Identification and characterization of azo dyes decolorizing and degrading native bacterial strains from industrial effluents

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1. A B S T R A C T

As evident from the scientific investigations, the textile industries are major enforcer for one of the biggest environmental pollution issues round the globe. The aquatic and terrestrial biota suffers from the negative effect of the discharge of untreated effluents. Thus, the environmental legislation frequently requires textile mills to cleanse these effluents before discharging them into sources of water bodies. There are prominent methods to treatment of textile effluents including bioremediation, catalytic oxidation, filtration, and sorption procedures. The most efficient and environmentally benign method of removing azo dyes from wastewater is biological treatment. The azo dyes decolorization effectiveness of acclimatedstrainfrom the textile effluent using the serial dilution pour plate method has been assessed. The six strains were isolated from the textile wastewater. Out of these six strains investigated, one showed the highest decolorization performance, hence, it was chosen for

further optimization and identification. *Exiguobacterium sp. BAB 5584* was identified and characterized using 16S rRNA sequencing and further used single strain for decolorizing Methyl Orange and Malachite Green dyes under optimized conditions of pH, temperature, and dyes concentrations. The native isolated bacterial strain showed decolorization of Methyl orange up to 96% at 100 mg/l concentration and 76% of malachite green for 500 mg/l concentration at 37^{o}C for 24 hrs. and 72 hrs. of incubation respectively with pH 7.5. The study explains the promising potentials of adapted *Exiguobacterium sp. BAB 5584* strain for eco-friendly removal of textile azo dyes.

Keywords: Textile wastewater, Isolation,Biochemical analysis, Molecular Identification, *Exiguobacterium sp*. BAB 5584.

2. INTRODUCTION

Azo dyes are frequently used, making up 60–70% of all known produced dye structures (Fu and Viraraghavan 2001; Bafana *et al.,* 2011; Hassaan and El Nemr 2017, Mostafa M. El-Sheekha *et al*., 2022). Approximately 700,000 tons of synthetic dyes are manufactured oversees each year (Anum Fareed *et al*., 2022). Textile dyeing is most water-intensive business, releasing persistent organic compounds, color difficulties, and high concentrations of BOD, COD, fibers, surfactants, detergents, and solvents, among other things (Ashwani Awasthi *et al*., 2018,Markandeya *et al*., 2022). A fraction of unfixed dyes is constantly discharged into the wastewater, and this pollutant is the most significant contaminant in the effluent (Chiong *et al*., 2016, Suman Shashank, Kumar Prashant, 2022), Utilization of azo dyes is prevalent leather tanning, paper and pulp, pharmaceutical, food, paint, plastics, cosmetics, and many more sectors in addition to the textile and carpet industries (S. Dhakshanamoorthy*et al*., C. Femina Carolin *et al.,* 2020). The unacceptable let out of dye effluents into the aquatic reservoirs has a negative impact on all living things and produces aesthetic annoyance, posing a serious concern for humans (S. Sharma*et al*., 2014, Pankaj Chowdhary*et al.,* 2019). The removal of azo dyes from textile wastewater has been investigated a lot. Several physicochemical approaches have been used; however, they have run into issues like as harmful by-product formation and economic infeasibility. Microbial degradation is cost-effective, environmentally benign, and do not carry out a big amount of

sludge. The use of pure culture to degradation of azo dyes was subject of some investigations (Kour *et al*., 2021). Because of the chemical complexity of these dyes, more efficient microbial decolorization techniques are required (Carolin *et al*., 2021). The objective of this work is highlighting the microbial decolorization of textile wastewater employing co-cultivated microorganisms. For many applications, biological treatment offers possible established technologies. Fungi, bacteria, algae, yeast and actinomycetes have been identified as microbiological sources based on microbial enzymes or entire cells and biomass (Indrani Jadhav *et al*., 2016,Erika J. Espinosa-Ortiz*et al*., 2021). The resilience and profligacy of selected microorganisms are boosted by their supplication under model conditions, therefore microbial decolorization is efficient. There be more large-scale manuscript narrating the degradation of azo dyes moderate by *Pseudomonasis sp*.was widely used to decolorization of azo dyes. Remazol Orange 3R by *Paeruginosa* strain BCH, which removed 98 % of the dye in 15 minutes, or Black B by *P. aeruginosa* strain BCH (Amarja H. Bhosale and Rahul M. Khobragade 2019, Mehvish Ajaz *et al*., 2019). The microbial integration is primarily used entirely as they can jointly carry out to no toxicity. By adapting to toxic wastes, microbes create new, resistant strains that subsequently change diverse harmful substances into less harmful ones (Adebayo, F.O., Obiekezie, S.O., 2018). Due to its greater versatility and toxicity tolerance, the native microbial consortia are possibly a better option for the breakdown of a wide range of industrial grade dyes, making it the greatest contender for the decolorization of textile effluent (Samuchiwal *et al*., 2021, Abiri *et al*., 2017; Popli and Patel, 2015). When such abiotic parameters are optimized, the microbial system becomes more effective and practical (Lal Babu Prasad Yadav, Ajay Singh, 2017). To improve the impact of environmentally friendly colour treatments on the landscape, innovative and long-lasting environmentally friendly colors treatment procedures are desired (Thangaraj *et al*., 2021).

3. Materials and Methods

3.1 Area of Studyand sample collection

The samples (untreated textile effluent) were collected (Left bank right bank and mid-stream) from the open drain from the drainage at Bhadohi District (UP), India, (25.3264^0N,82.4319^0N). Physicochemical parameters of the sample, including color,

temperature, pH, Ec, turbidity, Do, BOD, and COD were taken down, and sample was brought to the laboratory within 12 hours of collection. All samples were stored under prescribed condition (at 4^0c). The samples were screening for dyes degrading native bacterial strains.

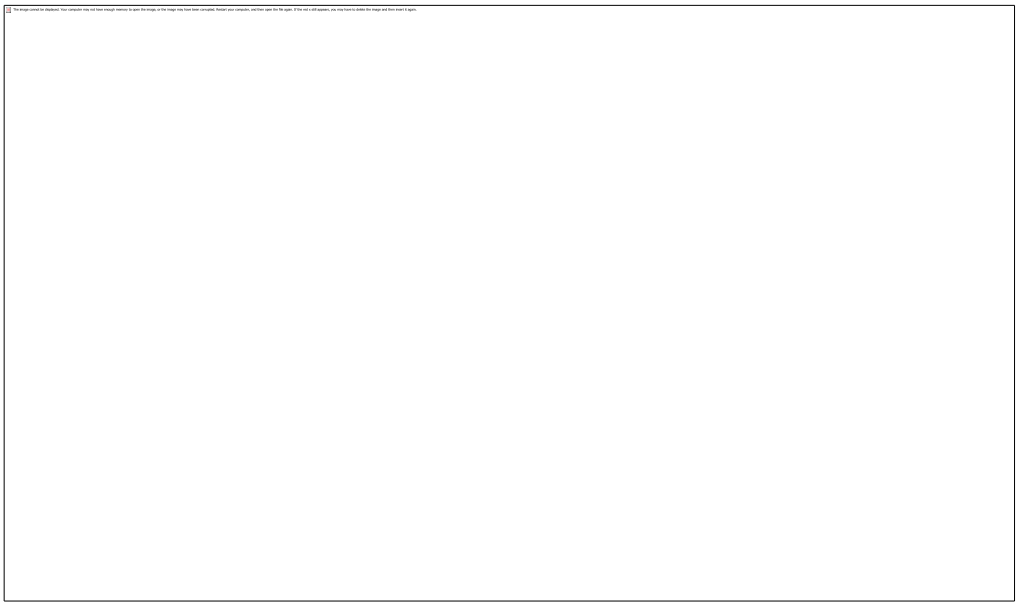

Fig 1: Sampling location

3.2 Azo dyes, Chemicals and Microbiological media

Azo dyes methyl orange (MO) and malachite green (MG) are procured from Sigma. Dyes were dissolved in distilled water and the stock solution was prepared (1000 mg/l) followed by filtration and sterilization and kept at 4°C for further use. At different concentrations (100 mg/l, 125 mg/l, and 500 mg/l) of selected Azo dyes, decolorization study was conducted using native isolated bacterial species.Moreover, nutrient medium (agar and broth) (Himedia) was used for maintenance of isolated bacterial strain.

3.3 Isolation and screening of azo dyes decolorizing bacterial strain

The strains were isolated from untreated textile effluents. Different bacterial strains were screened and characterized for their capacity to decolorize the dyes using morphological, physiological, and biochemical analysis (G. Vaijayanthi, *et al*., 2012,Kasana & Pandey 2018).In brief, the isolates that showed decolorization effectiveness were streaked on

enrichment agar medium containing dyes (2%). Bacterial isolate colonies with a clear achromatic zone around them were taken and stored for time a head uses.

3.4 Colony characteristics, morphological and biochemical analysis

The colony features and morphology of dye decolorizing bacterial strain were investigated. Colony parameters, gram response, and cell shape were studied using isolated cultures. The colony characteristics and morphological studies under the microscope included prepared smear and Gram's staining as express in (Manual of Basic, 2003, Engelirk and Duben Engelkirk, 2008).

3.5 Molecular identification of potential strain

The extra-pure Microbial DNA Isolation Kit (from Bogar Bio Bee Stores Pvt Ltd) was used for DNA isolation. Following that, Qubit 3.0 was used to quantify DNA concentrations. For later usage, isolated DNA was kept at 20°C. Using 1.5 L of Forwarding Primer (27F- 5′ AGA GTT TGATCMTGG CTC AG 3′) and Reverse Primer (1492R- 5′ TAC GGY TAC CTT GTT ACG ACTT 3′), 5 L of deionized water, and 12 L of Taq Master Mix, the isolated microorganism was subjected to the polymerase chain reaction (PCR).We used the Montage PCR Clean-up kit, the unincorporated PCR primers and dNTPs from the PCR products were removed (Millipore). The final PCR product was sequenced using an ABI PRISM ® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq ® DNA polymerase (FS enzyme) (Applied Biosystems). The 16S rRNA sequence was blasted using the NCBI blast similarity hunt tool. With the phylogenetic analysis of the query sequence and the nearly associated sequence of the blast findings, multiple sequence alignment was performed. In this experiment, fold scope was used to analyze to bacteria at a magnification of 140X and a resolution of 2.0 m. (Priyodip and Balaji, 2018).

3.6 Decolorization potential of Isolated native bacterial strain

A loopful of strain was taken from the cultured plate and further inoculated to 100ml media in a flask of 250ml and incubated at 37°C for 48 hrs. Two ml of culture inoculated in the 100 ml of media, supplemented with 10 to 500 mg/l concentration of methyl orange and malachite green dyes. After the incubation of 24hrs, centrifugation of the culture was done at 5000 rpm for 15 minutes. The supernatant was utilized to analyse the decolorization using a UV–Visible

double-beam spectrophotometer at maximum wavelength of 465 and 624 nm respectively with the help of percentage dye decolorization (Zhuang *et al.* 2020). Set of negative control also incorporated (Shah *et al*. 2013a, b).

$$\% \text{ Dye decolorization} = \frac{\text{Initial absorbance} - \text{Final absorbance}}{\text{Initial absorbance}} \times 100$$

4. Results

4.1 Colony characteristics, morphological and biochemical analysis

The appearance of colony morphology, observed among species, and colony traits were used to distinguish the bacteria. The native bacterial strain had colony characteristics similar to E*xiguobacterium sp.* BAB 5584 as evident from the fig. 2.

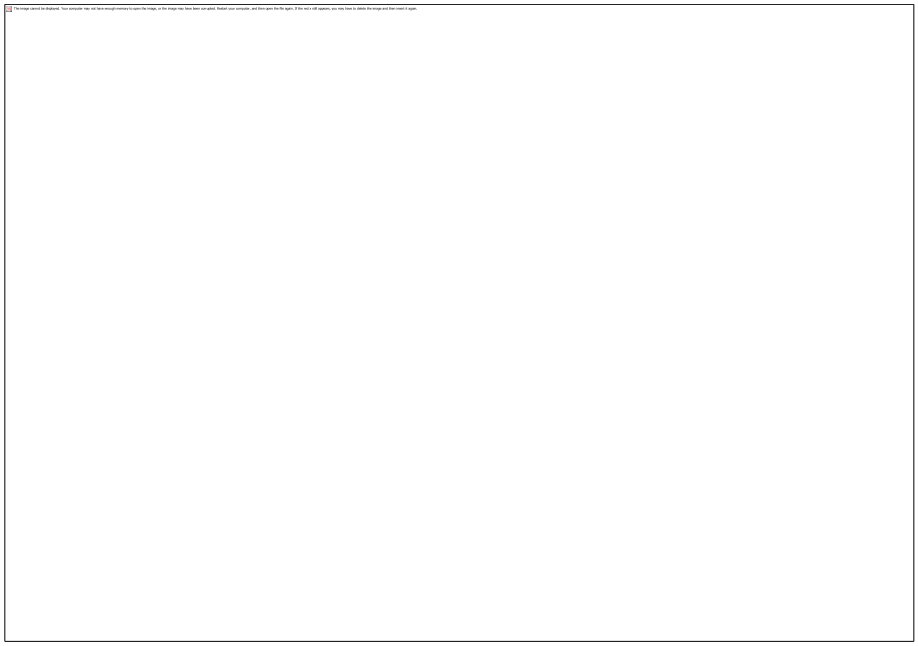

Fig 2: Colony characteristics of bacteria from textile industry wastewater

The colony features and morphology of dye decolorizing bacterial strain were investigated after it was isolated. Colony parameters, gram response, and cell shape were studied using isolate cultures. The colony characteristics were determined and the morphology of cells under the microscope including preparation of smear and Gram's staining which is mentioned in (Engelirk and Duben- Engelkirk, 2008).

Table1: Colony characteristics of the isolates

Parameter	**Results**
Shape	Round
Color	Yellow
Opacity	Opaque
Gram nature	Positive
Catalase	Negative
Temperature (4^0C)	Negative

4.2 Growth of isolated bacteria at different concentrations of MO and MC

Isolates were recovered by spreading the serially diluted samples on nutrient agar plates. The strains identified on the basis of morphology were utilized to remove the color from MO and MC dyes. The isolates from the wastewater were named as S1, S2, S3, S4, S5, and S6. Each strain's decolorization effectiveness was calculated up to96 hours using a UV-Visible spectrophotometer with a double beam (fig 5 and 6).The S3 strain was demonstrated to decolorize methyl orange and malachite green by up to 96 % in just under shaking conditions.Other strains, except for strain S3, were found to be ineffectual in the decolorization procedure.

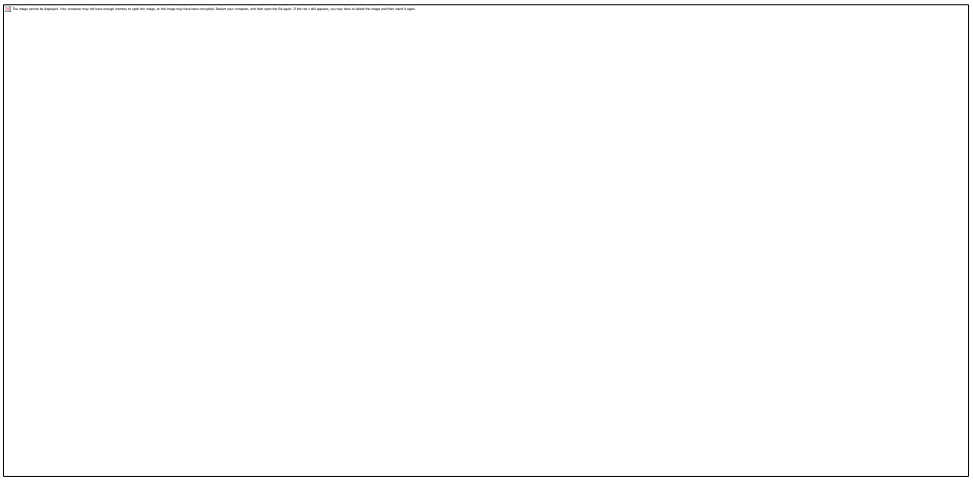

Fig 3: Plates show the growth of bacteria at different concentrations of MO (100mg/l, 125mg/l and500 mg/l) and MC (100mg/l, 125mg/l and500mg/l)

The results of earlier investigations indicate that as growth declines, clearance efficiency likewise falls. Fig 5 and 6 provide an examination of each strain's development at a methyl orange decolorization at 100 mg/l. The reason that of its strong decolorization effectiveness, the bacterium S3 was chosen for sequencing. *Exegobacterium sp.* BAB 5584 was identified by 16S rRNA sequence that was acquired by outcome.After being submitted to the Gene Bank, the isolate's nucleotide sequence was given the accession number KU697642. Figure 4 depicts the phylogenetic tree of S3. *Exegobacterium sp*. BAB 5584 was identified from a fold scope image of a gram-positive, round bacterium.

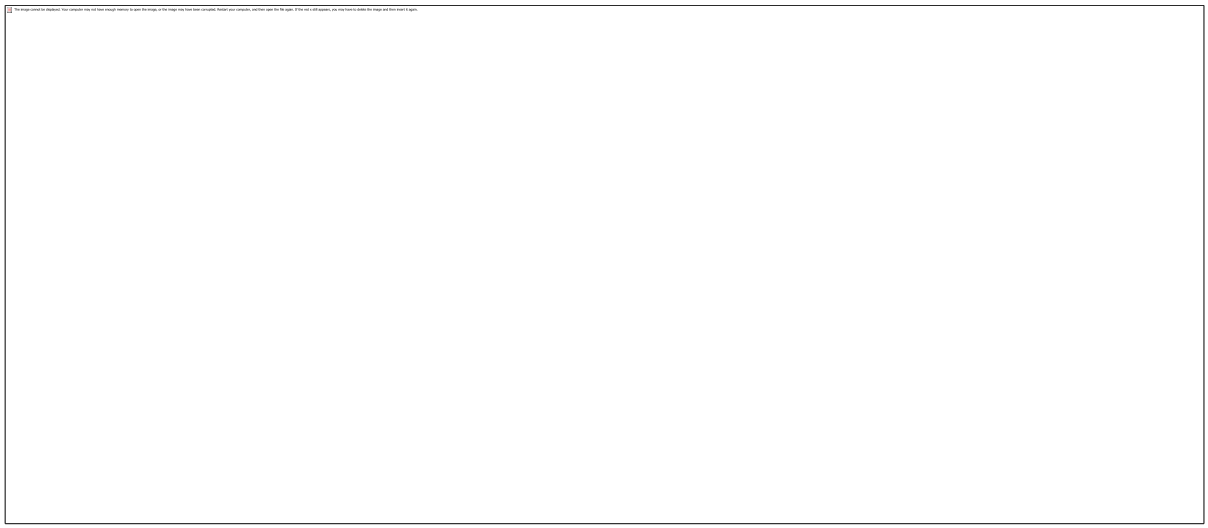

Fig 4: Phylogenetic tree of isolated native *Exiguobacterium sp. BAB 5584* based on 16s Rrna

4.3 Methyl orange dye decolorization

Preliminary result indicates the dye degrading properties of isolated Bacteria. It was observed that with passage of time, growth of bacterial cells increased accompanying the increase in decolorization percentage, but after 72hrs, decolorization percentage decreased when scanned at 465 nm by spectrophotometer based on the previous studies for the biomass production.

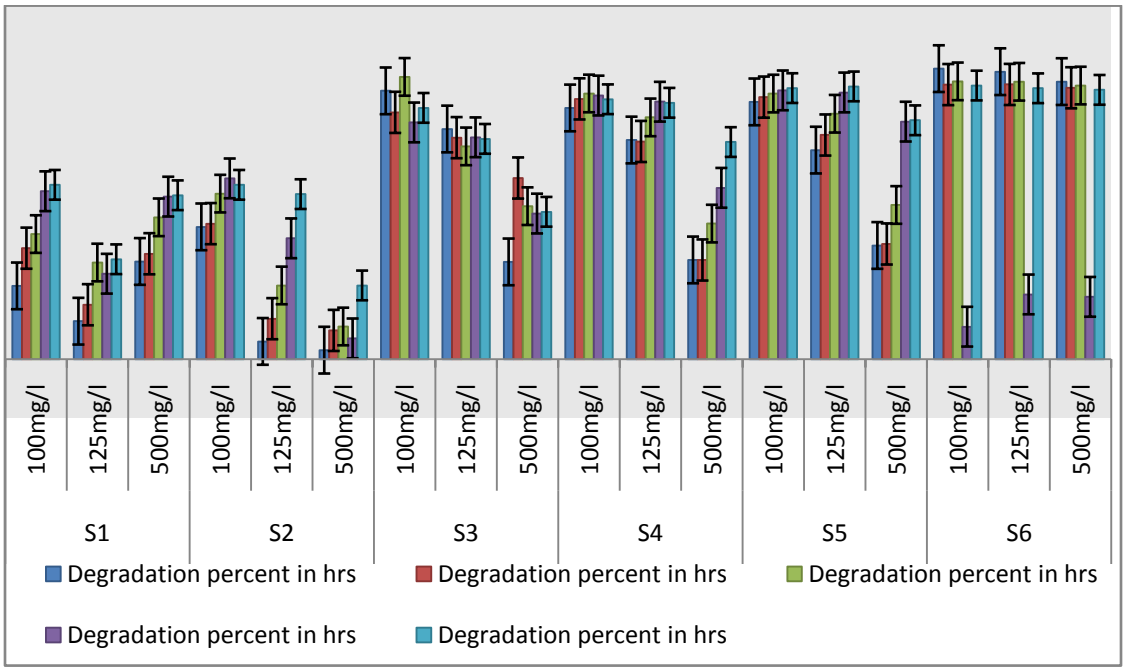

Fig 5: Degradation % age of MO in terms of optical density4.4 Malachite green dye decolorization

Our preliminary result indicates the dye degrading properties of Bacteria. Here is an Effective decolorization on the growth of bacterial isolates, it was determined that with the passage of time growth rate of bacteria increased along with the increase in decolorization percentage but after 48hrs decolorization percentage decreased, when scanned at 624 nm by spectrophotometer**.**

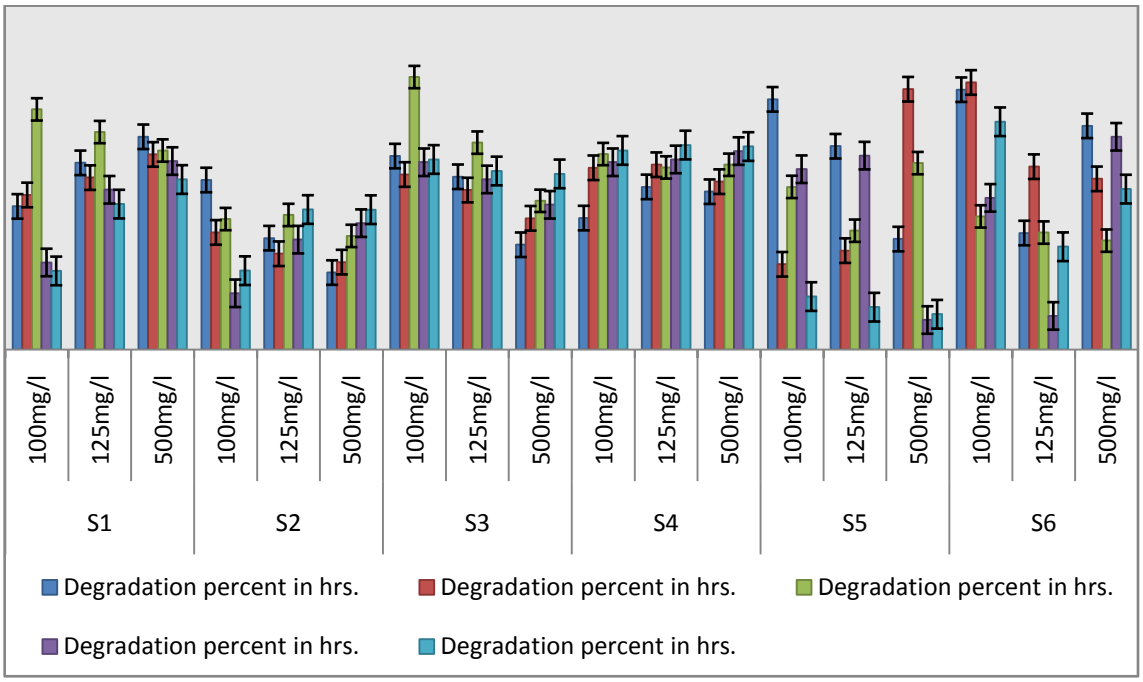

Fig 6: Degradation % age of MG in terms of optical density

4.5 Optimization of Factors

The native bacterial strain showed the highest decolorization efficiency parameters such as pH, temperature, time, Agitation speed and concentration of azo dyes.

4.5.1 Effects of Temperature

The decolorization of azo dyes is a temperature-dependent mechanism. The optimum temperature for maximum activity of *Exegobacterium sp.* BAB 5584 was determined by measuring azo dyes decolorization at different temperatures as shown in (fig7). Increasing trend in activity has been observed till 37^{o}C thereafter decreasing trend was observed due to hindering of bacterial activity at higher temperatures. The optimum temperature thus obtained was 37^{o}C (A. Tripathi *et al*., 2018).

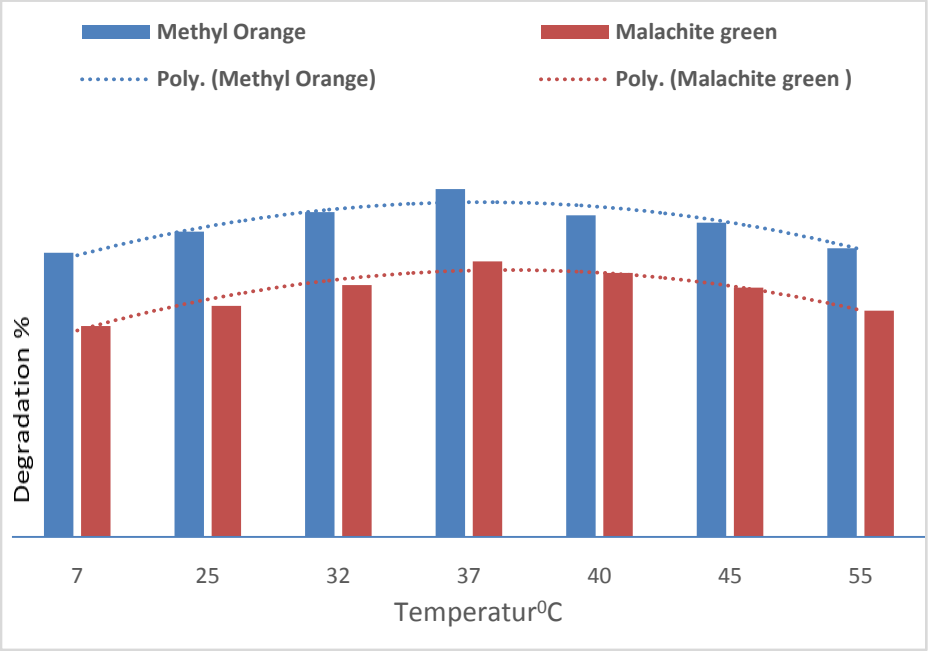

Fig 7: Percentage decolorization with Temperature

4.5.2 Effects of pH

The azo dye decolorization was affected due to pH on efficiency of *Exegobacterium sp.* BAB 5584 as shown in the fig8. These numbers demonstrate that pH 7.5 has produced the maximumdecolorization. The sensitivity ofbacterial strain to pH conditions can be linked to a decrease in azo dyes decolorization as bacterium got denatured above this pH.

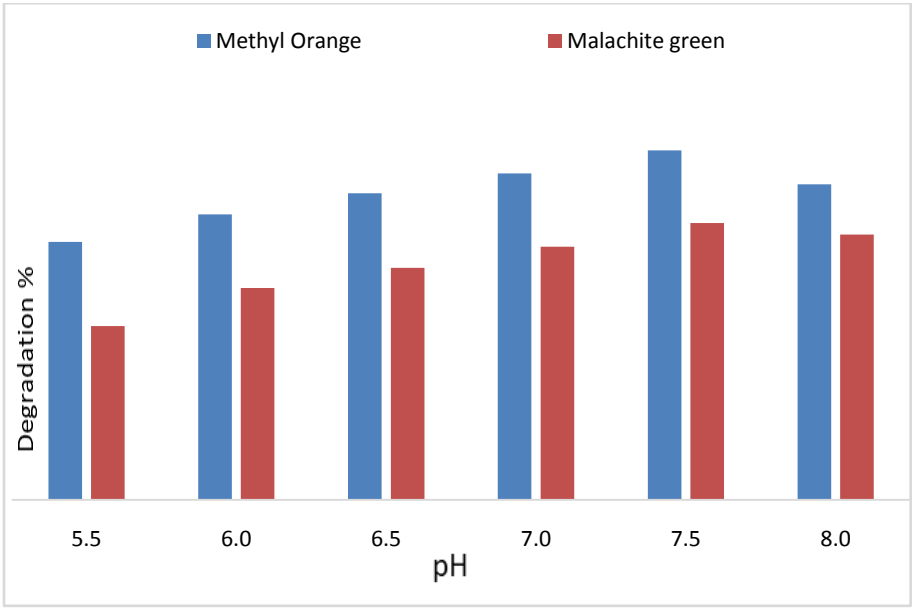

Fig 8: Percentage decolorization with pH

4.5.3 Effects of Agitation

The study showed % decolorization of MO and MG up to 96.01% and 76.01% at 48 hrs and 72hrs respectively, while under shaking conditions (120 rpm),achieved decolorization up to 95.22 % (Fig 9). The better decolorization under shaking conditions may be due to improved oxygen transport and even distribution of nutrients for all the individual colonies. The experiment has been performed under identical environmental conditions for 60hrs.

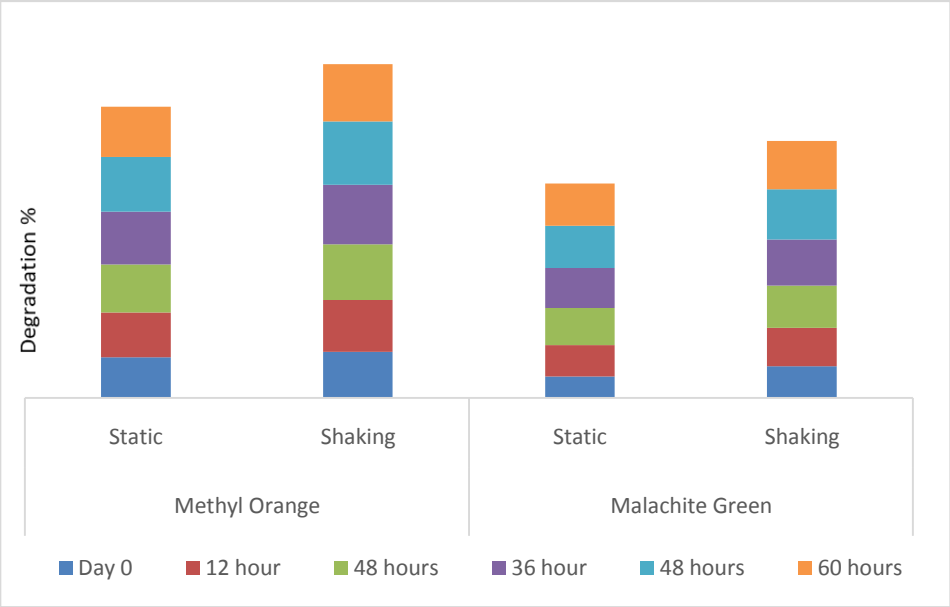

Fig 9: Percentage decolorization with effects of agitation

5. Discussion

The azo dyes are globally cast-off in the textile industries and major source of industrial effluent pollutants; (Asad *et al.*, 2007; Jadhav *et al.*, 2007; Kim *et al.*, 2008; Markandeya *et al.*, 2022), In the present investigation, two azo dyes namely MO and MG were targeted for their degradation potential. After 24 hours of incubation, *Exuobacterium sp.* BAB 5584 strain developed optimum growth which has also been delineated previously (Barragan *et al.*, 2007).Industrial textile dyes could be decolorized due to microorganisms in one of two ways: the dyes adsorb into the cell wall and hence depending on biomass and mass transfer reaction in medium (Hassan T. Abdul sahib*et al*., 2015, Lellis, B *et al.*, 2019) as well as biodegradation of dye occurs via the enzymatic responses of microorganisms which can be demonstrated by the development of yellow-colored colonies on the plates (Lucas *et al.*, 2006). Similarly, in our study yellow color colonies were seen in plates (Fig: 3). Researchers (Chen *et al*., 2003)

and (Yu and Wen, 2005) also reported similar findings. While the qualitative screening of decolorizing strains reveals, this might be used as an energy source for industrial dyes. The decolorization percentage for each strain was measured and assessed in liquid cultures, and the strain with the prominent capacity to remove color was chosen (Barragan *et al.,* 2007). It was discovered that they could thrive in a liquid media with dye serving as the only carbon source. **The isolated strains of native bacteria from textile industry effluents, the experiments with MO and MG, it was observed that with strains S1, S2, S3, S4, S5, and S6.** The decolorization was effective at low concentration (100mg/l) with strain S1, similar results shown by another research (C. Femina Carolin *et al.,* 2020), it was observed significant at the highest exposed concentration (500mg/l) within 48 hrs. Further, it was observed with all the strains that the percenrate of decolorization fluctuated and reached 92% but that was not stable at all the concentrations and with all the strains. All six bacterial strains showed a healthy decolorization within 48hrs range between 46.98 to 95.8 % which shows the native 6 strains can be used for effective decolorization of the dye and it can be used in the textile industry with minor modifications as per Roy, D. C. *et al.,* 2020. As the chemical stimulation is not acceptable, bio-augmentation may be a positive effort to integrate native species that may accelerate the decolorization of MO and MG in a shorter span (48 to 96 hrs), which is evident from the previous research conducted (Zubair *et al.,* 2018). Results of 16S RNA sequencing revealed the isolated culture similar to *Exegobacterium sp.* BAB 5584. Accordingly, (Kim *et al*., 2008), the rate of color removal rises as temperature rises, which makes the application of *Exegobacterium sp*. BAB 5584 for the industrial process of color removal even more attractive. Results have shown that the *Exuobacterium sp*. BAB 5584 *sp.* is a potent decolorizer of MO and MG (Ramaraju Kalpana*et al.,* 2020). At pH 7.5, 94% of MO and MG decolorization were observed in *Exuobacteriumsp*. BAB 5584 but, in acidic conditions, decolorization decreased. Azo dyes typically have one or more than one sulfonic acid groups in the aromatic rings, which prevent the growth of bacteria (Asad *et al.,* 2007, Neha potle, 2012).

6. Conclusion

The biodecolorization of textile industry wastewater is a strenuous process; A few microorganisms were lately been investigated as a possible textile dye and effluent scavengers.

The six native strains were studied for the decolorization of MO and MG dyes with bacterialstrainshow a specific eventuality of about 95% decolorization at the same time with the other strains. Studies have revealed that the *Exuobacterium sp*. BAB 5584is a potent decolorizer of selected dyes. The 96% decolorizationwas observed at optimized condition of pH 7.5, temperature 37^0C, however an increase in concentrations leads to drop in decolorization. our findings suggest that native bacterial strains may be employed in wastewater treatment facilities and presumably a pollution control biological contender for appropriate environmental management in future.

Declaration of Competing Interest

The authors don't claim any conflict of interest.

Author contribution

Kartikeya Shukla (Assistant professor) and Tanu jindal (Professor and Director) contributed to conception. Vartika Singh (PhD Scholar) designs the study, collected the sample, contributed to data analysis and interpretation, Jaswant Singh (Professor and HOI) and Ashutosh Tripathi reviewed the manuscript critically. All authors read and approved the final manuscript for publication.

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