and leather has increased due to their role in enhancing the aesthetic appeal of products. However, while some industries discharge pre-treated, non-toxic effluents, others

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release untreated wastewater, which possess serious environmental and health concerns (Ali *et al.* 2021a ,Yaseen & Scholz 2019a). These dye-laden effluents can adversely affect aquatic ecosystems, alter soil fertility, reduce crop productivity, and introduce toxic, mutagenic and carcinogenic substances into the environment (Katheresan *et al*. 2018).

The rapid expansion of the textile industry has particularly contributed to this issue, as it extensively uses synthetic dyes, many of which are released into water bodies without proper treatment (Lellis *et al.* 2019). Textile wastewater typically contains a high biological oxygen demand (BOD) and chemical oxygen demand (COD), reflecting its high organic load and toxicity (Mishra & Maiti 2019). Additionally, the presence of non-biodegradable dye compounds exacerbates environmental degradation, leading to eutrophication, disruption of aquatic life, and long-term ecological imbalances (Shahid *et al.* 2021).

To address these environmental challenges,various dye removal techniques have been developed, including physical, chemical, nanoparticle-based, and biological methods (Saratale *et al*. 2011a). Physical methods such as adsorption, ion exchange, membrane filtration, and precipitation are frequently applied, yet they often fall short due to high operational costs, regeneration issues, and limited dye removal efficiency (Crini & Lichtfouse 2019). Chemical methods like coagulation-flocculation, ozonation, advanced oxidation, and electrochemical treatments are relatively effective but can lead to the generation of toxic by-products and excessive sludge, posing disposal and secondary pollution challenges (Forgacs *et al.* 2004, Robinson *et al.* 2001).

Nanotechnology-based approaches using materials such as iron oxide, hematite, magnetite and titanium dioxide have emerged as innovative alternatives. While these nanoparticles offer improved adsorption and photocatalytic degradation capabilities, they are expensive, require controlled conditions, and raise concerns about nanoparticle toxicity and persistence in the environment (Ahmed *et al*. 2021).

In contrast, microbial bioremediation presents an eco-friendly, cost-effective, and efficient solution for dye degradation. Microorganisms such as bacteria, fungi and actinomycetes possess enzymatic machinery capable of breaking down complex dye molecules into less toxic forms. These enzymes, including azo-reductases, laccases, peroxidases, and oxidases, play a vital role in decolorization and detoxification of dyes through enzymatic pathways (Jadhav *et al.* 2016a, Singh *et al.* 2021). Bacterial species like *Bacillus subtilis* and *Pseudomonas putida* are especially promising due to their resilience, fast growth rates, and enzymatic versatility under diverse environmental conditions.

This study focuses on enhancing the microbial decolorization of textile dyes using a bacterial consortium of *Bacillus subtilis* MTCC-2414 and *Pseudomonas putida* MTCC-2445. Specifically, it investigates the influence of different substrate types and concentrations on dye decolorization efficiency under optimized environmental conditions, including temperature, pH, and incubation period.

To analyze the interactions among various parameters and optimize the bioremediation process, Response Surface Methodology (RSM) was employed. RSM is a statistical technique that facilitates the modeling and analysis of problems in which a response is influenced by several variables, allowing the optimization of operational conditions (Box & Draper 2007a). Within this framework, the Central Composite Design (CCD) was selected to evaluate the effects of multiple factors simultaneously, enhancing the precision of experimental results and enabling the scalability of the process.

In addition to RSM, various statistical tools were employed to validate the significance of independent variables on dye decolorization. Analysis of Variance (ANOVA) was used to determine the statistical relevance of parameters such as substrate concentration, incubation period, and temperature (Montgomery, 2017a). Further refinement was achieved using Tukey's Honest Significant Difference (HSD) test, which identified statistically significant differences between treatment groups. Principal Component Analysis (PCA) was applied to reduce dimensionality and highlight the most influential variables affecting the decolorization process (Hair *et al*. 2019a).

Contour plots were constructed to provide a visual representation of the interactions among independent variables and their effect on dye removal efficiency. These plots aid in identifying optimal experimental conditions and facilitate a deeper understanding of variable relationships.

By integrating microbial bioremediation with advanced statistical and modeling tools, this study aims to contribute to the development of a sustainable, effective, and scalable solution for the treatment of dye-laden textile wastewater. The findings are expected to offer valuable insights into the enhancement of dye decolorization efficiency through substrate optimization and microbial synergy.

MATERIALS AND METHODS

Microbial cultures used

Bacillus subtilis (MTCC-2414) and *Pseudomonas putida* (MTCC-2445), cultures were obtained from Microbial Type Culture Collection. The bacterial strains were inoculated on nutrient agar slants and were sub-cultured when required. For the experiment the cultures grown in nutrient broth were used.

Preparation of dye solution

The dye used in the experiment is a dark pink colored dye that is obtained from Kadam Chemicals Pvt Ltd 1% dye solution was used in the experiment. 1g of dye powder was dissolved in 100 ml of sterile water.

Medias used: All the culture medias used were obtained from HIMEDIA.

Absorption maxima of the dye: The absorption maxima (λmax) of the dye was determined using a previously prepared 1% dye solution. The dye solution was placed in a clean quartz cuvette, and its absorbance was measured across the 400–700 nm wavelength range using a UV-Vis spectrophotometer. The highest absorbance peak was observed at 520 nm, further indicating it is the dye's maximum absorption wavelength (Ali *et al*. 2021b, Yaseen & Scholz 2019b).

Response surface methodology (RSM): A separate experimental setup was maintained for each bacterial strain to ensure consistency and eliminate process efficiency variations. The study involved six different substrate concentrations for *Bacillus subtilis* MTCC-2414 and *Pseudomonas putida* MTCC-2445. The experimental design incorporated glucose, sugarcane bagasse, and rice bran as substrates, each tested at varying concentrations based on a central composite design (CCD) within RSM (Box & Draper 2007b).

Decolorization assay

Decolorization efficiency was assessed for both bacterial strains using the following steps (Jadhav *et al.* 2016b, Saratale *et al*. 2011b). In brief Aliquots of 2 mL were collected from cultures at specific time intervals (0, 24, 48, 72 and 96 hrs) under sterile conditions, in Eppendorf tubes, centrifuged at 6000 rpm for 10 minutes to separate bacterial biomass, and the supernatant was transferred to fresh, labeled tubes and absorbance was measured at 520 nm using a UV-Vis spectrophotometer to evaluate dye decolorization efficiency.

The percentage of dye decolorization was calculated using the following formula:

$$\% \text{ dye decolorization} = [(A0 - At) / A0] \times 100$$

Where,

A0 = Initial absorbance of dye without bacterial culture.

At = Final absorbance of dye with bacterial culture.

Statistical analysis

Contour plots

Contour plots were used to visualize the interaction between independent variables (substrate concentration and incubation period) and the dependent variable (percentage of dye decolorization). (Montgomery 2017b).

Analysis of variance (ANOVA)

One-way ANOVA was performed to assess the significant differences in dye decolorization across different substrate concentrations (Hair *et al.* 2019b).

Tukey's honestly significant difference (HSD) test

Tukey's HSD test was used as a post-hoc analysis after ANOVA to compare multiple groups and identify the optimal substrate concentration for microbial dye degradation. This test ensured that statistically significant differences were correctly identified, reducing the likelihood of false results (Montgomery 2017c).

Principal component analysis (PCA)

PCA was employed to identify key factors influencing microbial decolorization efficiency (Hair *et al.* 2019c).

RESULTS AND DISCUSSION

Optimization of pink dye decolorization by *Bacillus subtilis* MTCC-2414 using central composite design (CCD).

The optimization of pink dye decolorization by *Bacillus subtilis* MTCC-2414 was investigated using Response Surface Methodology (RSM) with Central Composite Design (CCD). Various concentrations of three carbon substrates—glucose, sugarcane bagasse, and rice bran—were tested at different incubation intervals (0, 24, 48, 72 and 96 hrs). The substrates were incorporated into M9 minimal medium, and decolorization efficiency was measured as a percentage (Table 1).

Glucose as substrate

When glucose was used as the carbon source, decolorization was relatively low at lower concentrations (0.5% and 1%) during early time points. However, at higher concentrations (≥ 3%) and longer incubation times (72–96 hrs), decolorization efficiency increased significantly, peaking at 93.36% at 3.5% glucose concentration at 72 hrs (Table 1). This trend indicates that glucose acts as a good carbon source when provided in higher concentrations and allowed sufficient incubation time. However, sharp increases were noted post-48 hrs, suggesting enzyme induction or late-phase metabolic activation.

Sugarcane bagasse as substrate

Sugarcane bagasse, a lignocellulosic biomass, demonstrated superior performance in dye decolorization across all time points. The highest efficiency was observed between 72 and 96 hrs, with a peak decolorization of 94.57% at 1% and 3.5% concentrations at 72 hrs (Table 1). Interestingly, even at the lower end

Table 1. Percentage of pink dye decolorization by *Bacillus subtilis* MTCC-2414.

Substrate	Time in hours	Substrate concentrations 0.5% (Axial point-alpha)	1% (Low level -1)	2% (Center point 0)	3% (High level +1)	3.5% (Axial point +alpha)
M9+Glucose	0	56.5	61.58	58.29	36.29	32.13
	24	8.81	-3.13	10.01	7.92	18.23
	48	34.97	60.68	47.53	49.47	57.54
	72	29.53	32.22	32.22	28.33	30.12
	96	34.31	54.94	81.96	79.2	62.41
M9+Sugarcane	0	19.81	40.59	46.52	65.76	67.86
	24	-5.82	36.02	23.76	42	36.17
	48	40.5	32.96	59.49	52.01	68.9
	72	43.94	81.91	71.44	81.01	84.15
	96	53.31	86.54	84.6	80.56	55.09
M9+Ricebran	0	19.81	45.93	42.45	24.66	35.72
	24	8.96	10.46	7.02	4.03	8.37
	48	40.8	32.73	3.13	63.67	44.69
	72	39.61	41.4	48.13	58.59	63.52
	96	65.17	60.83	54.19	78.77	79.52

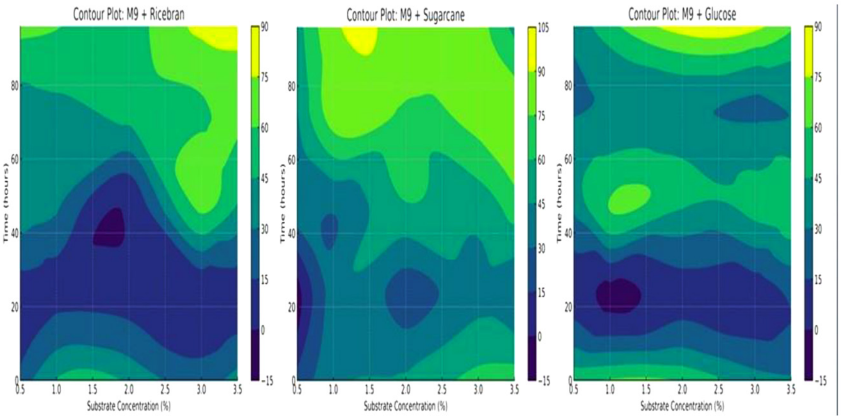

Fig. 1. Contour plots of substrates indicating the interactions between variables in *Bacillus subtilis* MTCC-2414 .

(1%), sugarcane achieved better results than glucose or rice bran. This efficiency could be attributed to the mixed sugar profile in sugarcane (simple and complex sugars), promoting sustained microbial growth and enzyme production.

Rice bran as substrate

Rice bran exhibited a different decolorization pattern. While the initial time points (0–24 hrs) showed very low efficiency across all concentrations, a remarkable increase was observed from 48 to 72 hrs. At 3% rice bran, 94.29% decolorization was recorded at 72 hrs, the highest among all treatments for *Bacillus subtilis* (Table 1). However, efficiency dipped slightly at 96 hrs. These fluctuations suggest complex enzymatic adaptation, possibly due to the requirement for inducible enzymes to metabolize rice bran's complex components.

Contour plots

With M9 and Glucose, Decolorization is low at lower concentrations and shorter times. Higher concentrations (>3%) and longer incubation periods (>60 hrs) show maximum decolorization (~ 80%). M9 with

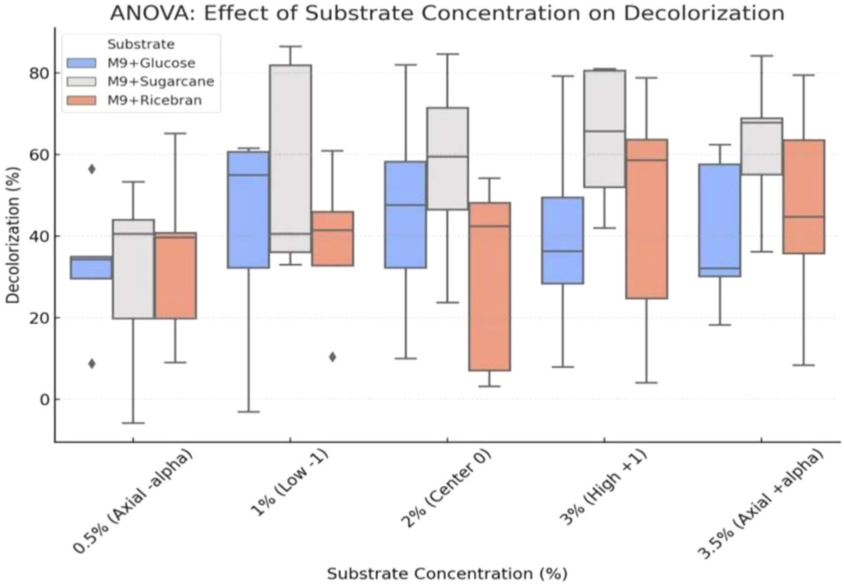

Fig. 2. Box plot indicating the effect of substrates on dye decolorization in *Bacillus subtilis* MTCC-2414.

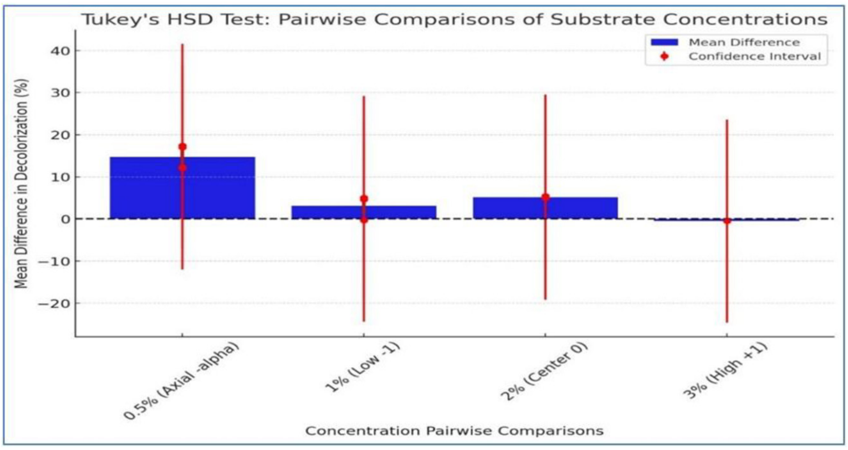

Fig. 3. Box plot showing the effect of different substrate concentrations in *Bacillus subtilis* MTCC-2414.

sugarcane bagasse showed higher decolorization efficiency peaking at ~90%. Higher concentrations (>2%) with extended incubation periods lead to optimal decolorization. M9 and rice bran displayed a different pattern, with decolorization efficiency varying across time. A significant drop in efficiency around 24–48 hrs, followed by an increase at later stages was observed (Fig. 1).

One-way ANOVA

The one-way ANOVA results demonstrate that the substrate concentration alone does not significantly affect dye decolorization ($p > 0.05$), though observable trends exist. The analysis suggests that incubation time plays a more crucial role. Particularly, the effect of higher substrate concentrations is maximized only when paired with extended incubation (Fig. 2).

Tukey's HSD-based box plot

The box plot comparing different substrate concentrations showed overlapping confidence intervals, indicating statistically insignificant differences between most concentration pairs. However, a trend

Table 2. Percentage of pink dye decolorization by *Pseudomonas putida* MTCC-2445.

Substrate	Time in hours	Substrate concentrations 0.5% (Axial point-alpha)	1% (Low level –1)	2% (Center point 0)	3% (High level +1)	3.5% (Axial point +alpha)
M9+Glucose	0	56.5	61.58	58.29	36.29	32.13
	24	8.81	–3.13	10.01	7.92	18.23
	48	34.97	60.68	47.53	49.47	57.54
	72	29.53	32.22	32.22	28.33	30.12
	96	34.31	54.94	81.96	79.2	62.41
M9+Sugarcane	0	19.81	40.59	46.52	65.76	67.86
	24	–5.82	36.02	23.76	42	36.17
	48	40.5	32.96	59.49	52.01	68.9
	72	43.94	81.91	71.44	81.01	84.15
	96	53.31	86.54	84.6	80.56	55.09
M9+Ricebran	0	19.81	45.93	42.45	24.66	35.72
	24	8.96	10.46	7.02	4.03	8.37
	48	40.8	32.73	3.13	63.67	44.69
	72	39.61	41.4	48.13	58.59	63.52
	96	65.17	60.83	54.19	78.77	79.52

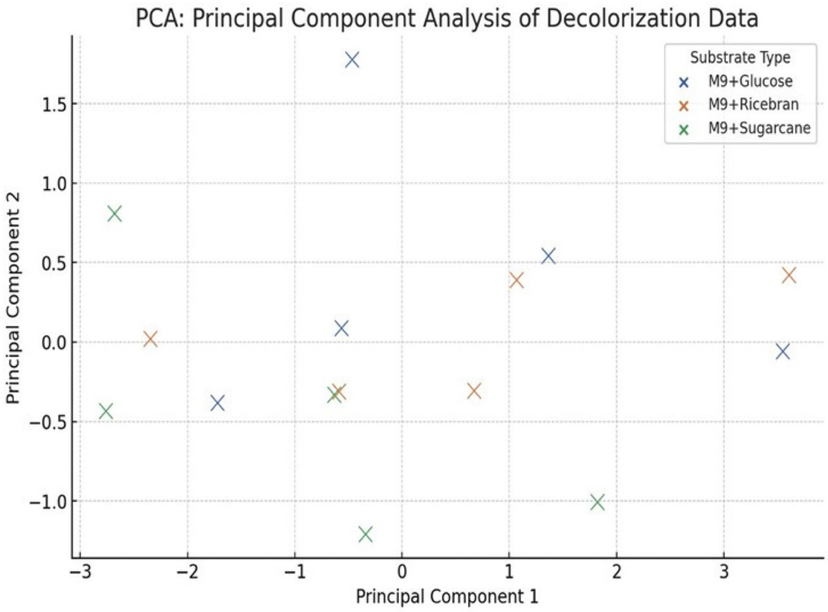

Fig. 4. Scatter plot showing the distribution of different substrates effecting decolorization in *Bacillus subtilis* MTCC-2414.

of improved efficiency at 3%–3.5% was observed, especially with rice bran and sugarcane substrates (Fig. 3).

PCA scatter plot

Principal Component Analysis (PCA) revealed that the first two components accounted for 76.06% (PC1) and 10.09% (PC2) of the variance, respectively. The scatter plot indicated distinct clustering of the three substrates. Glucose showed a broader spread, reflecting variable performance. In contrast, sugarcane and rice bran formed tighter clusters at high decolorization efficiencies, suggesting consistent performance (Fig. 4).

Optimization of pink dye decolorization by *Pseudomonas putida* MTCC-2445

Similar to the earlier experiment, *Pseudomonas puti-*

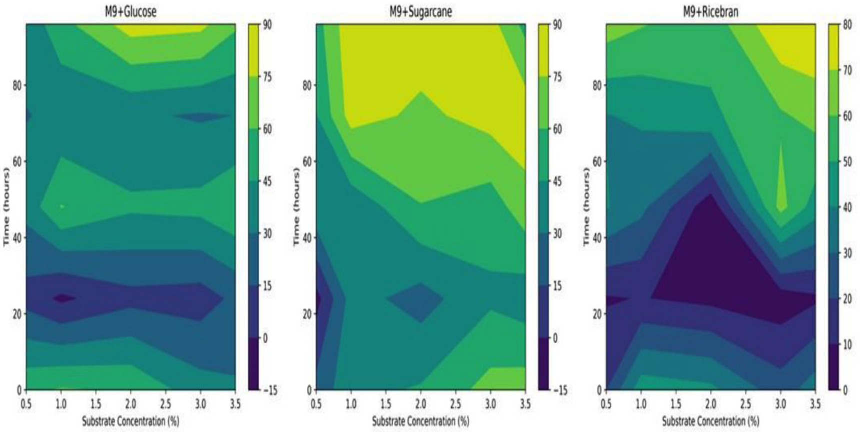

Fig. 5. Contour plots of substrates indicating interactions between variables in *Pseudomonas putida* MTCC-2445.

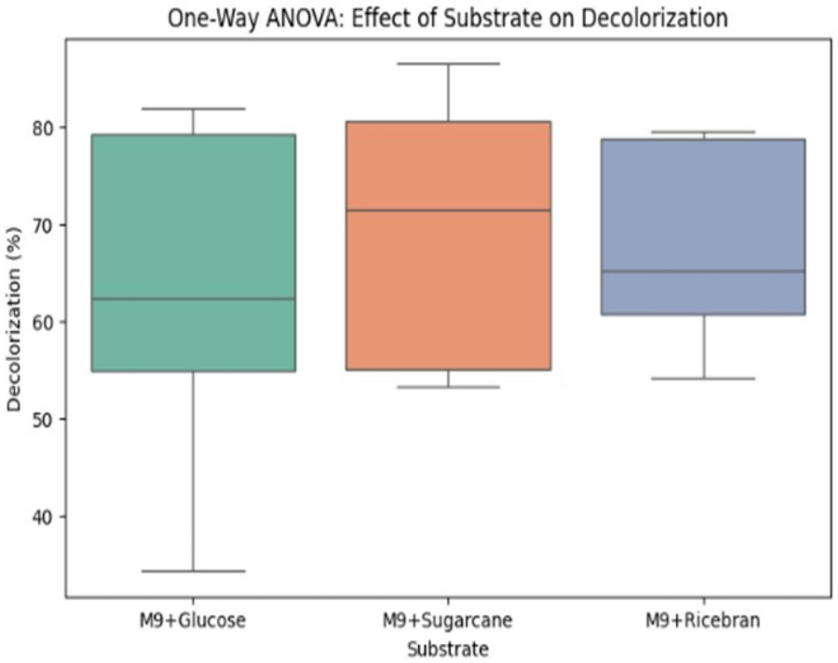

Fig. 6. Box plot indicating effect of substrate on dye decolorization in *Pseudomonas putida* MTCC-2445.

da was evaluated for dye decolorization in M9 media supplemented with glucose, sugarcane bagasse, and rice bran at concentrations ranging from 0.5% to 3.5%, across a 96-hour time course (Table 2).

Glucose as substrate

Glucose yielded moderate decolorization, with values ranging from 32.13% to 81.96%. The highest efficiency was observed at 96 hrs with 2% glucose (81.96%) (Table 2). Interestingly, very low or even negative values were recorded at 24 hrs for some concentrations, indicating possible metabolic inhibition or delayed enzyme induction in early phases.

Sugarcane bagasse as substrate

Sugarcane bagasse again showed superior performance. At 72 and 96 hrs, decolorization peaked between 80–86%, particularly at higher concentrations (Table 2). The dual sugar profile of sugarcane likely promotes stable bacterial metabolism and continuous enzyme activity, facilitating more effective dye breakdown.

Rice bran as substrate

Rice bran showed moderate performance, with increasing decolorization observed from 48 to 96 hrs, achieving a maximum of 79.52% at 3.5% concentration (Table 2). The slower initial response suggests that enzyme systems involved in rice bran degradation may take time to express optimally.

Contour plot

The contour plot for glucose confirms that maximum decolorization was achieved after 72 hrs, particularly at 2%–3% concentrations. The less prominent contour lines indicate minor improvements beyond this point, suggesting saturation (Fig. 5).

One-way ANOVA

The one-way ANOVA showed significant differences ($p < 0.05$) in decolorization efficiency among the substrates. Glucose had the highest variability and the lowest efficiency, while sugarcane showed the most consistent and highest decolorization. Rice bran performed intermediately. The presence of lower

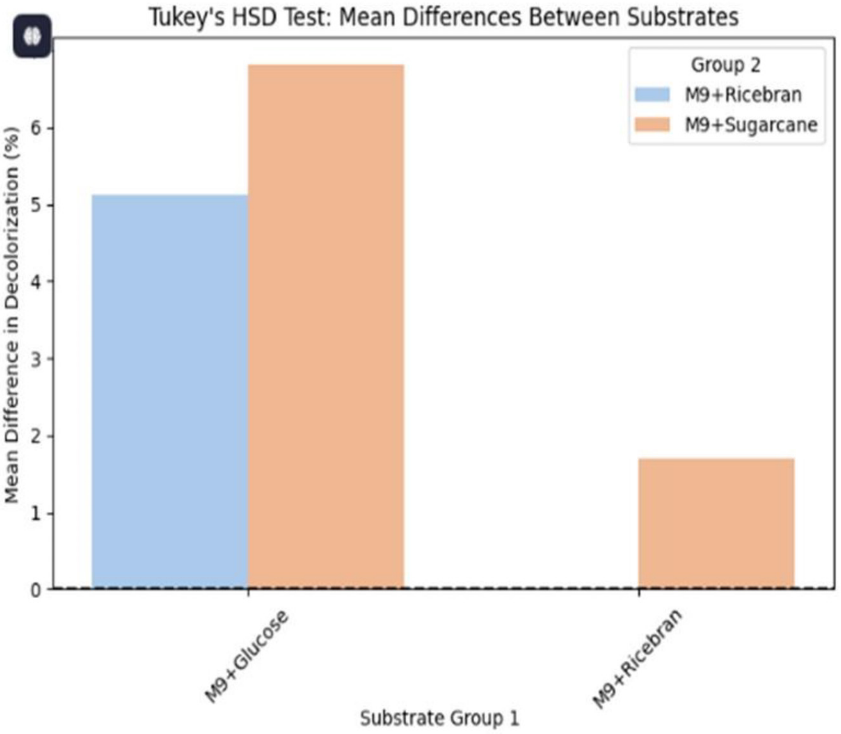

Fig. 7. Box plot showing different substrates influence decolorization in *Pseudomonas putida* MTCC-2445.

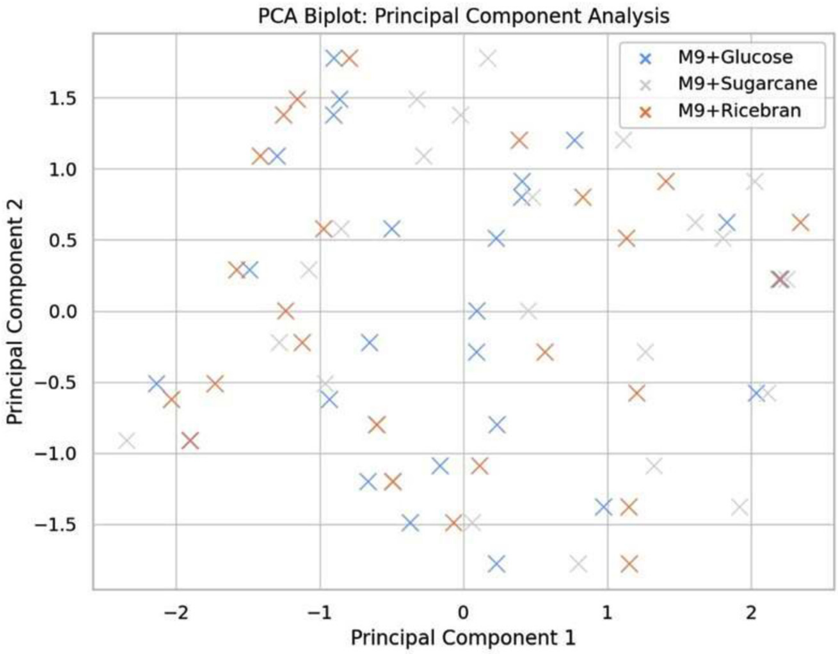

Fig. 8. Scatter plot showing distribution of different substrates influencing decolorization in *Pseudomonas putida* MTCC-2445.

Table 3. The comparative analysis between two organisms.

Parameter	*Pseudomonas putida*	*Bacillus subtilis*
Peak decolorization	72–96 hrs	>60 hrs
Best substrate	Sugarcane bagasse	Sugarcane bagasse
Contour plot	High substrate concentrations required	Incubation periods play an important role
ANOVA	Significant effect of substrate	Incubation period is important
Tukey's HSD	Sugarcane> Rice-bran>Glucose	No significant difference between types of substrates
PCA clustering	Sugarcane and rice-bran have similar effect	Distinct groups of clusters were observed for all the different substrates

whiskers in glucose treatment indicates suboptimal activity (Fig. 6).

Tukey's HSD test

The bar plot comparing mean differences confirmed that sugarcane and rice bran had similar decolorization effects. However, glucose performed significantly worse. The confidence intervals suggest sugarcane as the most effective substrate overall (Fig. 7).

PCA scatter plot

The PCA scatter plot confirmed that glucose samples were widely distributed, indicating variable metabolic responses. Sugarcane formed a distinct cluster with high decolorization values, while rice bran formed a moderately effective group. PCA demonstrated that the type of carbon source significantly affected bacterial metabolic behavior (Fig. 8).

The comparative study using RSM suggests that while both microorganisms are efficient in decolorizing dye, their working mechanisms are different (Table 3). *Pseudomonas putida* is most efficient with high substrate loads and is enhanced by sugarcane bagasse, while *Bacillus subtilis* is uniformly efficient across substrates but utilizes increased incubation times for maximum efficiency. Such findings hold importance in the design of bioreactors and optimizing wastewater treatment, where microbial selection can be driven by substrates available and treatment time.

CONCLUSION

Both *Bacillus subtilis* and *Pseudomonas putida* demonstrated their potential in pink dye decolorization, with substrate type and incubation time being critical factors. Sugarcane bagasse consistently outperformed other substrates, especially at higher concentrations and prolonged incubation periods. Glucose, though rapidly metabolized, showed inconsistent results and lower overall efficiency, particularly with *Pseudomonas*. Rice bran, due to its complexity, had delayed but ultimately high decolorization, indicating the necessity for longer incubation for optimal results.These findings underscore the value of using lignocellulosic wastes like sugarcane and rice bran as sustainable, cost-effective substrates for bioremediation applications. Future work should explore enzyme expression profiles and kinetics in response to substrate complexity for better industrial optimization.

ACKNOWLEDGMENT

The authors sincerely thank the Principal and Management of Bhavan's Vivekananda College of Science, Humanities and Commerce for providing Seed Money (BVC MRP Seed Money 2022-23/11) to carry out this research project. We also thank them for their continued support and encouragement.

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