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STRATEGIC OPTIMIZATION OF FERMENTATION PARAMETERS FOR *BACILLUS SUBTILIS*-MEDIATED AZO DYE TREATMENT

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Abstract: Azo dyes, widely used in the textile industry, pose significant environmental concerns due to their toxicity, potential carcinogenicity, and impact on aquatic ecosystems. Similarly, coffee dye, a natural pigment released from coffee-processing industries, contributes to organic pollution, oxygen depletion, and discoloration of water bodies. *Bacillus subtilis* has emerged as a promising microorganism for the bioremediation of both azo and coffee dyes, offering a sustainable and eco-friendly solution. This study provides a comprehensive overview of the current knowledge on optimizing cultural conditions for dye decolorization by *B. subtilis*. Key factors such as pH, temperature, dye concentration, carbon and nitrogen sources, redox mediators, and incubation time are discussed for enhancing decolorization efficiency. The enzymatic mechanisms involved—including azoreductases, laccases, and peroxidases—are also examined. *B. subtilis* demonstrates effective degradation of complex dye structures in both synthetic azo dyes and natural coffee dyes, making it a versatile agent for industrial bioremediation. The study further explores future research directions, including genetically engineered strains, immobilization techniques, and integrated treatment systems, along with the importance of phytotoxicity and microbial toxicity assessments. Overall, this study highlights the bioremediation potential of *Bacillus subtilis* for treating diverse dye-containing effluents.

Keywords: *Bacillus subtilis*, Azo dyes, Bioremediation, Optimization, Enzymatic mechanisms, Industrial-scale applications

I. INTRODUCTION

The textile industry plays a crucial role in the global economy but is also a major source of environmental contamination, particularly due to the release of synthetic azo dyes like Congo Red and Reactive Black 5. These dyes are characterized by azo bonds (–N=N–) and aromatic structures that contribute to their color stability but also their resistance to conventional treatment methods (1,2). Their persistence, mutagenicity, and toxicity pose significant threats to aquatic ecosystems and human health (3,4). In response, microbial bioremediation has gained attention as a sustainable and cost-effective solution. Among the microorganisms studied, *Bacillus subtilis*, a Gram-positive, spore-forming bacterium, has demonstrated significant potential due to its robustness, rapid growth, and ability to produce dye-degrading enzymes like azoreductases and laccases (5,6). These enzymes cleave azo bonds under aerobic or anaerobic conditions, converting toxic dyes into less harmful compounds (7,8). Submerged fermentation (SmF) offers a controlled environment to optimize various fermentation parameters, including pH, temperature, dye concentration, carbon sources, and incubation time, all of which influence the efficiency of microbial degradation(9,10). For instance, a neutral pH (around 7.0) and moderate dye concentration enhance azoreductase activity, while extreme conditions hinder enzyme performance (11,12).Additionally, replacing costly carbon sources with agro-industrial by-products such as banana sap and coffee powder has shown promise in improving both biodegradation and economic feasibility (13,14). These alternatives offer nutrient-rich substrates that support bacterial growth and align with sustainable waste reuse practices. This study focuses on optimizing fermentation parameters for *Bacillus subtilis* to efficiently decolorize azo dyes, aiming to scale microbial treatment for textile wastewater. By integrating phenotypic, molecular, and environmental approaches, the research provides a framework for applying *B. subtilis* as a practical bioremediation agent in industrial effluent treatment systems (15,16).

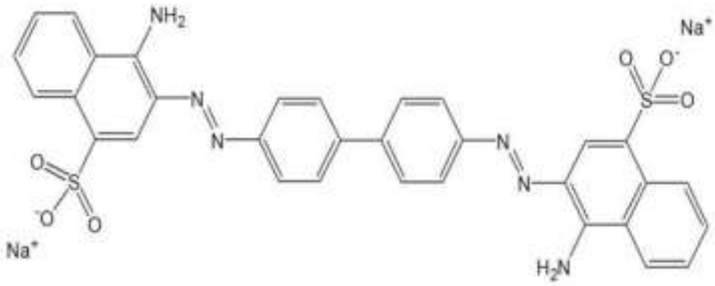


Fig 1.1: Molecular Structure of Congo Red (17)

AIM AND OBJECTIVE

AIM - The goal was to find the best conditions for Bacillus subtilis to break down the textile dyes Congo Red and coffee dye in liquid culture, aiming for an eco-friendly way to treat textile wastewater**.**

OBJECTIVES

1. To isolate and identify *Bacillus subtilis* from textile waste dumping site soil using plating and molecular techniques.
2. To determine the optimal pH (5.0, 6.0, 7.0, 8.0, 9.0) for Congo Red decolorization by measuring absorbance at 520 nm.
3. To assess the effect of Congo Red concentrations (20, 40, 60, 80, 100 mg/L) on decolorization efficiency.
4. To compare decolorization efficiency using different carbon sources (sucrose, starch). And to compare between chemical dye (Congo red) and natural dye (Coffee powder).
5. To evaluate time-dependent decolorization (24, 48, 72 hours) under optimized conditions for maximum dye degradation.

II. MATERIAL AND METHOD

2.1: Material

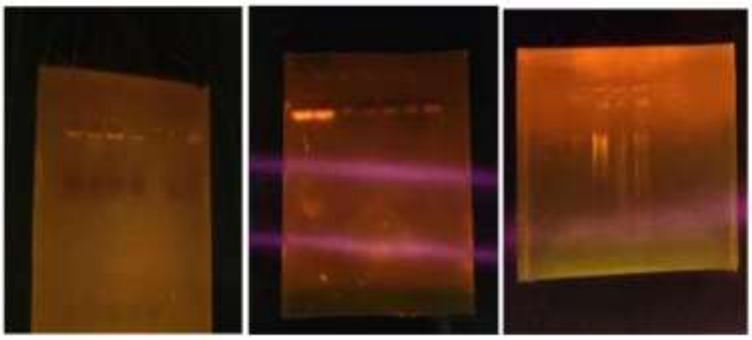
Biological sources: *Bacillus subtilis* (soil isolate), coffee powder.
Chemicals: Congo red, NaOH, HCl (for pH adjustment), distilled water, nutrient broth/agar, minimal medium, glucose, sucrose, starch

2.2: Methods

2.2.1 Methodology:

Soil samples were collected from a textile waste dumping site using sterile spatulas, targeting the top 5–10 cm of the soil surface to obtain microbial populations adapted to dye contamination. Approximately 100 grams of soil were transferred to sterile polyethylene bags and stored at 4°C to maintain microbial viability until processing. Isolation of azo dye-degrading bacteria was carried out using the spread plate method on nutrient agar medium. NA was prepared by dissolving 2.8 g in 100 mL distilled water, autoclaved at 121°C for 15–20 minutes, followed by the addition of 2.5 g agar and further heating. After cooling in a laminar flow hood under UV light, the medium was poured into sterile Petri dishes. The soil samples were gradually diluted from 10^{-1} to 10^{-6} using sterile distilled water to reduce the concentration for further analysis. Then, 0.1 mL from the 10^{-3} to 10^{-6} dilutions was carefully spread onto plates using flame-sterilized glass spreaders. Plates were incubated at 37°C for 24 hours. To obtain pure isolates, the streak plating method was used. Colonies from spread plates were streaked across quadrants of fresh TSA plates with an inoculation loop sterilized between streaks. After 24-hour incubation at 37°C, isolated colonies were subjected to Gram staining (18). Gram-positive rod-shaped bacteria retaining crystal violet were identified as bacteria and sent for gene sequencing where it was confirmed that the isolated bacteria is *Bacillus subtilis*. For broth culture preparation, single colonies were inoculated into 120 mL of autoclaved nutrient broth and incubated at 37°C in a rotary shaker for 24 hours. DNA was isolated from the culture by lysis with lysozyme and TE buffer, followed by ethanol precipitation. The extracted DNA was verified by running it on an agarose gel, and its quality was further assessed using a spectrophotometer (19). PCR amplification further validated the identity of *Bacillus subtilis* (20). A stock solution of Congo Red was prepared by dissolving 1 g dye in 10 mL acetone and stored in a dark container. A simple nutrient medium was prepared for dye decolorization, containing glucose (1 g/L), yeast extract (0.5 g/L), NaCl (0.5 g/L), KH_2PO_4 (0.5 g/L), and $MgSO_4 \cdot 7H_2O$ (0.2 g/L). The pH was set to 7.0, and the medium was sterilized by autoclaving to ensure it was free from contaminants. Optimization experiments included varying pH (5.0–9.0), dye concentrations (20–100 mg/L), carbon sources (sucrose, starch), and incubation temperatures (25°C, 30°C, 40°C). The samples were kept at 37°C for 24, 48, and 72 hours, and the level of dye decolorization was measured using a spectrophotometer at 520 nm. To test decolorization of natural dyes, coffee extract was added to minimal medium under similar conditions. Absorbance was measured between 420–450 nm, and decolorization percentage was calculated(21,22).

Fig 2.1: The bacterial culture was sub-cultured on TSA media



DNA Run After Isolation DNA Run After PCR DNA with Ladder Post-PCR

Fig 2.2: DNA Band Pattern Analysis Post-Isolation via Agarose Gel

Fig 2.3: Optimization of pH (5,6,7,8,9) at time intervals

Fig 2.4: Optimization of concentration of azo (20%, 40%,60%, 80%, 100%)

Starch (sample 1 & 2) and sucrose(1 & 2) 24 hours

Starch (sample 1 & 2) and sucrose(1 & 2) 48 hours

Fig 2.5: Optimization of different sources like starch and sucrose

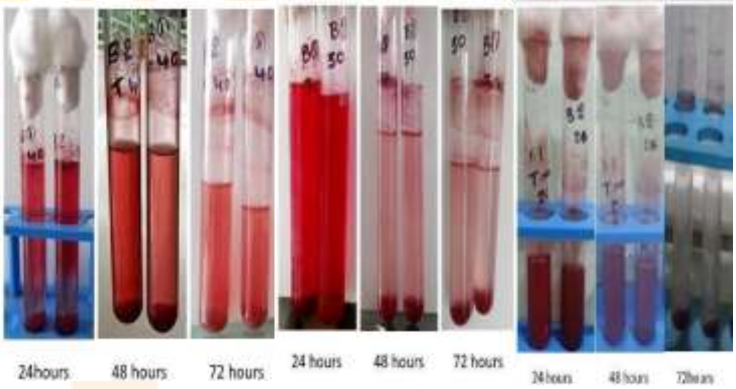


Fig 2.6: Optimization of temperature at 40^0C, 30^0,25^0 C

Fig 2.7: Coffee powder replacing azodye

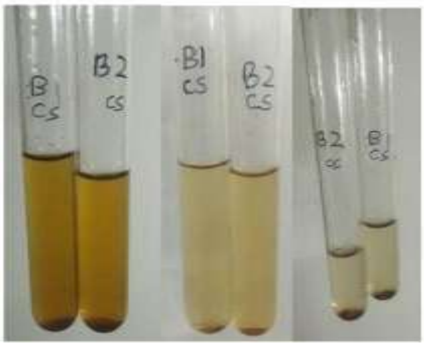
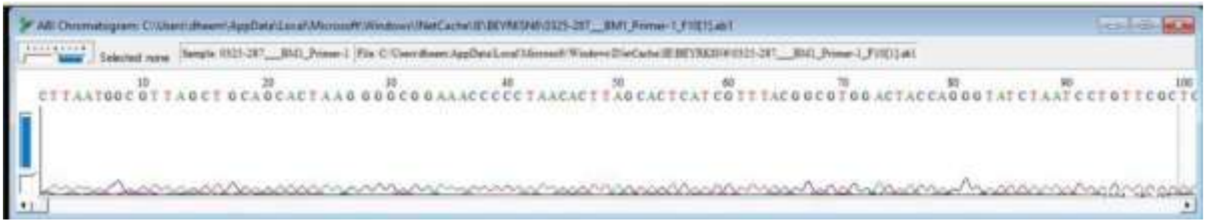


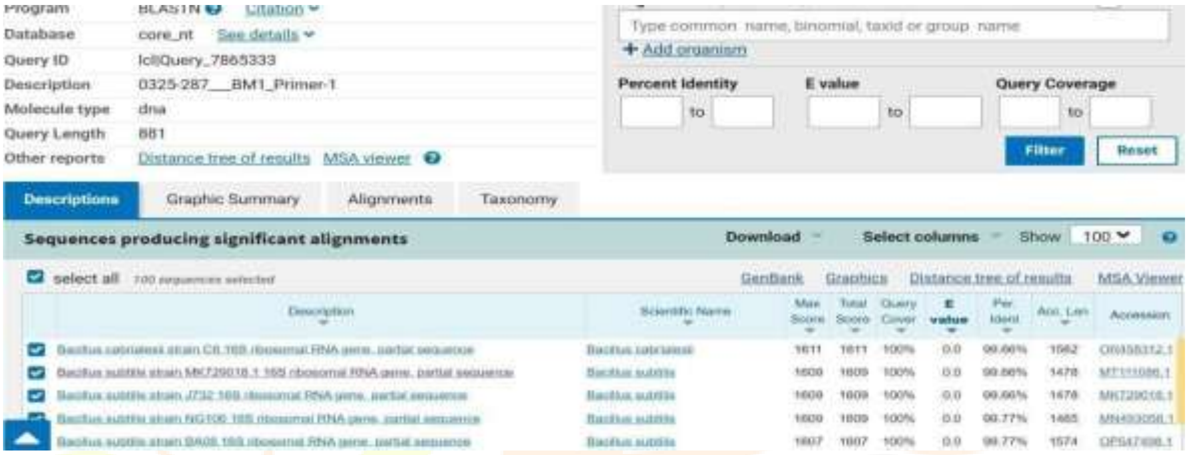
Fig 2.8: Decolorization of Natural Pigments from Coffee Powder replacing azodye

III. RESULT AND DISCUSSION

3.1 DNA sequencing report:

3.1.1 Sample-1





Program	BLASTN Citation
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Query ID	lcl|Query_7865333
Description	0325-287__BM1_Primer-1
Molecule type	dna
Query Length	881
Other reports	Distance tree of results MSA viewer

Sequences producing significant alignments

☑ select all 100 sequences selected

	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☑	Bacillus cabrialesii strain C8 16S ribosomal RNA gene, partial sequence	Bacillus cabrialesii	1611	1611	100%	0.0	99.66%	1562	OR358312.1
☑	Bacillus subtilis strain MK720018.1 16S ribosomal RNA gene, partial sequence	Bacillus subtilis	1609	1609	100%	0.0	99.66%	1478	MT111086.1
☑	Bacillus subtilis strain J732 16S ribosomal RNA gene, partial sequence	Bacillus subtilis	1609	1609	100%	0.0	99.66%	1478	MK720018.1
☑	Bacillus subtilis strain NG106 16S ribosomal RNA gene, partial sequence	Bacillus subtilis	1609	1609	100%	0.0	99.77%	1465	MN433058.1
☑	Bacillus subtilis strain BA08 16S ribosomal RNA gene, partial sequence	Bacillus subtilis	1607	1607	100%	0.0	99.77%	1574	OP547498.1

>0325-287BM1_Primer-1

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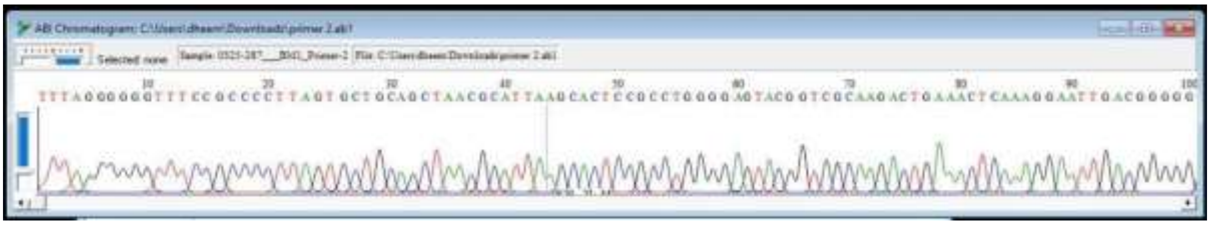
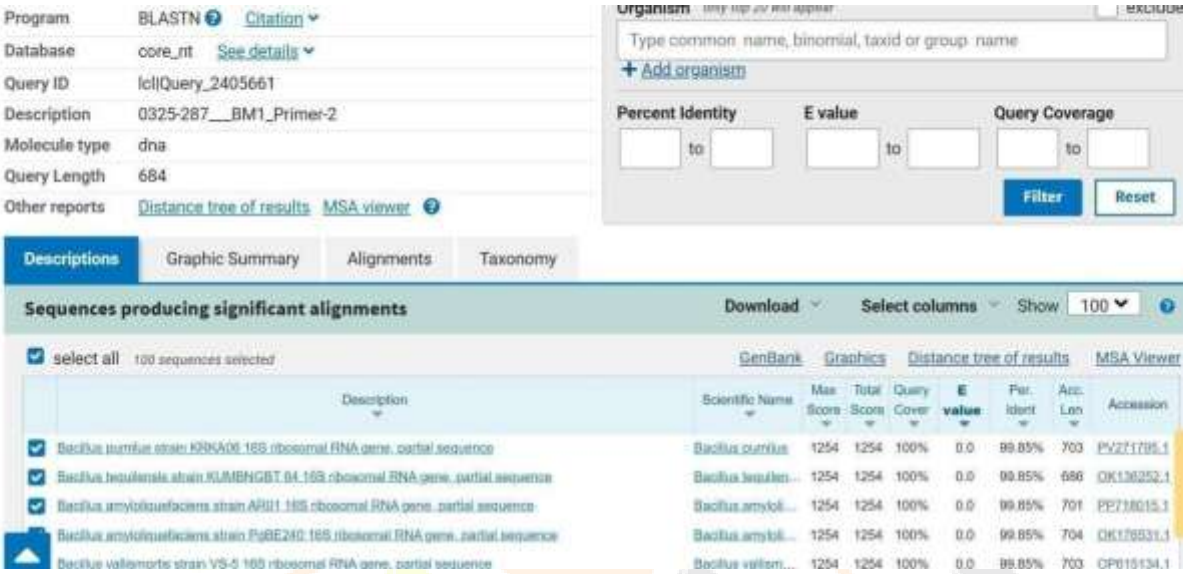
3.1.2 Sample-2





Program	BLASTN Citation
Database	core_nt See details
Query ID	lcl|Query_2405661
Description	0325-287__BM1_Primer-2
Molecule type	dna
Query Length	684
Other reports	Distance tree of results MSA viewer

Sequences producing significant alignments

☑ select all 100 sequences selected

	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☑	Bacillus pumilus strain KRKA06 16S ribosomal RNA gene, partial sequence	Bacillus pumilus	1254	1254	100%	0.0	99.85%	703	PV271795.1
☑	Bacillus tequilensis strain KUMBNGBT 84 16S ribosomal RNA gene, partial sequence	Bacillus tequilen...	1254	1254	100%	0.0	99.85%	686	OK136252.1
☑	Bacillus amyloliquefaciens strain AR01 16S ribosomal RNA gene, partial sequence	Bacillus amyloli...	1254	1254	100%	0.0	99.85%	701	PP718015.1
☑	Bacillus amyloliquefaciens strain PgBE240 16S ribosomal RNA gene, partial sequence	Bacillus amyloli...	1254	1254	100%	0.0	99.85%	704	OK176531.1
	Bacillus vallismortis strain VS-5 16S ribosomal RNA gene, partial sequence	Bacillus vallism...	1254	1254	100%	0.0	99.85%	703	CP015134.1

>0325-287BM1_Primer-2

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CGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTT
TTAGGAGCCAGCCGCCGAAGGTGGGACAGATGATTGGGGTGAAGTCGTACA
AGGGTAACCGAA**

3.1.3 Report: on gene sequencing of sample 1 and 2 :

Parameter	**Primer 1**	**Primer 2**
Strain name ID	**0325-287 _ BM1_Primer-1**	**0325-287 _ BM1_Primer-2**
Accession number	**PV875830**	**PV875834**
Primer Used	**BM1_Primer-1 (Partial 16S rRNA)**	**BM1_Primer-2 (Partial 16S rRNA)**
Length of Sequence	**881 bp**	**866 bp**
Sequence ID (Query)	**lcl|Query_0325-287_ BM1_Primer-1**	**lcl|Query_0325-287 _ BM1_Primer-2**
Best Match Organism	***Bacillus subtilis***	***Bacillus subtilis***
Reference Sequence ID	**NR_112116.1**	**NR_112116.1**
Taxon ID	**1423**	**1423**
Sequence Identity (%)	**98% (865/881 bp)**	**98% (851/866 bp)**
Remarks	**The isolate matched closely with *Bacillus subtilis*, confirming species-level identity.**	**The high sequence similarity confirms the identification and supports the reliability of the molecular data.**

Molecular Identification of the Isolated Strain via 16S rRNA Gene Sequencing Two partial 16S rRNA gene sequences of the isolated strain were amplified using two different primers (BM1_Primer-1 and BM1_Primer-2) and subjected to NCBI BLAST analysis to identify the organism at the molecular level. The sequencing results were aligned with reference sequences from the NCBI database, as shown in the (fig-18 & 19).

Both primers targeting different regions of the 16S rRNA gene independently confirmed that the isolated bacterium belonged to the species *Bacillus subtilis*. The consistent 98% sequence identity with the reference strain (NR_112116.1) from NCBI, and taxon ID 1423, validates the molecular identification. These results ensure the credibility of using this strain in further studies related to azo dye decolorization.

3.2 Optimization studies:

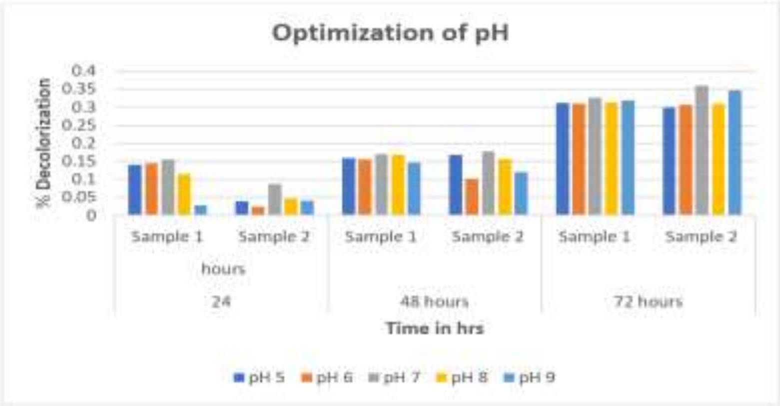
3.2.1 The Optimization of pH:

The effect of pH on the decolorization of Congo Red by *Bacillus subtilis* was shown in the (Fig-2.3) studied across a pH range of 5.0 to 9.0. The results show a clear variation in dye degradation efficiency based on pH and incubation time. At 24 hours, the highest decolorization was observed at pH 7.0, with absorbance values of 0.156 (sample 1) and 0.087 (sample 2), indicating early dye breakdown. At 48 hours, pH 7 and pH 8 showed relatively higher decolorization compared to acidic or alkaline conditions. The trend continued at 72 hours, where pH 7 (sample 1: 0.327; sample 2: 0.359) and pH 8 (sample 1: 0.313; sample 2: 0.31) maintained high degradation levels. In contrast, pH 5 and pH 9 showed significantly lower activity, suggesting that extreme acidic or alkaline environments reduce bacterial efficiency, possibly due to enzyme instability or reduced growth.

The bar graph supports this trend, showing peak activity around neutral pH, with gradual decreases at both extremes. These findings confirm that *Bacillus subtilis* performs best under near-neutral conditions for azo dye degradation.

condition	24 hours		48 hours		72 hours	
pH	Sample 1	Sample 2	Sample 1	Sample 2	Sample 1	Sample 2
pH 5	0.140	0.040	0.159	0.169	0.311	0.299
pH 6	0.144	0.025	0.156	0.102	0.309	0.306
pH 7	0.156	0.087	0.170	0.178	0.327	0.359
pH 8	0.115	0.047	0.168	0.158	0.313	0.310
pH 9	0.028	0.042	0.146	0.120	0.320	0.347

Table-3.1



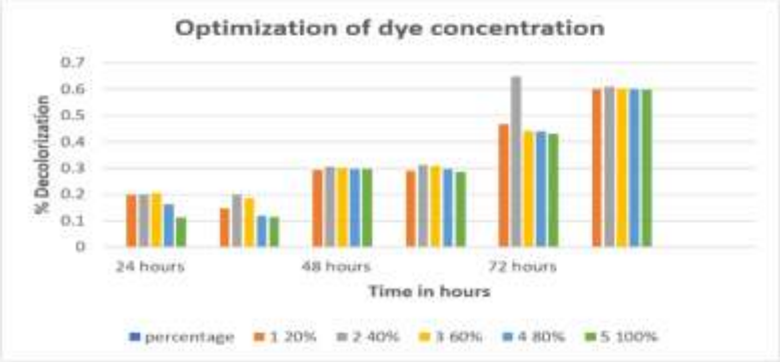
Graph-1

3.2.2 Optimization of dye Concentration

The data shown in the table and graph (Fig-2.4) highlights how Bacillus subtilis responds to different Congo Red concentrations (20%, 40%, 60%, 80%, and 100%) over 24, 48, and 72 hours of incubation, helping to determine the most effective conditions for dye decolorization. At 24 hours, decolorization efficiency was relatively low across all concentrations, with sample 1 ranging from 0.112 to 0.206 and sample 2 from 0.115 to 0.198, suggesting limited bacterial activity at early incubation. Upon extending the incubation to 48 hours, a marked increase in decolorization was observed, particularly at 40% and 60% concentrations, where values approached 0.313. The most significant results were recorded at 72 hours, where sample 1 and sample 2 reached maximum decolorization values of 0.649 and 0.611 at 40%, and 0.442 and 0.604 at 60%, respectively. These findings demonstrate that *Bacillus subtilis* exhibited the highest efficiency in the mid-range concentrations. Conversely, at the highest concentration (100%), a slight decline in decolorization was noted, likely due to dye toxicity or saturation, which can inhibit enzymatic activity or microbial growth. Thus, 40–60% dye concentration was identified as the optimal range for maximum biodegradation.

condition	24 hours		48 hours		72 hours	
Dye concentration	Sample 1	Sample 2	Sample 1	Sample 2	Sample 1	Sample 2
20%	0.197	0.147	0.293	0.290	0.467	0.601
40%	0.201	0.198	0.307	0.313	0.649	0.611
60%	0.206	0.187	0.301	0.309	0.442	0.604
80%	0.164	0.119	0.297	0.298	0.441	0.600
100%	0.112	0.115	0.298	0.285	0.430	0.598

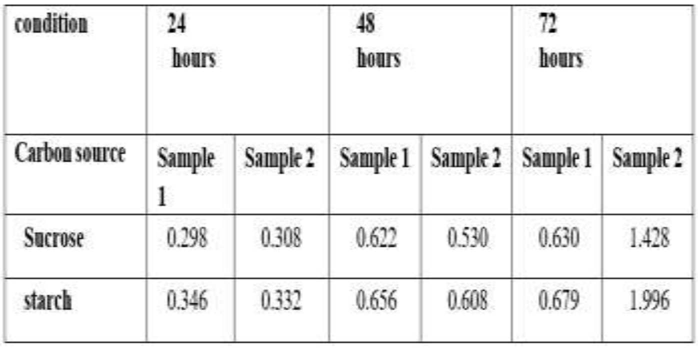
Table-3.2



Graph-2

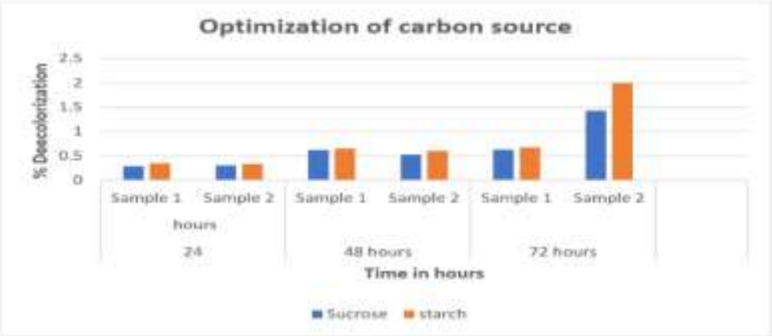
3.2.3 Optimization of Carbon sources

The optimization of carbon sources for dye decolorization by *Bacillus subtilis* was evaluated using two carbon sources: sucrose and starch, across three-time intervals—24, 48, and 72 hours—for two samples (fig-2.5). The results demonstrated a clear preference and higher efficiency with starch over sucrose across all time points. In sample 1, the decolorization efficiency with sucrose increased from 0.298 (24 h) to 0.622 (48 h), reaching 0.630 at 72 hours. In comparison, starch showed a consistently higher activity: 0.346 at 24 hours, 0.656 at 48 hours, and 0.679 at 72 hours. Likewise, in sample 2, sucrose levels gradually increased from 0.308 at 24 hours to 1.428 at 72 hours. However, starch consistently performed better, starting at 0.332 at 24 hours, rising to 0.608 at 48 hours, and reaching the highest value of 1.966 at 72 hours. The bar graph further emphasizes this trend, highlighting that starch consistently promoted higher dye decolorization rates. The results suggest that starch, being a complex polysaccharide, may act as a more effective inducer of microbial enzymatic systems involved in azo dye degradation compared to simple sugars like sucrose.



condition	24 hours		48 hours		72 hours	
Carbon source	Sample 1	Sample 2	Sample 1	Sample 2	Sample 1	Sample 2
Sucrose	0.298	0.308	0.622	0.530	0.630	1.428
starch	0.346	0.332	0.656	0.608	0.679	1.996

Table-3.3

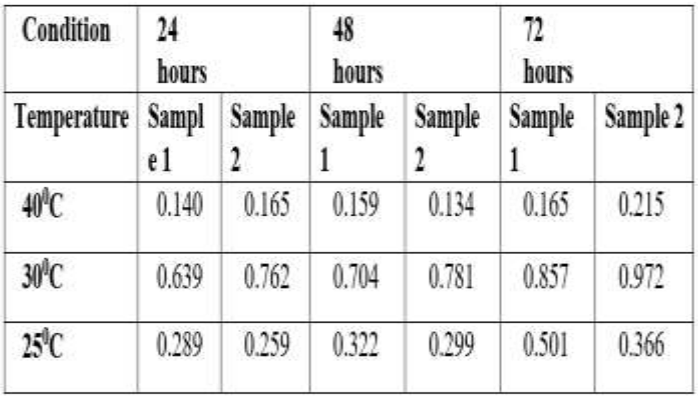


Graph-3

3.2.4 Optimization of Temperature

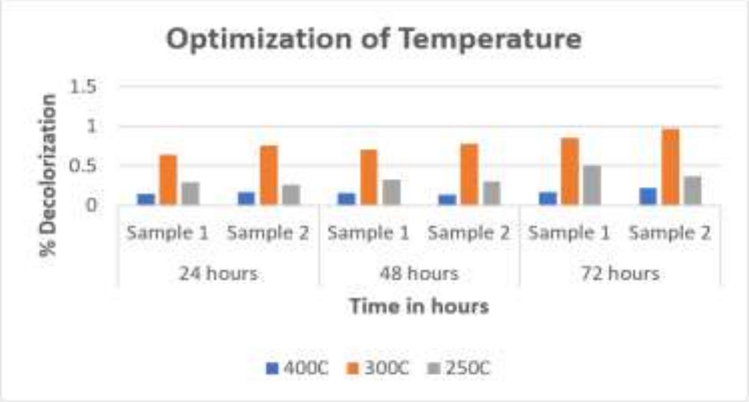
The effect of temperature on dye decolorization by *Bacillus subtilis* was assessed at three different levels—25°C, 30°C, and 40°C—across 24, 48, and 72-hour incubation periods for two samples (fig-2.6). The results indicate that temperature has a significant impact on microbial decolorization efficiency. At 25°C, the decolorization was relatively moderate, with sample 1 increasing from 0.289 (24 h) to 0.501(72 h), and sample 2 from 0.259 to 0.366.

However, at 30°C, the highest decolorization efficiency was recorded: sample 1 rose from 0.639 (24 h) to 0.857,(72 h), and sample 2 from 0.762 to 0.972. In contrast, 40°C showed the least performance, where sample 1 peaked only at 0.140 and sample 2 at 0.165, indicating a decline in bacterial efficiency at higher temperatures. The bar graph clearly supports this trend, showing adistinct peak at 30°C across all time points. This suggests that 30°C offers optimal thermal conditions for enzyme activity and microbial metabolism, while temperatures above or below this range reduce effectiveness, possibly due to reduced enzymatic efficiency at lower temperatures and heat stress at higher temperatures.



Condition	24 hours		48 hours		72 hours	
Temperature	Sample 1	Sample 2	Sample 1	Sample 2	Sample 1	Sample 2
40^{0}C	0.140	0.165	0.159	0.134	0.165	0.215
30^{0}C	0.639	0.762	0.704	0.781	0.857	0.972
25^{0}C	0.289	0.259	0.322	0.299	0.501	0.366

Table-3.4



Graph-4

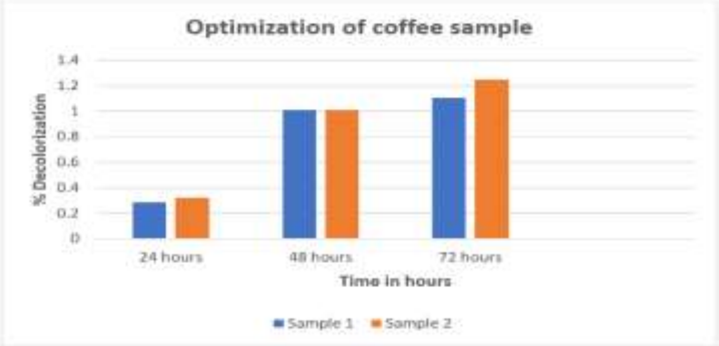
3.2.5 Optimization of Coffee sample

Under optimized conditions (pH 7.0, 40 mg/L dye concentration, and sucrose as carbon source), *Bacillus subtilis* demonstrated effective degradation of coffee powder pigments, which served as a natural dye model (Fig-2.7 & 2.8). As shown in the chart, Sample 1 showed an increase in absorbance from 0.289 to 1.01 at time interval of 24hrs to 48 hours, and 1.103 at 72 hours respectively Sample 2 exhibited a similar trend, with absorbance rising from 0.322 to 1.25 over the same period. This increase reflects the microbial breakdown of coffee pigments and the release of soluble compounds into the medium.These findings are consistent with the degradation pattern observed in Congo Red azo dye decolorization, where *Bacillus subtilis* showed maximum degradation efficiency by 72 hours under the same optimized conditions. The similar kinetic trend suggests that the bacteria possess broad-spectrum dye-degrading enzymes capable of acting on both synthetic azo dyes and complex natural pigment compounds. The slightly higher absorbance in Sample 2 indicates greater pigment solubilization or degradation, highlighting variability in microbial activity across replicates.

The ability of *Bacillus subtilis* to degrade both synthetic (azo dyes) and natural (coffee-based) colorants emphasizes its versatility and potential application in treating a wide range of dye- containing effluents like textile and agro-industrial sources. These research findings reinforce the organism's role in eco-friendly and scalable bioremediation strategies.

Coffee sample	**24 hours**	**48 hours**	**72 hours**
Sample 1	0.289	1.010	1.103
Sample 2	0.322	1.013	1.250

Table-3.5



Graph-5

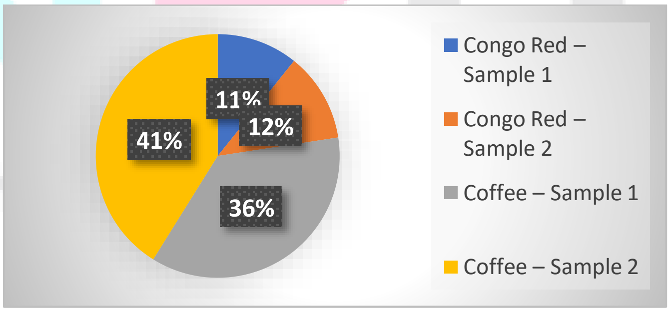
3.2.6 Optimum Conditions for Congo Red Decolorization by *Bacillus subtilis*

- The optimal pH for decolorization was 7.0, which is neutral and ideal for bacterial enzyme activity.
- A 40% concentration of Congo Red showed the highest degradation, indicating that this level strikes a good balance between providing enough dye for the microbes to act on and not overwhelming their ability to tolerate it.
- Starch as a carbon source significantly enhanced decolorization, possibly due to its complex structure supporting prolonged metabolic activity.
- The optimal temperature was 30°C, where enzyme kinetics and bacterial metabolism appeared most efficient.

3.2.7 Comparative Analysis of Congo Red (Azo Dye) and Coffee Pigment Degradation by *B.subtilis* :

Time	**Sample 1**	**Sample 1 (abs)**	**Sample 2 (abs)**
72	Congo red	0.649	0.64
72	Coffee pigment	1.103	1.250

Table-3.6



Graph (pie chart)- 6

Result-

Under optimized conditions, *Bacillus subtilis* demonstrated effective degradation of both Congo Red (synthetic azo dye) and coffee pigment (natural dye). Congo Red showed lower absorbance values, indicating efficient decolorization, while coffee pigment exhibited higher absorbance, suggesting solubilization of pigment compounds. This contrast highlights *B. subtilis* as a versatile microbe capable of degrading both synthetic and natural dyes through distinct mechanisms, making it suitable for various dye-contaminated wastewater treatments.

IV. DISCUSSION AND CONCLUSION

The findings of this study confirm the bioremediation potential of *Bacillus subtilis* in the effective decolorization of synthetic azo dyes, particularly Congo Red, under submerged fermentation conditions. The strain was isolated from a textile waste-contaminated environment, suggesting its natural adaptation to dye-laden conditions. This aligns with previous studies by (5,23), who demonstrated that *Bacillus subtilis* isolated from polluted soils exhibits enhanced enzymatic activity against textile dyes. The morphological and molecular confirmation of the isolate adds reliability to the study, with PCR results indicating the presence of genes possibly encoding azoreductases, as supported by (8). The optimization of fermentation conditions was critical in enhancing dye removal efficiency. Among the parameters tested, pH 7.0 emerged as optimal, consistent with earlier findings by (11), which emphasized the stability of azoreductases under neutral conditions. Lower and higher pH levels likely induced enzyme inhibition or stress-related metabolic suppression. Similarly, a Congo Red concentration of 40 mg/L provided the best decolorization results, avoiding the substrate inhibition observed at higher concentrations, a phenomenon noted by (12,24). The impact of carbon sources was also notable, with sucrose supporting the highest bacterial activity and decolorization efficiency. These results mirror those of (25), who reported enhanced microbial performance with easily metabolizable carbon sources. An innovative aspect of this study was the use of coffee powder as a natural dye source. Though not serving as a carbon supplement, it functioned as a complex pigment model, demonstrating 65–75% decolorization under optimized conditions. This suggests *Bacillus subtilis*'s potential applicability beyond synthetic dyes, extending to natural waste-based pigments, as also suggested by (13,26). These insights are valuable for developing low-cost, environmentally sustainable dye remediation systems.

Compared to above research findings our results establish *Bacillus subtilis* as a robust and efficient biological agent for the decolorization of azo dyes, particularly Congo Red. The bacterium's adaptability to polluted environments and its enzymatic capacity for dye degradation make it a strong candidate for sustainable wastewater treatment. Through the optimization of fermentation parameters—such as pH, dye concentration, temperature and carbon source—high levels of decolorization were achieved, with starch and neutral pH, temoerature at 30^0C and 37^0C offering the best results. The exploration of coffee powder as a natural dye model further demonstrated the organism's ability to act on complex pigment compounds, highlighting its broader applicability. The study contributes to the growing body of research advocating microbial bioremediation as a practical alternative to conventional treatment methods. Overall, this work lays a strong foundation for future industrial-scale applications, offering a cost-effective and eco-friendly approach to mitigate the environmental impact of textile dye effluents.

V. References

1. Selvaraj, V., & Swarna Karthika, T. Microbial remediation and microbial communities for azo dye degradation, *Journal of Environmental Management*, 2020, *272*, 111086.
2. Al-Tohamy, R., Sun, J., Fareed, M., Kenawy, E., & Ali, S. Ecofriendly biodegradation of Reactive Black 5 by newly isolated *Sterigmatomyces halophilus* SSA1575, valued for textile azo dye wastewater processing and detoxification, *Scientific Reports*, 2020, *10*, 12370.
3. Wadhawan, G., Kalra, A., & Gupta, A. Potential of halophiles and alkaliphiles in bioremediation of azo dyes-laden textile wastewater: A review, *3 Biotech*, 2024, *14*(9), 194.
4. Khan, R., Bhawana, P., & Fulekar, M. Microbial decolorization and degradation of azo dyes: A review, *International Journal of Environmental Science and Technology*, 2020, *17*, 345–356.
5. Gunti, H., Maruthiramaih, V., & Shivanandappa, T. Bio-degradation of azo dye Acid Orange-10 by a new isolate of *Bacillus subtilis* isolated from soil sample around textile industry in South Karnataka, *Biosciences Biotechnology Research Asia*, 2020, *17*(4).
6. Zhao, X., Liu, Z., & Zhang, Y. Azoreductase and laccase activity in *Bacillus* sp. for methyl red degradation, *Biotechnology Journal*, 2020, *15*, 1900345.
7. El-Bendary, M., Ezzat, S., Ewais, E., & Al-Zalama, M. Optimization of spore laccase production by *Bacillus amyloliquefaciens* isolated from wastewater and its potential in green biodecolorization of synthetic textile dyes, *Preparative Biochemistry & Biotechnology*, 2021, *51*, 16–27.
8. Abbas, A., Mushtaq, A., & Cheema, A. Heterologous expression of azoreductase-encoding gene *azrS* of *Bacillus* sp. MR-1/2 for enhanced azo dye decolorization and wastewater treatment, *Archives of Microbiology*, 2020, *202*(8), 2135–2145.
9. Carolin, C., Kumar, P., & Joshiba, G. Sustainable approach to decolourize methyl orange dye from aqueous solution using novel bacterial strain and its metabolites characterization, *Clean Technologies and Environmental Policy*, 2021, *23*, 173–181.
10. Rathod, J., Patel, K., & Dhameliya, H. Overexpression of azoreductase and formate dehydrogenase genes for enhanced azo dye degradation, *Biotechnology Letters*, 2021, *43*, 567–576.
11. Goud, B., Cha, H., Koyyada, G., & Kim, J. Augmented biodegradation of textile azo dye effluents by plant endophytes: A sustainable, eco-friendly alternative, *Current Microbiology*, 2020, *77*(11), 3240–3255.
12. Qiu, H., Shen, F., & Yin, A. Biodegradation and detoxification of azo dyes by halophilic/halotolerant microflora isolated from the salt fields of Tibet Autonomous Region, China, *Frontiers in Microbiology*, 2022, *13*, 877151.
13. Khaled, M., Shete, S., & Kumar, A. Decolorization of textile azo dyes T-blue, Yellow GR, and Orange 3R by *Bacillus cereus* and *Pseudomonas parafulva*, *Journal of Environmental Sciences*, 2022, *115*, 91–102.
14. Sun, Y., Shi, X., & Xing, Y. Co-culture of *Aspergillus sydowii* and *Bacillus subtilis* induces the production of antibacterial metabolites, *Fungal Biology*, 2022, *126*(4), 320–332.
15. Ikram, M., Naeem, M., & Zahoor, M. *Bacillus subtilis* as an efficient bacterial strain for the reclamation of water loaded with textile azo dye, Orange II, *International Journal of Molecular Sciences*, 2022, *23*(18), 10637.
16. Vilchis-Carmona, J., & Sánchez-Martínez, A. Decolorization of textile dyes by *Stenotrophomonas* strains isolated from textile effluent, *Journal of Chemical Technology and Biotechnology*, 2021, *96*, 1234–1243.
17. Liu, J., Guo, D., Zhou, Y., Wu, Z., Li, W., Zhao, F., & Zheng, X. (2011). Identification of ancient textiles from Yingpan, Xinjiang, by multiple analytical techniques. *Journal of Archaeological Science*, *38*, 1763–1770
18. Smith, A. C., & Hussey, M. A. Gram stain protocols, *MicrobeLibrary*, 2005, September 30. Retrieved from

19. Nishiguchi, M. K., Doukakis, P., Egan, M., Kizirian, D., Phillips, A., Prendini, L., Rosenbaum, H. C., Torres, E., Wyner, Y., DeSalle, R., & Giribet, G. DNA isolation procedures, In *Techniques in molecular systematics and evolution*, 2002, Birkhäuser Verlag, pp. 249–287.
20. Lorenz, T. C. Polymerase chain reaction: Basic protocol plus troubleshooting and optimization strategies, *Journal of Visualized Experiments*, 2012, *(63)*, e3998.
21. Saharan, B. S., & Ranga, P. Enhanced decolourization of Congo Red dye under submerged fermentation (SMF) process by newly isolated *Bacillus subtilis* SPR42, *Journal of Applied and Natural Science*, 2011, *3*(1), 51–53.
22. Moshafi, M. H., Forootanfar, H., Ameri, A., Shakibaie, M., Dehghan Noudeh, G. R., & Razavi, M. Antimicrobial activity of *Bacillus* sp. strain FAS1 isolated from soil, *Pakistan Journal of Pharmaceutical Sciences*, 2011, *24*(3), 269–275.
23. Sarim, K., Kukreja, K., Shah, I., & Choudhary, C. Biosorption of direct textile dye Congo Red by *Bacillus subtilis* HAU-KK01, *Bioremediation Journal*, 2020, *23*, 185–195.
24. Srinivasan, S., & Sadasivam, S. Biodecolorization and degradation of textile azo dyes using *Lysinibacillus sphaericus* MTCC 9523, *Frontiers in Microbiology*, 2022, *13*, 933939.
25. Soni, B., Kumar, S., & Sharma, A. Optimization of soil conditions for enhanced azo dye degradation by *Bacillus* sp., *Journal of Soil Science and Plant Nutrition*, 2020, *20*, 1234–1243.
26. Purnomo, A., Hairunnisa, F., & Misdar, M. Anionic dye removal by immobilized bacteria into alginate-polyvinyl alcohol-bentonite matrix, *Journal of Environmental Management*, 2020, *268*, 110670..