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Comparative Evaluation of *Pseudomonas putida* and *Lysinibacillus sphaericus* in Submerged Fermentation for Microbial Degradation of Dyes Present in Industrial Effluents

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Abstract—*Dye degradation is imperative for industries seeking to mitigate the release of polluted effluents into the environment. Notably, Pseudomonas putida, Acid Yellow 42, and Direct Red 28 dyes constitute significant pollutants in industrial effluents, posing severe environmental risks. Primarily utilized in textile and leather industries as colouring agents, these dyes pose challenges for removal due to their dispersal and resistance to eradication or reduction in effluents. The complexity of dye composition further complicates degradation efforts. The current study aims to address this challenge by employing microbial degradation, utilizing pure cultures of Pseudomonas putida and Lysinibacillus sphaericus in separate monoculture submerged fermentation setups. By investigating the comparative degradation of these dyes by two distinct bacterial cultures, the study seeks to pave the way for future research into combined co-culture submerged fermentation setups. Three concentrations of each dye (100 ppm, 300 ppm, and 500 ppm) were subjected to degradation studies. Pseudomonas putida demonstrated remarkable efficacy in degrading Direct Red 28, achieving maximum colour degradation of 100% within 24 hours, while Lysinibacillus sphaericus achieved similar results between 24 to 48 hours. Pseudomonas putida also demonstrated significant dye degradation, with 96.43% and 98.86% for Acid Yellow 42 and Pseudomonas putida, respectively, at 100 ppm concentration. Observations over 144 hours, at 24-hour intervals, revealed optimal colour degradation occurring between 24 to 48 hours for both bacterial strains, even at higher dye concentrations (500 ppm). These findings underscore the effectiveness of both bacterial strains in dye degradation, highlighting the potential for developing a multi-microbial degradation mechanism to address industrial dye pollution. Through biological treatment approaches, this work offers promise in mitigating environmental dye contamination arising from industrial activities.*

Keywords: *colouring agents; industrial effluents; submerged fermentation; multi-microbial degradation.*

1. INTRODUCTION

Dye degradation has emerged as a critical environmental imperative due to its deleterious effects on ecosystems. Approximately 10-15% of dyes are discharged directly into water bodies via industrial processes, adversely affecting water quality [1, 2]. These dyes disrupt photosynthesis, reduce water transparency, and compromise gas solubility by interacting with water molecules, releasing metals and chlorides such as rhodamine 110 chloride and other metal dyes [3]. Moreover, they pose significant challenges in textile machinery operations due to deposition, leading to clogs and disruptions. Furthermore, concerns regarding the carcinogenic and mutagenic properties of dye constituents heighten the urgency for effective degradation methods [4]. Addressing these challenges necessitates innovative approaches. Various physical and chemical treatments, such as precipitation, coagulation, flocculation, ion-exchange adsorption, membrane separation, and ozonation, are employed to mitigate dye toxicity. Chemical oxidation and reduction processes break down dyes into simpler fragments, aiding in effluent color removal [5-8]. Biological treatments, utilizing aerobic and anaerobic digestion, offer effective mitigation of dye toxicity. Microorganisms, including bacteria, algae, and fungi, play pivotal roles in decolorization processes, facilitating dye remediation from effluents [9-11]. Researchers have identified several microorganisms for dyes decolorization, including *Coriolus versicolor, Mycelia sterilia, Aspergillus fumigates, Phanerochaete chrysosporium, Trametes versicolor*, and *Pseudomonas putida* [12-17]. Notably, *Pseudomonas putida* and *Lysinibacillus sphaericus* have shown promise in this regard. These organisms demonstrate rapid growth on various substrates and possess capabilities for efficient dye degradation. Certain dyes, such as *Pseudomonas putida*, Acid Yellow 42, and Direct Red 28, exhibit particularly harmful environmental implications when present in industrial effluents. *Pseudomonas putida* is known to be toxic to aquatic life and may cause long-term damage to ecosystems. Acid Yellow 42 can have adverse effects on

aquatic organisms and disrupt the balance of aquatic ecosystems. Direct Red 28, similarly, can pose risks to aquatic life and may lead to the deterioration of water quality. Their presence necessitates enhanced degradation strategies [18-23].

Enzymes like laccases, produced by microorganisms like *Pseudomonas putida* and *Lysinibacillus sphaericus*, offer promising avenues for dye degradation. Laccases, belonging to the group of blue oxidases, catalyze the oxidation of various organic and inorganic compounds, including dyes, through enzymatic action. Notably, *Pseudomonas putida* and *Lysinibacillus sphaericus* have demonstrated the production of ligninolytic enzymes such as laccase, lignin peroxidase, and manganese peroxidase, which are instrumental in dye decolorization.

In this study, we aim to biologically degrade or decolorize dyes, specifically *Pseudomonas putida*, Acid Yellow 42, and Direct Red 28, utilizing microorganisms such as *Pseudomonas putida* and *Lysinibacillus sphaericus*. Additionally, investigating the comparative degradation of these dyes by two distinct bacterial cultures, the study seeks to pave the way for future research into combined co-culture submerged fermentation setups.

2. MATERIALS AND METHODS

2.1 Microbial strain used for study

Pseudomonas putida and *Lysinibacillus sphaericus* were obtained from the Microbial Type Culture Collection (MTCC) in Chandigarh, India. The strains were revived and sub-cultured on growth medium 3 (nutrient agar media), comprising Beef Extract 1.0 g, Peptone 5.0 g, NaCl 5.0 g, and Agar 15.0 g per liter of distilled water. *Pseudomonas putida* culture was incubated at 30°C for 24 hours, while *Lysinibacillus sphaericus* culture was incubated at 37°C for 48 hours. The subsequent colonies were sub-cultured again and the incubation temperatures were maintained [24].

2.2 Dyes selected for the study

The dyes selected for the biodegradation were *Pseudomonas putida* (RR), Acid Yellow 42 (AY), and Direct Red 28 (DR). All the three selected dyes are highly toxic and cause serious problems. These dyes were selected for our degradation studies, as they are widely used in textile and leather industries, due to which they directly affect humans. Three different concentrations of selected dyes for the study were 100 ppm [*Pseudomonas putida* (RR1), Acid Yellow 42 (AY1), and Direct Red 28 (DR1)], 300 ppm [*Pseudomonas putida* (RR3), Acid Yellow 42 (AY3), and Direct Red 28 (DR3)] and 500 ppm [*Pseudomonas putida* (RR5), Acid Yellow 42 (AY5), and Direct Red 28 (DR5)]. The dyes used were of high purity purchased from Himedia, India.

2.3 Inoculum preparation

The Nutrient Broth (NB) media of composition 13 g/l, was used as media to prepare inoculums. The cultures from the plates were transferred under the aseptic conditions using sterile distilled water into 5 ml inoculum media separately. The *Pseudomonas putida* inoculum was kept in incubator at 30^{o}C for 72 h and similarly, *Lysinibacillus sphaericus* inoculum was kept in incubator at 37^{o}C for 72 h.

2.4 Fermentation condition

The dye degradations were carried out in submerged fermentation. The fermentation media composition (FMC) in g/l was glucose 5.0, KH2PO4 2.0, MgSO4.7H2O 0.5, Urea 0.036 and CaCl2 0.09930 [25]. In the fermentation media different concentrations of dyes were added to observe dye degradation. The fermentation condition was selected to provide the minimum nutrient media to the *Pseudomonas putida* and *Lysinibacillus sphaericus* for growth in presence of dyes. The total 18 sets of experiments were designed in triplicate for three different dyes, each in 100 ml Erlenmeyer flask. In each flask 50 ml fermentation media was taken to carry out degradation process. Then 5ml of previously prepared inoculum was added to each fermentation flask. The fermentation as carried out in an orbital shaker incubator at 100 rpm and incubation temperature of 30^{o}C for *Pseudomonas putida* and 37^{o}C for *Lysinibacillus sphaericus* for 144 h.

2.5 Kinetic study of dye degradation and its estimation

The kinetic study was carried out at the time interval of 24 h up to 144 h. The colour detection of *Pseudomonas putida* 620 nm [26], Acid Yellow 42 at 450 nm [27], and Direct Red 28 at 498 nm [28]. The protein estimation was done by Lowry's method34, glucose estimation by DNS method35 and color degradation by spectrophotometry using a double beam UV-visible spectrophotometer spectrometer36.

3. RESULTS AND DISCUSSION

The kinetic study was carried out for degradation of dyes indicated by colour reduction by *Pseudomonas putida* and *Lysinibacillus sphaericus* separately.

3.1 Kinetic study of color degradation of dyes

The kinetic study aimed to evaluate the percentage color removal of selected dyes by *Pseudomonas putida* and *Lysinibacillus sphaericus* separately. The investigation revealed effective color degradation in all three chosen dyes, with superior performance observed at lower dye concentrations compared to higher concentrations for both bacteria. In the supernatant post-filtration and centrifugation, Pseudomonas putida exhibited 100% degradation in Direct Red 28 (DR1), with 97.72% and 91.32% in DR3 and DR5, respectively. Similarly, *Lysinibacillus sphaericus* demonstrated 100% degradation in DR1, with 96.89% and 93.56% in DR3 and DR5, respectively, albeit slightly less efficient than DR1 for both bacteria [29].

For Acid Yellow 42 (AY1) and *Pseudomonas putida* (RR1) at 100 ppm, Pseudomonas putida achieved 96.43% and 98.86% color removal, while at 300 ppm, it achieved 86.33% and 88.9% for AY3 and RR3, and at 500 ppm, 83.45% and 90.12% for AY5 and RR5, respectively. *Lysinibacillus*

sphaericus recorded the highest color degradation (92.71%) at 300 ppm AY3. These results indicate that both Pseudomonas putida and *Lysinibacillus sphaericus* effectively degrade dyes at low concentrations, with maximum color reduction observed in Direct Red 28 dye [30].

In 100 ppm Direct Red 28, an optical density of 0.0081 at 498 nm was measured at time zero, with complete color removal observed within 24 hours. Conversely, maximum color reduction in Acid Yellow 42 and *Pseudomonas putida* was observed after 48 hours, with color removal remaining stable between 48 and 144 hours during the kinetic study. Interestingly, while previous studies reported maximum decolorization after at least 5 days of incubation, we observed maximum color removal within 48 hours, showcasing promising results. For instance, the 0.1% w/v Direct Red 28 was reported to decolorize by 74.63% after 10 days of incubation, whereas we achieved 100% degradation in 100 ppm Direct Red 28 after just 24 hours [31].

3.2 Kinetic study of substrate utilization during dyes degradation and laccase production

Glucose was added as a carbon source and urea as a nitrogen source to facilitate the production of laccase by *Pseudomonas putida* and *Lysinibacillus sphaericus*, crucial for dye degradation. Previous studies have indicated that the availability of carbon and nitrogen sources, as well as the concentration of microelements, significantly affect laccase production by bacterial strains. In our experiments, maximum substrate utilization was consistently observed within 48 hours across all experimental conditions. Notably, glucose consumption was nearly complete within this timeframe, particularly evident in fermentation media containing 100 ppm dyes as shown in **Table 1**. These findings underscore the importance of optimizing carbon and nitrogen sources to enhance laccase production and subsequent dye degradation efficiency.

Table 1: Glucose Consumption during biodegradation of selected three dyes.

Incubation time (h)	Glucose content, mg/ml in selected dyes of different concentration for *Pseudomonas putida*								
	RR1	RR3	RR5	AY1	AY3	AY5	DR1	DR3	DR5
0	1.34	1.48	1.44	1.81	1.57	1.72	1.85	1.65	1.55
24	0.49	1.32	0.56	0.65	1.34	1.63	0.59	0.93	0.80
48	0.09	0.29	0.31	0.16	0.59	1.42	0.08	0.57	0.47
72	0.07	0.33	0.26	0.17	0.61	1.33	0.09	0.36	0.27
96	0.06	0.24	0.19	0.05	0.64	1.29	0.05	0.19	0.27
144	0.001	0.19	0.14	0.02	0.42	0.87	0.002	0.15	0.13

Incubation time (h)	Glucose content, mg/ml in selected dyes of different concentration for *Lysinibacillus sphaericus*								
	RR1	RR3	RR5	AY1	AY3	AY5	DR1	DR3	DR5
0	1.51	1.66	1.62	1.77	1.52	1.90	1.55	1.47	1.86
24	0.43	1.42	0.54	0.56	1.34	1.78	0.64	0.76	0.89
48	0.12	0.43	0.43	0.23	0.69	1.58	0.07	0.57	0.28
72	0.08	0.23	0.31	0.16	0.71	1.37	0.09	0.45	0.16
96	0.09	0.20	0.27	0.09	0.73	1.23	0.05	0.29	0.15
144	0.002	0.17	0.13	0.04	0.52	0.89	0.001	0.11	0.09

3.3 Kinetic study of protein content during dye degradation and laccase production

In all experimental sets, protein content showed a significant increase up to 48 hours, after which negligible changes were observed. However, in cases of RR1, RR3, and RR5, protein content continued to increase until 72 hours before declining steadily until 144 hours for *Pseudomonas putida*. Similarly, for AY1, AY3, and AY5 of *Lysinibacillus sphaericus*, protein content peaked at 48 hours, followed by a decline thereafter. Interestingly, in Direct Red 28 dye treatments, protein content remained relatively constant throughout the degradation process, with only a slight increase observed in DR5 at 48 hours, followed by a decline for *Lysinibacillus sphaericus*. It's noteworthy that protein concentration tended to increase with higher dye concentrations, escalating from 100 ppm to 500 ppm as shown in **Table 2**. These observations shed light on the dynamics of protein production during the degradation process and underscore the potential influence of dye concentration on protein synthesis.

Table 2: Protein Content during Biodegradation of selected three dyes.

Incubation time (h)	Protein Content, mg/ml in selected dyes of different concentration for *Pseudomonas putida*								
	RR1	RR3	RR5	AY1	AY3	AY5	DR1	DR3	DR5
0	0.18	0.42	0.63	0.42	0.54	0.78	0.47	1.34	1.78
24	0.23	0.35	0.74	0.45	0.71	0.82	0.48	1.32	1.80
48	0.23	0.56	0.75	0.47	0.68	0.89	0.42	0.88	1.83
72	0.24	0.58	0.79	0.42	0.59	0.86	0.39	0.89	1.76
96	0.25	0.61	0.64	0.39	0.46	0.62	0.32	0.92	1.68
144	0.28	0.53	0.49	0.26	0.32	0.56	0.41	0.82	1.59
Incubation time (h)	Protein Content, mg/ml in selected dyes of different concentration for *Lysinibacillus sphaericus*								
	RR1	RR3	RR5	AY1	AY3	AY5	DR1	DR3	DR5
0	0.14	0.56	0.60	0.46	0.71	0.90	0.67	1.45	1.84
24	0.25	0.59	0.67	0.47	0.72	0.87	0.56	1.33	1.81
48	0.27	0.64	0.69	0.56	0.79	0.90	0.78	0.89	1.94
72	0.27	0.67	0.46	0.53	0.65	0.98	0.79	0.93	1.91
96	0.25	0.58	0.37	0.46	0.32	0.76	0.54	0.95	1.78
144	0.22	0.51	0.33	0.32	0.21	0.45	0.34	0.91	1.36

3.4 Comparative degradation of the selected dyes by two distinct bacterial cultures *Pseudomonas putida* and *Lysinibacillus sphaericus*

Pseudomonas putida and *Lysinibacillus sphaericus*, both display notable effectiveness in degrading all three selected dyes, with *Pseudomonas putida* demonstrating slightly superior degradation efficiency, particularly evident at lower dye concentrations. Interestingly, both bacteria exhibit a pattern of increased degradation efficiency at lower dye concentrations, indicating that the degradation process is more effective when initial dye concentrations are lower. While both bacteria exhibit high degradation efficiency across all dyes, slight variations exist in their performance. For instance, *Pseudomonas putida* achieves higher color removal percentages for Acid Yellow 42 and Reactive Red 198, while *Lysinibacillus sphaericus* demonstrates superior degradation efficiency for Acid Yellow 42 at 300 ppm. Additionally, *Pseudomonas putida* exhibits faster degradation kinetics, with maximum color reduction typically observed within 24-48 hours, whereas

Lysinibacillus sphaericus displays slightly slower kinetics, with peak degradation occurring within 48-72 hours. Notably, the rapid color removal observed in this study, particularly within 24-48 hours, surpasses timelines reported in previous studies, indicating enhanced efficiency. For instance, complete degradation of Direct Red 28 at 100 ppm was achieved within 24 hours in this study, compared to previous reports of maximum decolorization occurring after 5 days or more. In summary, both *Pseudomonas putida* and *Lysinibacillus sphaericus* demonstrate effective degradation of the selected dyes, with *Pseudomonas putida* showing slightly higher efficiency and faster kinetics, especially at lower dye concentrations. Nonetheless, *Lysinibacillus sphaericus* exhibits competitive performance, particularly evident in the degradation of Acid Yellow 42 at 300 ppm, underscoring the potential of both bacterial strains for industrial applications in dye degradation processes.

CONCLUSION

The study underscores the remarkable efficacy of both *Pseudomonas putida* and *Lysinibacillus sphaericus* in degrading the selected dyes, offering promising prospects for industrial applications in dye degradation processes. Notably, *Pseudomonas putida* exhibits slightly superior degradation efficiency, particularly evident at lower dye concentrations. The pattern of increased degradation efficiency at lower dye concentrations suggests the importance of initial dye concentration in determining the effectiveness of the degradation process. By elucidating the comparative degradation of these dyes by two distinct bacterial cultures, the study lays the groundwork for future research into combined co-culture submerged fermentation setups, offering potential avenues for further advancements in dye degradation technologies.

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Data availability The authors confirm that the data supporting the findings of this study are available within the article.

DECLARATIONS

Ethical approval Not applicable.

Competing interests The authors declare no competing interests.

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