Decolorization of Orange G using Exiguobacterium spp. BAB 5584: A Sustainable Approach for Textile Dye Remediation

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Abstracts: The unregulated discharge of untreated effluents from textile industries poses a significant environmental threat to both aquatic and terrestrial ecosystems. In response, this study investigates the efficacy of a biological remediation approach employing an adapted native bacterial strain exhibiting superior decolorization capabilities compared to other isolated strains. The isolated bacterial strain, demonstrating remarkable decolorization potential, undergoes further optimization and identification processes. The native bacterial strain is identified as Exiguobacterium spp. BAB-5584 through 16S rRNA sequencing. This strain is subsequently employed for the degradation of Orange G azo dye under optimized conditions of pH, temperature, and dye concentration. The adapted bacterial strain showcases its maximum degradation efficiency, achieving a remarkable 98% degradation of Orange G at a concentration of 100mg/l with in a 24-hours incubation period at 370C and pH 7. Characterization of the degradation products is carried out using gas chromatography-mass spectrometry (GC-MS). To assess the ecological impact of the treated wastewater, germination assays are performed using chickpea and mung seeds. Notably, there is no observed inhibition of germination when compared to the control group, affirming the non-toxic nature of the degraded metabolites.

Keywords: Textile Wastewater, Molecular Identification, GC-MS, Exiguobacterium *spp*. BAB 5584.

1. INTRODUCTION

Azo dyes, characterized by azo bonds (-N=N-), constitute the largest group of synthetic dyes, utilized across diverse industrial sectors. These compounds are notorious for their water solubility and resistance to biodegradation, posing significant environmental and health concerns. Consequently, their removal from industrial wastewater is imperative. This study focuses on the biodegradation of Orange G, a representative azo dye, using the isolated bacterial strain Exiguobacterium *spp*. BAB 5584. This gram-positive, rod-shaped bacterium, isolated from textile industrial effluent contaminated with various pollutants, exhibits remarkable resilience in harsh environments. Recent studies have highlighted its prowess in degrading a spectrum of pollutants, including polycyclic aromatic hydrocarbons (PAHs) and heavy metals.

Azo dyes are integral to various industries but have garnered notoriety for their recalcitrance to degradation, attributed to the stubborn azo bonds. However, select bacterial strains have exhibited the capability to cleave azo bonds and degrade azo dyes. Biodegradation of azo dyes typically relies on enzymes like azo reductase, which reduce the azo bond, leading to the formation of aromatic amines that can undergo further enzymatic degradation Exiguobacterium *spp*. BAB 5584 has emerged as a promising candidate for azo dye biodegradation due to its unique enzymatic repertoire. Biodegradation of Orange G by Exiguobacterium *spp*. BAB 5584 has been extensively studied in recent years. Ina study by Puvaneswari *et al*. (2021), degradation reached 85% within 48hours. Similarly, Waseem *et al.,* 2022 reported a 78% degradation of Orange G in 72 hours, primarily attributed to the action of azo reductase enzymes. This degradation pathway involves a complex cascade of enzymatic environment (Dwivedi Naveen *et al*., 2021). Furthermore, Singh *et al*. (2017) reported an impressive 97.5% degradation of Orange G within 24 hours under optimized conditions. This study not only underscored the potential of Exiguobacterium *spp*. BAB 5584 but also elucidated the biodegradation pathway, wherein the azo bond of orange G is cleaved to yield 4-amononaphthalene-1-sulfonic acid, further degrading into simpler, less toxic compounds (Liang Tan *et al*., 2009; R. Palani Velan *et al*., 2019). Factor influencing biodegradation efficiency several factors impact the efficiency of

biodegradation, including pH, temperature, and initial dye concentration. Optimization of theses conditions is crucial form maximizing the efficiency of Orange G biodegradation by Exiguobacterium *spp*. BAB 5584. Future research should aim to comprehensive delineate the mechanisms underlying this degradation process (Singh, P., Kumari, M., Lal, B. *et al*., 2017; Anamika Kumari *et al*., 2021). The biodegradation of Orange G by Exiguobacterium *spp*. BAB 5584 represents a promising approach for treating wastewater contaminated with this recalcitrant azo dye, The versatility of Exiguobacterium *spp.* BAB 5584, capable of degrading various pollutants, including azo dyes, suggests its potential application for treating wastewater containing mixed pollutants. However, further investigations are essential to fine-tune the operational conditions and comprehensively elucidate the intricate mechanisms governing this biodegradation process.

2. MATERIALS AND METHODS

2.1 Area of Study and Sample Collection

The textile industry effluent samples were collected from the Bhadohi District (UP), India. The physical parameters of the samples, including color, temperature, and pH, were analyzed on-sites, and the samples were brought to the laboratory within 12 hours of collection. All the samples were stored at 4^0C until further use.

2.2 Dyes, Chemicals, and Nutrient Media

Azo dye Orange G was procured from Sigma. The dye was assessed for properties such as color, solubility in water, and maximum absorption. The stock solution was prepared (1000 ppm) using distilled water, followed by filtration, sterilization, and stored at 4°C for further use. Dyes at different concentrations (100 ppm, 250ppm, and 500 ppm) were utilized to study their impact on bacterial growth and adsorption when added to the culture media. Nutrient agar medium served for bacterial culture maintenance.

2.3 Isolation and Screening of Dyes Decolorizing Bacteria

A dye-degrading bacterial strain was isolated from this sample. The bacterial strains were screened and assessed for their capacity to decolorize the dye. The isolates that showed decolorization effectiveness were streaked on enrichment agar medium (2%) containing dyes. Bacterial isolate colonies with a clear colorless zone were collected and cultured for future studies. The strain that showed the maximum degradation was identified by 16s RNA sequencing.

2.4. Molecular Identification of Potential Strain

DNA isolation was performed using the extra-pure Microbial DNA Isolation Kit From Bogar Bio Bee Store Pvt. Ltd. (an Iso: 2015 Certified Company). DNA concentration was quantified using the Qubit 3.0 system. Isolated DNA was stored at -200C for subsequent use. Polymerase chain reaction (PCR) amplification was carried using 1.5µL of forward primer (27F-5´ AGA GTT TGATCMTGG AG3´). 5µL of deionized water, and 12µL of Taq Mas Kit (Mix. Unincorporated PCR primers and dNTPs were removed from the PCR products using the Montage PCR Clean-up Kit (Millipore). The final PCR product was subject to sequencing using the ABI PRISM Big Dye TM Terminator Cycle Sequencing Kit with AmpliTaq DNA polymerase (FS enzyme) from applied Biosystems. The resulting 16SrRNA sequence was analyzed using the NCBI BLAST tool for sequence similarity. Phylogenetic analysis involved multiple sequence alignments with the query sequence and closely related sequences identified through the BLAST search. Morphological analysis of the identified bacteria was conducted using a foldscope at a magnification of 140X and a resolution of 2.0 µm (Priyodip and Balaji, 2018).

2.5. Degradation experiment of Orange G

Pre-inoculum was prepared in a 250 ml Erlenmeyer flask and a loopful of culture was aseptically transferred from the cultured plates and inoculated into 100 ml of nutrient broth media. Turbidity as a sign of microbial growth, 2 ml of culture spike in 100 ml of media, media was supplemented with 100 to 500 mg L^{-1} concentration of Orange G

Following 48 h of incubation 48hrs, the centrifugation was done at 5000 rpm for 15 minutes. The supernatant was used to determine the decolorization using a UV–Visible double-beam spectrophotometer (Model No: LI UV-7000) at 485 nm with the help of percentage dye decolorization (Zhuang *et al.* 2020). A control was also included in the experiment (Shah *et al*., 2013a, b).

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

2.6. Optimization Process

The native bacterial strain showed the highest decolorization and degradation efficiency with pH, temperature, time, agitation speed, and concentrations of azo dye.

2.6.1. Impact of pH

The pH of the medium supplemented with Orange G (100 mg L^{-1}) was adjusted to 4, 5, 6,7,8, and 9 using 1 M HCL or 1 M NaOH. The incubation temperature was maintained at 37°C for five days.

2.6.2 Impact of Temperature

The decolorization performance of the isolate was analyzed at different incubation temperatures range from to 25-55°C. The effect of temperature on decolorization was determined spectrophotometrically.

2.6.3 Impact of Agitation

The study showed % decolorization of MO up to 96.01% at 48 h, whereas under shaking conditions (120 rpm), achieved decolorization up to 95.22 % (Fig 9). Better decolorization under shaking conditions may be due to improved oxygen transport and even distribution of nutrients for all individual colonies. Experiments were performed under identical environmental conditions for 60 h.

2.7. Analysis Using Gas Chromatography-Mass Spectrometry (GC–MS

To elucidate the metabolites generated throughout the degradation process, the supernatant was subject to GC-MS analyses. Metabolites existing in the supernatant were extract using an equal volume of ethyl acetate via solvent extraction. The residual components were subsequently dissolved in methanol and employed for GC-MS analyses, as previously described (Bharagava *et al*., 2018).

3. RESULTS

3.1 Colony Characteristics, Morphological and Biochemical Analysis

The colony characteristics, morphology, and biochemical analysis revealed variation among species and colony traits to distinguish gram-positive bacteria *Exiguobacterium spp.* BAB 5584 isolated from different samples for the study, had the same colony characteristics as evident from the fig.1.

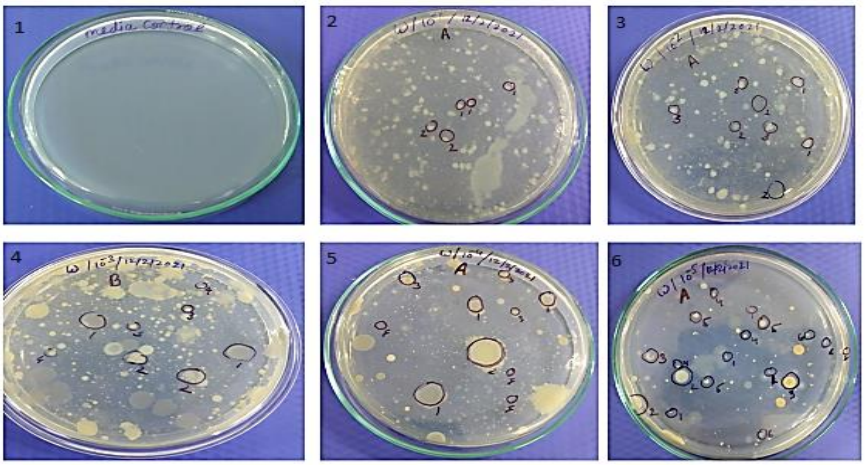

Fig 1: Colony characteristics of bacteria from textile industry wastewater

Table1: Colony characteristics of the isolates

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

4. RESULTS AND DISCUSSION

4.1. Strain Screening, Isolation and Identification

The samples were serially diluted and spread on nutrient agar in petri dishes. After growth on the Petri plate, three strains of bacteria were isolated. Isolated bacterial strains were used for OG degradation. The degradation efficiency was performed for 48hrs with isolated strains and analyzed by a double-beam UV–VIS spectrophotometer at 510 nm, at this wavelength, there is a maximum absorbance peak shown in figure. Approximately 98% degradation was observed within 48 h. However, after 48 h, the efficiency increased more than after 48 h, which might be due to the growth of microbial colonies as well as the limiting of nutrients. The UV–Vis spectra obtained before and after degradation are shown in Figure S1(c). The strain was analyzed by 16s rRNA sequencing and the obtained sequence and using ClustalW phylogenetic tree was constructed with the name 'Query' and from the phylogenetic tree, the species was identified as Exiguobacterium *spp*. BAB 5584 and given in Fig.2.

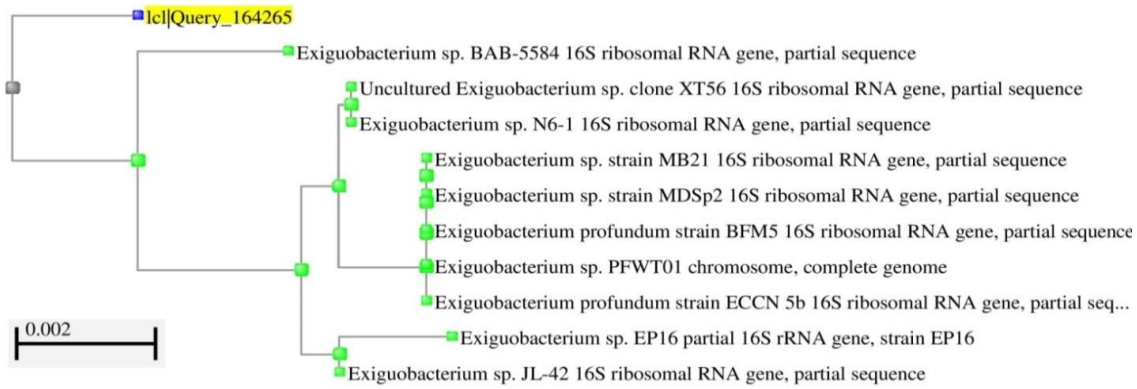

Fig :2 Phylogenetic tree of isolated native *Exiguobacterium spp. BAB 5584* based on 16sRNA

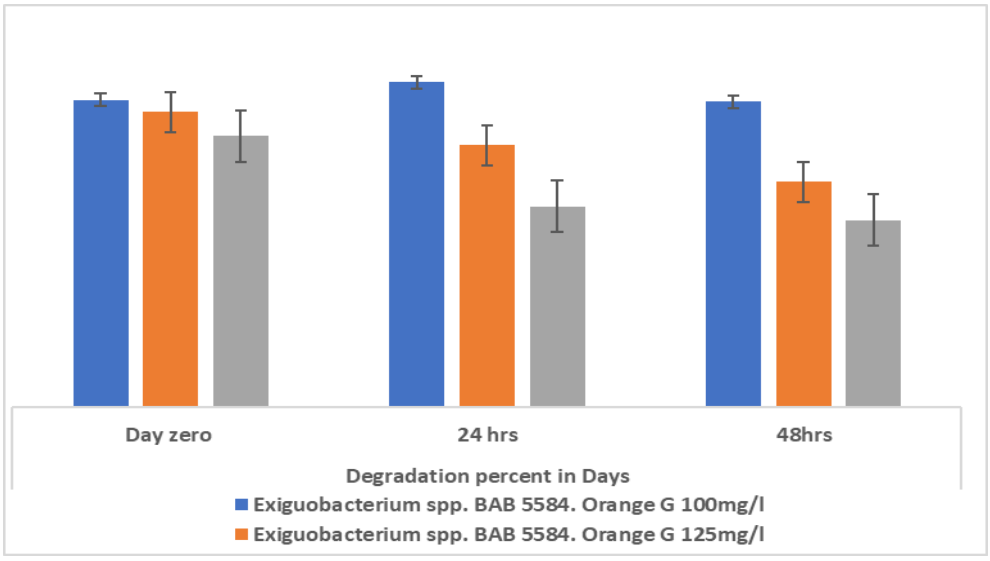

Fig:3 Decolorization % age of azo dyes in terms of concentration

4.2. Optimization Process

The native bacterial strain showed the highest decolorization and degradation efficiency with pH, temperature, agitation speed.

4.3. Impact of pH

Azo dye decolorization was affected by pH in the efficiency of *Exegobacterium sp.* BAB 5584, as shown in Fig. 8. These numbers demonstrate that pH 7 produced maximum decolorization. The sensitivity of bacterial strain to pH conditions can be linked to a decrease in azo dyes denatured above this pH

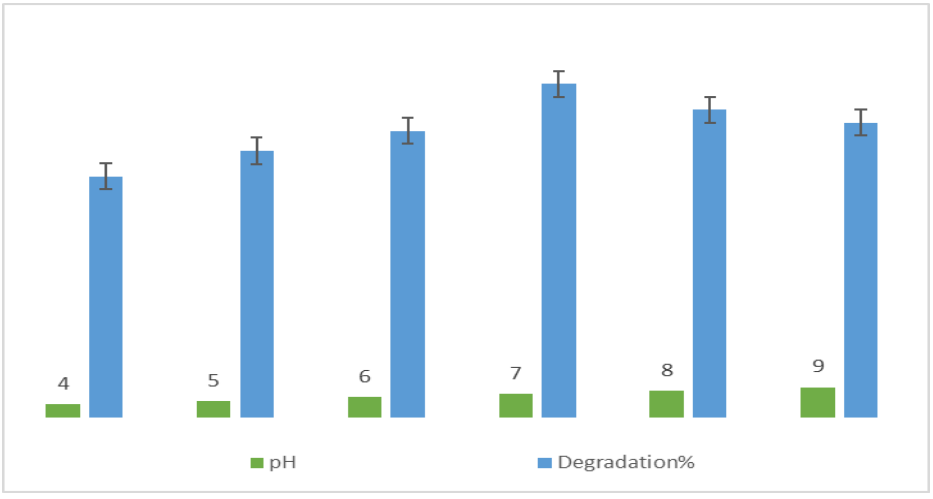

Fig:4 Decolorization % age of azo dyes in terms of pH

4.3.1. Impact of Temperature

The decolorization of azo dyes is a temperature dependent mechanism. The optimum temperature for the maximum activity of *Exegobacterium sp.* BAB 5584 was determined by measuring the azo dye decolorization at different temperatures as shown in (fig 7). The maximum activity was observed at 37 °C.

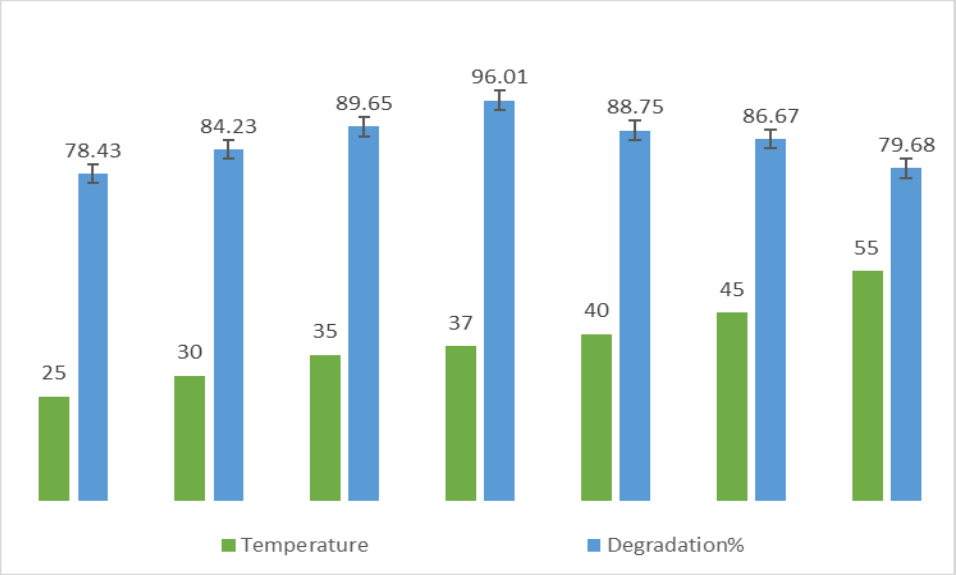

Fig: 5 Percentage decolorization of azo dyes in terms of varying Temperature (^{0}C)

4.3.2. Impact of Agitation

The study showed % decolorization of OG up to 96.01% at 48 h, whereas under shaking conditions (120 rpm), achieved decolorization up to 95.22 % (Fig 9). Better decolorization under shaking conditions may be due to improved oxygen transport and even distribution of nutrients for all individual colonies. Experiments were performed under identical environmental conditions for 60 h.

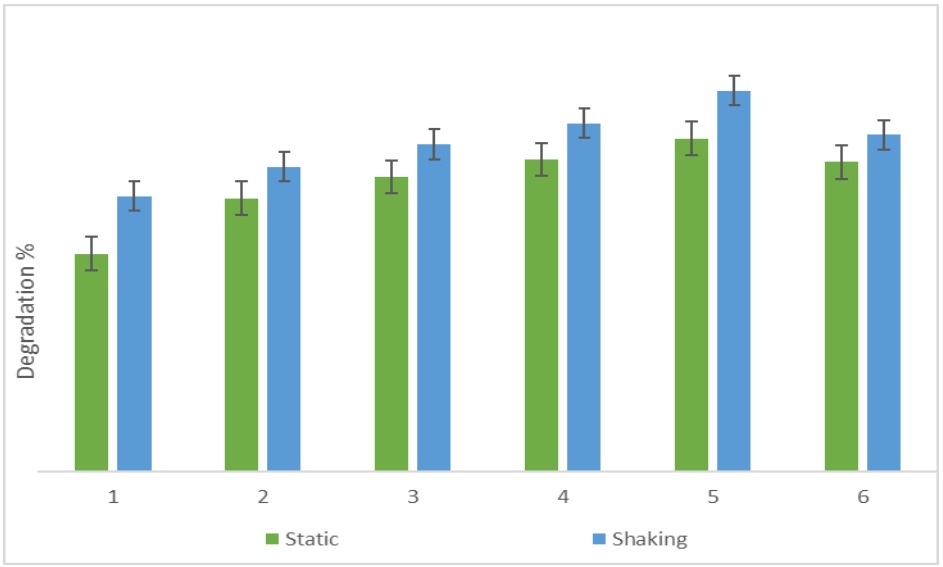

Fig:6 Percentage decolorization of azo dyes in terms of agitation speed

4.3.3. GC-MS Analysis

The extracted sample from the supernatant obtained after 96 h of treatment with Aeromonas hydrophila was identified by GC–MS analysis. The elution profile is shown in Fig.4. The peaks obtained from the chromatograms were analyzed. The molecule was recognized as 4-amino sulfonic acid with a retention time of 16.092 min and N, N-dimethyl p-phenylenediamine with a retention time of 19.109 min, as previously reported, by comparing the retention time of the NIST library in the instrument (Ali and Nabi, 2005; Parshetti *et al*., 2010). A pathway for forming

N, N-dimethyl p-phenylenediamine and 4-amino sulfonic acid has been proposed (Parshetti *et al*., 2010). The degradation pathway is catalyzed by the enzyme reductase, particularly azo reductase. Initially, the reduction of methyl orange was catalyzed by azo reductase. The symmetric cleavage of the azo bond resulted in the formation of the products

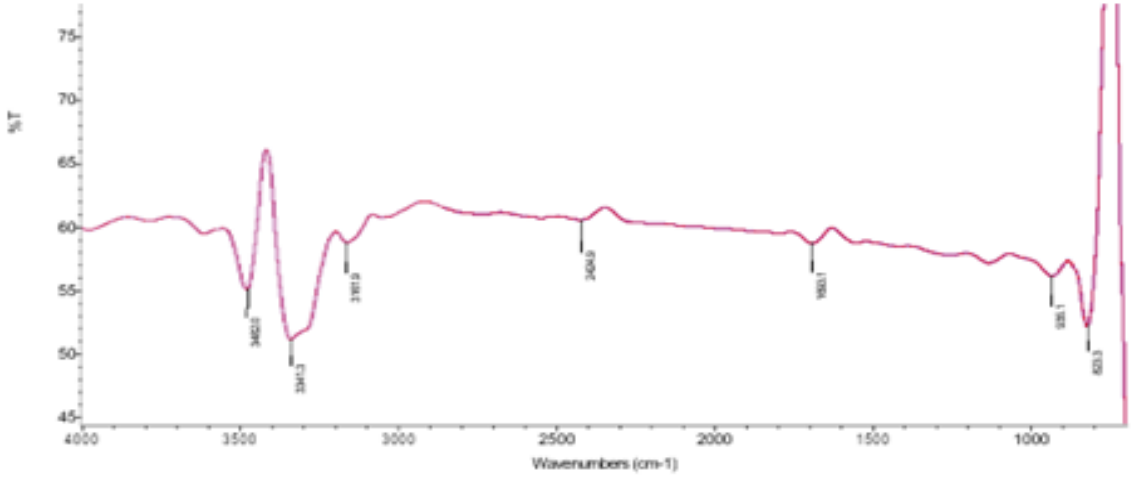

Fig:7 Graphical interpretation of GC-MS

5. DISCUSSION

The azo dyes are widely used in the textile industries and constitute a substantial source of industrial effluent contamination; (Asad *et al.,* 2007; Kim *et al.,* 2008; and Markandeya *et al.,* 2022), In the present investigation, OG was targeted for degradation potential of the isolate. After 24 h of incubation, *Exuobacterium sp.* BAB 5584 strains developed optimum growth which was also reported by Barragan *et al.,* 2007. Most textile dyes can be decolorized by microorganisms through bioaccumulation, in which the dyes are adsorbed onto the cell walls of microorganisms. This is dependent on biomass and mass transfer effects in the medium (Lellis *et al.,* 2019). The biodegradation of dyes occurs via the enzymatic response of microorganisms. This can be demonstrated by the development of colonies on the plates (Lucas *et al*., 2006), which were visible in our study plates (Fig:1). Qualitative screening of decolorizing strains revealed that they may be used as an energy source for industrial dyes. The decolorization rate of the strain was measured and assessed during quantitative selection in liquid cultures. A strain with a prominent capacity to remove color was chosen (Barragan *et al.,* 2007). It was discovered that it could thrive in a liquid medium with a dye serving as the only carbon source. Exposing the isolated strain from textile industry effluents, experiments with OG showed that decolorization was effective at low concentrations (100 mg/L^{-1}). Similar results have been reported in another study (C. Femina Carolin *et al.,* 2020), where it was observed significant decolorization at the highest expose concentration (100mg/l) within 24 hrs. Furthermore, it was observed that the rate of decolorization fluctuated and reached 98%, but it was not stable at all concentrations and with time. The isolated strain showed healthy decolorization within 24 h, ranging from 46.98 to 97.8 %, indicating that the native strain could be used for effective decolorization of the dye. It can also be used in the textile industry with minor modifications as reported by Roy *et al. (2020)*. As chemical stimulation is not acceptable, bio-augmentation may be an effort to integrate native species that may accelerate the decolorization of the azo dye (OG) in a shorter span (48–96 h), which is evident from the previous research conducted (Zubair *et al.,* 2018). The results of 16S RNA sequencing revealed an isolated culture similar to *that of Exegobacterium sp.* BAB 5584. *Exegobacterium sp.* BAB 5584 may continue to degrade the dye if it is exposed for a longer time. According to (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 is even more attractive. Results have shown that the *Exuobacterium sp*. BAB

5584 *sp.* is a potently decolorize OG (Ramaraju Kalpana *et al.,* 2020). At pH 7, 98 % of OG decolorization was observed by *Exuobacterium sp*. BAB 5584. But, in acidic conditions, decolorization showed decrease. Azo dye, typically has one or more than one sulfonic acid groups in the aromatic ring, which can function as detergents and prevent the growth of bacteria (Asad *et al.,* 2007). Dyes hinder nucleic acid synthesis or cell development; these that may impact DNA synthesis (Chen *et al.,* 2003).

CONCLUSION

A bacterial strain, *Exiguobacterium sp.* BAB 5584 was shown to be capable of decomposing Orange G as a single source of carbon with minimum nutritional needs, when the cultured in static condition. Because of its capacity to decolorize Orange G across a broad range of pH, temperature, agitation and dye concentrations, the strain has potential to be used in a variety of commercial applications. The findings of the analytical analysis have revealed that *Exiguobacterium sp.* BAB 5584 destroys the aromatic character of the dye Orange G. This makes the strain a very promising bacterium that having the potential to be employed in the treatment of textile industry effluents containing different dyes. Considering the nitrogen and carbon sources, 1% sucrose showed approximately 95% degradation, whereas 1% peptone showed 90% degradation. The GC–MS fragment pattern represented N, N-dimethyl p-phenylenediamine and 4-amino sulfonic acid.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Author Contribution

Kartikeya Shukla (Assistant professor) and Ashutosh Tripathi (Associate professor and HOD) contributed to design the study. Vartika Singh (PhD Scholar) collected the data, contributed to data analysis and interpretation, Jaswant Singh (Professor and HOI) reviewed the manuscript critically. All the authors have read and approved the final version of the manuscript.

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