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OPTIMAL BIODEGRADATION OF METHYL VIOLET ACHIEVED BY ENTEROBACTER STRAIN

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Methyl Violet (MV), Enterobactersp, Bioremediation, Ganges River

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ABSTRACT: The most widely used cationic dye, methyl violet (MV), has significant carcinogenic potential. The textile, cosmetic, printing, pharmaceutical, and food processing sectors all make extensive use of artificial dyes. They are typically referred to as xenobiotics because of their strong resistance to biodegradation. Microbes are beneficial because of their rapid growth and ability to break down and mineralise dyes. Methyl violet-degrading *Enterobacter* sp. was isolated from the Ganges River. It degrades up to 180 ppm of methyl violet in 24 hours when shaken at 25°C - 45ºC and pH 1 -12; optimum degradation occurs at 40 ppm in 24 hours at pH 7.0. The effects of temperature, initial pH, and dye concentration were investigated. The capacity of bacteria to degrade was demonstrated by experimental results. The deterioration of methyl violet was validated by UV-Vis spectroscopy, SEM, and FTIR examination of samples both before and after dye breakdown in culture media.

INTRODUCTION: Water serves as a crucial entity to our existence. About 71% of the Earth's surface is surrounded by water, with 97.5% being seawater and just 2.5% being freshwater [1]. The rapid increase in the global population, climate change, and industrial expansion have greatly impacted water quality, leading to a growing freshwater crisis worldwide. Dyes rank among the most significant sources of ecological pollution, originating from various industries [2]. It can be categorised according to its chemical structures into various categories, including nitro dyes, azo dyes, diphenylmethane dyes, triphenylmethane dyes, xanthene dyes, phthaleins dyes, indigoid and thionidigoid dyes, and anthraquinoid dyes [3].

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Triphenylmethane dyes, like crystal violet, methyl violet, malachite green, and brilliant green, are extensively used in various industries, including textiles, cosmetics, pharmaceuticals, food, leather, households, paper, paints, and varnishing. They are also commonly employed as biological stains, such as in the Gram stain procedure [4, 5]. MV (methyl violet), a cationic dye, is dispersible in water, methanol, diethylene glycol, ethanol, and dipropylene glycol [6]. The unusual discolouration of water leads to neurological and sensory harm, metabolic strain, and mortality among fish.

It also disrupts photosynthetic processes within aquatic plants by restricting light penetration, slows down germination rates, reduces biomass accumulation, and hampers the respiratory capacity of plants because of the buildup of unstable aromatic compounds [7]. Furthermore, human exposure to this dye poses significant risks due to their toxicological and carcinogenic impacts on the immune and respiratory systems [4, 8]. Methyl violet dye can cause genetic mutations, sterility, skin

inflammation, and perforation of the nasal septum [9]. Effluents from industries containing dyes pose environmental hazards, and their accumulation in the human body can impact human life through the food chain. Direct contact causes pain, congestion, and irritation of the skin and eyes [6].

Various techniques have been developed and studied for eliminating dyes from wastewater, encompassing physical, chemical, and biological techniques. These include adsorption, membrane filtration, ion exchange, electrocoagulation and flocculation, precipitation, ozonation, UV irradiation, and advanced oxidation technique [7]. The drawbacks associated with physical and chemical techniques, including high costs, inability to eliminate persistent dyes, and generation of substantial sludge volumes, which restrict their broader application [4, 10]. Biological processes are viewed as promising alternatives, garnering more interest due to their cost-effectiveness, environmental friendliness, and minimal sludge production compared to physical and chemical methods [11, 12]. Bacteria offer advantages due to their high growth rates and their capability to degrade and mineralize dyes [13]. Bioremediation presents an environmentally friendly and cost-effective approach for eliminating a wide variety of organic and inorganic pollutants from contaminated environments. Advanced treatment methods utilizing microorganisms can enhance the efficiency of remediation processes, provided that suitable environmental conditions are maintained to ensure effective bioremediation [1].

Fungi like *Phanerochaete chrysosporium*, *Cyathus bulleri, and Trametes versicolou*r, along with bacteria like *Bacillus subtilis, Enterobacter cloacae, Pseudomonas mendocina, Stenotrophomonas maltophilia, Sphingomonas paucimobilis,* and *Rhizobium radiobacter*, have been studied for their capacity to biodegrade triphenylmethane dyes [11]. Recently, Actinobacteria have shown promise in utilising carbon sources from toxic compounds and have also been investigated for remediating pesticides, heavy metals, hydrocarbons, and dyes [9]. This study mainly focused on microbial biodegradation of methyl violet by SSAU6 isolated from the Ganges under various optimum conditions, and degradation was analysed by UV-Vis spectrophotometer. The characterisation of this microbe was mainly done by SEM and optical microscopy, while the product of degradation is characterised by FTIR. This microbe can prove to be a paradigm shift in the methyl violet-related environmental pollution and its remediation.

MATERIALS AND METHODS:

Chemicals and Media: For bacterial culturing and other degradation processes, nutrient broth with the composition Peptone (5 g/L), NaCl (5 g/L), and beef extract (1.5 g/L) was utilised. Additionally, various chemicals and materials were obtained from Himedia, including methyl violet, ethanol, dimethyl sulfoxide, NaCl, KOH, glycerol, indole Reagent, Simmons Citrate Agar, Mannitol Salt Agar, Mac Conkey Agar, OsO4, Glutaraldehyde, HCl, Nutrient Agar, Glucose, Lactose, Sucrose, Mannitol, Fructose, Dextrose, Urea, Ammonium sulfate, Ammonium iron III citrate, Luria Broth, Ammonium nitrate, Sorbitol, Ammonium chloride, Crystal Violet, Iodine solution, Safranin, K2HPO4, KH2PO4, MgSO4, CaCl2, Potassium chloride. All chemicals used were of analytical grade. A stock solution of methyl violet (MV) was prepared by dissolving 0.1 g of MV in 10 mL of distilled water and stored in a falcon tube for further use.

Extraction, Characterization, and Maintenance of Dye-Degrading Species: In the current research, bacteria were obtained from the river Ganges in Prayagraj, Uttar Pradesh, India. Initially, bacterial isolates demonstrating methyl violet decolorizing activity were obtained and purified through repeated sub-culturing on nutrient agar (NA) plates containing Methyl Violet (Composition: Peptone 5 g/L, NaCl 5 g/L, Beef Extract 1.5 g/L, Agar 15 g/L), and designated as S-1 to S-8.

These isolates were maintained at 4°C and sub cultured weekly on fresh nutrient agar slants. Additionally, a pure culture was preserved at 20°C in 20% (v/v) glycerol stocks. According to their ability to decolorize methyl violet, isolates were screened, and S-6 was selected for further experimentation. The S-6 isolate was cultured in a 250 mL Erlenmeyer flask containing 50 mL of nutrient broth (NB) and incubated on a rotary shaker at 180 rpm and 35°C to promote cell growth for inoculum preparation.

Morphological Characterization: The features of colonies and cell morphology was examined based on color, shape, margin, elevation, surface, and bacterial arrangement [14]. The isolates were morphologically characterized using the standard gram staining techniques [15] Scanning electron microscopy [16].

Genomic Characterization: The DNA of the bacterial isolate was extracted using a standard method and analyzed through molecular characterization via 16S rRNA sequencing. The PCR amplification of the 16S rRNA gene included an initial denaturation at 94° C for 2 minutes, followed by 35 cycles of amplification at 94° C for 45 seconds, 55° C for 60 seconds, 72° C for 60 seconds, and a final extension at 72° C for 10 minutes, using universal forward (5′-GGATGA GCCCGCGGCCTA-3′) and reverse (5′-CGGTGTGTA CAAGGCCCGG-3′) primers. After electrophoresis, the PCR products were cut from a 1 % agarose gel and purified with a QIAquick gel extraction kit (Qiagen), then sequenced directly using a Biotech Diagnostics Big-Dye sequencing kit on an ABI 377 sequencer (Applied Biosystems, Foster City, Calif.). The isolates were tentatively identified through 16S rDNA sequence analysis using the NCBI 16S BLAST database.

Biochemical Characterization:

The S-6 was Characterized Based on Following Biochemical Tests: Indole test [17], Simmons Citrate Agar test [17], Methyl Red test [17], MacConkey agar test, Mannitol salt agar test [17], Casease test [18, 19], motility test, catalase test [20, 21], and sulfur reduction test [22] to examine the distinctive features of the isolate.

Dye Degradation Studies: 10 µl loopful of microbial culture was introduced into a 250 ml Erlenmeyer flask with a volume of 100 mL of nutrient broth and then incubated at 35° C for a duration of 24 hours. After this initial incubation period, dyes were added to each flask at a various concentration of 10 mg/L to 200 mg/L. Subsequently, 3 ml of the culture medium was withdrawn from each flask at different time intervals. These samples were centrifuged at 5000 × g for 20 minutes to separate the bacterial cell mass. The clear supernatant was then used to determine decolorization by measuring the change in absorbance of the culture supernatants at the 540 nm wavelength. The following formula was used to estimate the degradation [23, 13].

$$\% \text{ Decolourization} = \text{Initial absorbance} - \text{Observed absorbance} / \text{Initial absorbance} \times 100$$

Optimization regarding dye degradation conditions: Optimization was performed by varying one parameter while others were maintained at a constant level. Various environmental parameters such as salinity (5 g/L, 10 g/L, 20 g/L, 40 g/L, 60 g/L, 80 g/L and 100 g/L), pH (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12), temperature (25º C, 30º C, 35º C, 40º C and 45º C), initial dye concentration (10 ppm, 20 ppm, 40 ppm, 60 ppm, 80 ppm, 100 ppm, 120 ppm, 140 ppm, 160 ppm, 180 ppm and 200 ppm), carbon sources (sucrose, glucose, lactose, fructose, sorbitol, dextrose and mannitol), nitrogen sources (ammonium nitrate, ammonium iron III citrate, ammonium sulfate, peptone, urea and ammonium chloride), static/shaking(10 ppm, 20 ppm, 50 ppm, 80 ppm and 100 ppm) and inoculum size (0.5 %, 1 %, 2 %, 4 %, 6 %, 8 % and 10 %) were optimized for the decolorizing of Methyl Violet. The experiment involved inoculating the mixture with a bacterial culture that had been incubated for 24 hours. Uninoculated solutions of Methyl Violet dye were utilized as controls [24, 25]. Based on the observation, optimum degradation condition was selected for the further experimentation.

Antibiotic Susceptibility Test: Antibiotic sensitivity was assessed using the ring diffusion method. In this research, twelve antibiotic rings, including amoxicillin (AMX-10mcg), amikacin (AK-30mcg), augmentin (AMC-30mcg), ciprofloxacin (CIP-5mcg), cefotaxime (CTX-30mcg), ceftriaxone (CTR 10mcg), colistin (CL-10mcg), co-trimoxazole (COT-25mcg), furazolidone (FR-50mcg), gentamicin (GEN-10mcg), norfloxacin (NX-10mcg), and netillin (NET-30mcg), were used. The sensitivity and resistance patterns of the isolated bacteria were tested on a nutrient agar medium. After overnight incubation at 35º C, the diameter of the inhibition zone was measured [26].

SEM Studies: The standard SEM protocol involves several key steps: first, fixation is carried out using 2.5% glutaraldehyde in PBS at a pH of

7.4, followed by post-fixation in a 1% OsO_4 solution in water. Next, the sample undergoes dehydration via a sequence of ascending alcohol concentrations. After dehydration, critical point drying is performed using CO_2 substitution. Finally, the sample is coated with a platinum sputter [16, 27]. Alter the coating, the sample was observed with SEM-JOEL (JXA-8100) at multiple resolutions.

FTIR: The bacterial cells were extracted using centrifugation at 5000 rpm, 20 minutes, then washed three times with a Phosphate buffer solution and dried at 45° C for 24 hr [28, 29]. Following three sets were used in this study: control (without MV), MV, SSAU-6 (after degradation). The samples were characterized by using FTIR spectrum two (C96219).

Growth and Degradation Kinetics: To study the degradation kinetics, freshly prepared 2% inoculums was introduced into 20 mL of NB media. Samples were collected at different intervals of time and processed by centrifugation at 5000 RPM for 15 minutes. The supernatant was then analyzed for the reduction using a spectrophotometer at 540 nm, and the proportion of degradation was calculated using an equation. The degradation kinetic curve, showing the proportion of degradation over time, was then used to determine the rate constant for the growth kinetics; sample growth was analyzed after every 2 hr at 600 nm by using UV-Vis spectrophotometer.

% Decolourization = Initial Absorbance - Observed Absorbance / Initial Absorbance × 100

Statistical Analysis: All tests were conducted along with their corresponding determinations, and experimental errors were represented and evaluated using standard error (depicted as error bars). Data from the previous experiments were gathered and analyzed using an ANOVA test with a 95% confidence interval, performed on SPSS software for statistical analysis.

RESULTS AND DISCUSSION:

Morphological, Biochemical and Molecular Characterization:

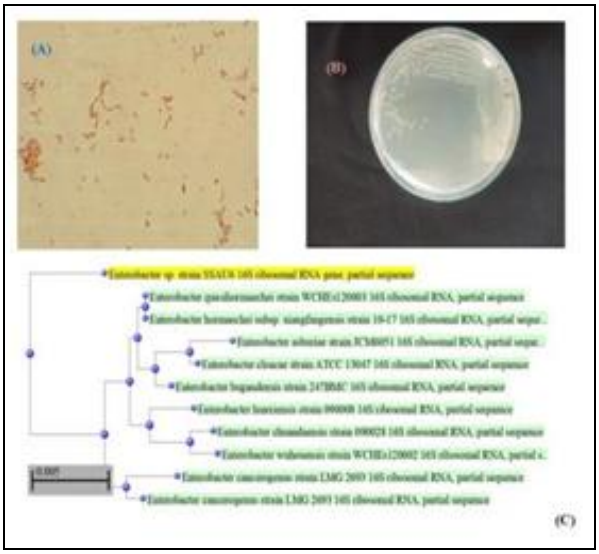

FIG. 1: (A) REPRESENT THE MICROSCOPIC OBSERVATION OF SSAU-6 STRAIN. (B) REPRESENT THE MORPHOLOGICAL CHARACTERISTIC OF SSAU-6 STRAIN. (C) PHYLOGENETIC TREE OF SSAU-6. THE PHYLOGENETIC TREE REVEALED THAT THE BACTERIAL SPECIES WAS ENTEROBACTER STRAIN SSAU-6.

On solid agar medium supplemented with methyl violet dye, eight morphologically distinct colonies displaying prominent clearing zones were selected. Samples from the river Ganges were labeled with codes (S-1 to S-8) corresponding to their respective plates. The isolates were purified five times using the streaking method, selecting colonies from the designated plates. Among all the isolates, two purified strains were chosen based on their performance in the degradation assay. The analysis revealed that the strain SSAU-6 exhibited the highest degradation potential among the bacterial

strains extracted from the river Ganges. Consequently, SSAU-6 was selected for further characterization and evaluation. The morphological, colony characteristics, and other biochemical characteristics of the isolate SSAU-6 were shown in figure and table. According to microscopic investigations, SSAU-6 cells were slightly curved, rods, and Gram-negative. Thangaraj *et al.*, 2021 [30] showed that *Enterobacter hormaechei* SKB16 Gram-negative a rod-shaped bacterium with elongated cells.

Campbell and Roth 1975 [31] reported that Enterobacter based on colonial morphology will be useful across both the clinical laboratory and in the area determination of water quality from the bacteriological standpoint. Poddar *et al.*, 2021 [32] showed that the strain PWN1 was Gram-negative, oval-shaped, and did not form endospores. The observed colonies were round, small in size, translucent in opacity, regular in margin, raised in elevation, and pigmentation is cream white. The texture of isolated colonies was smooth.

Thangaraj *et al.*, 2021 [30] Identified as *Enterobacter hormaechei* SKB16, a facultative anaerobe, with rapid growth and smooth in texture, circular shape. The 16S rRNA gene sequences were submitted through the 'BANKIT' option on the NCBI GenBank webpage to obtain accession numbers for the nucleotide sequences (PP600149). The new strain underwent genomic relatedness analysis from a publicly accessible database to verify its classification within the genus. Phylogenetic assessment of strain S-6, based on core 16S rRNA genome data, revealed over 95.5% similarity within the Enterobacteriaceae family.

Various biochemical tests were conducted to describe the nature about bacteria that has been isolated, and named as SSAU6. The tests that get positive results are like macconkey, citrate, sulfur reduction, motility, methyl red, as well as catalase, whereas the tests with negative results are mannitol, casease, and indole, summarized in **Table 1** Poddar *et al.*, 2021 [32] reported negative results for the indole test, methyl red test, and citrate test. Azis *et al.*, 2019 [33] showed that the genus Enterobacter, strain AA26, was oxidase-negative, indole negative, and Voges-Proskauer-positive.

TABLE 1: REPRESENT THE BIOCHEMICAL TEST OF SSAU-6 STRAIN

Biochemical	Positive/Negative
Mac conkey	+
Mannitol	-
Citrate	+
Casease test	-
Indole	-
Sulfurreduction	+
Motility	+
Methylred	+
Catalase	+

Optimization of Degradation Condition: Effect of Salinity:

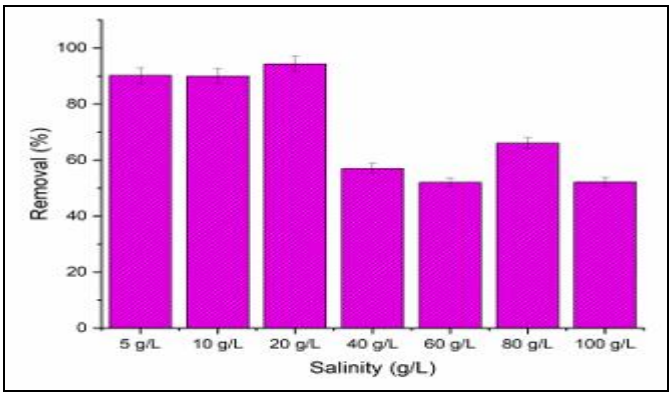

FIG. 2: VARIATION OF PERCENTAGE DEGRADATION (Y- AXIS) ON VARYING NACL CONCENTRATIONS (X-AXIS)

The study examined the impact of different salinity levels while maintaining a dye concentration constant at 40 ppm. Salinity ranging from 5 g/L, 10 g/L, 20 g/L, 40 g/L, 60 g/L, 80 g/L, and 100 g/L. The results, depicted in **Fig. 2**, showed that the rate of degradation was high at a concentration of 5 g/L to 20 g/L salinity. While increasing the level of salinity, it was found that the rate of degradation was reduced. SSAU6 could degrade up to 94.3% of methyl violet within 24 hours when exposed to 20 g/L of NaCl. However, the rate of degradation is 90.23%, 89.95%, 94.36%, 57.03%, 52.00%, 66.01%, and 52.17% at salinity levels of 5, 10, 20, 40, 60, 80, and 100 g/L, respectively. Masarbo *et al.*, 2018 [34] stated that salt concentrations above 3 g/L can cause obstruction of bacterial metabolism. Guo *et al.*, 2020 [35] investigated that high salinity has been revealed to induce a significant decrease in enzyme activity, even leading to total inactivation. Al-Tohamy *et al.*, 2020 [36] observed that another reason for the reduction in terms of dye removal at high salt concentrations could be the reduction in dye solubility.

Effect of pH:

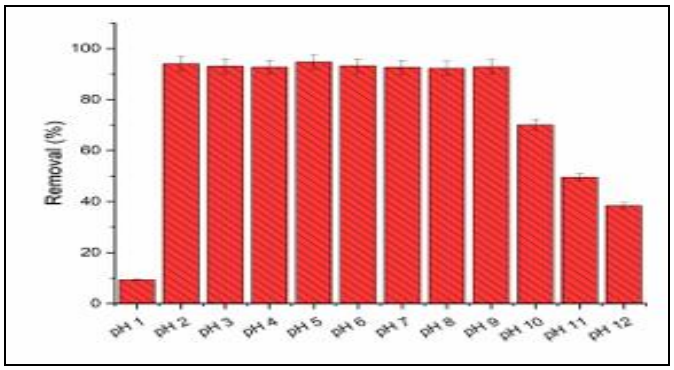

FIG. 3: VARIATION OF PERCENTAGE DEGRADATION (Y-AXIS) ON INITIAL PH VARIATION (X-AXIS)

The study examined the impact of different pH levels while maintaining dye concentration constant at 40 ppm. pH ranging from 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12. The results, depicted in the **Fig. 3**, indicated that the rate of degradation is constant from pH 2 to 9, and after increasing the pH, the removal effectiveness is reduced, this reduction is due to the negative influence of transmembrane transport of dye. SSAU6 could degrade up to 9.37%, 94.02%, 93.08%, 92.68%, 94.69%, 93.19%, 92.63%, 92.24%, 92.80%, 70.08%, 49.55%, 38.38% and 92.13% at pH 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 and control, respectively.

The bacteria can tolerate and function efficiently across a wide pH scale, ranging from acidic to basic conditions. This broad pH tolerance means that the bacteria have adaptive mechanisms, such as pH homeostasis, that allow them to maintain stable intracellular conditions necessary for dye destruction even in extreme pH environments. Li *et al.*, 2014 [4] studied that the removal of crystal violet color and methyl violet remained constant over an extensive range of pH (6.0 to 9.0), which would be favorable for bioprocessing dye-containing wastewater and useful for industrial purposes. Adenan *et al*., 2021 [9] observed that the low decolorization efficiency exhibited by live cells at pH 3 and 5 could be linked to suboptimal conditions for their growth and enzyme synthesis. At higher pH levels, the surface of No cardiopsisc ells carries a negative charge, leading to greater adsorption of positively charged dye molecules (MG, MV, and CV). This suggests that the treatment of TPM dyes with dead cells was impacted by their ionic interactions. Kumar *et al*., 2011 [11] showed similar finding that the pH conditions needed for the highest decolorization of MV by *Aspergillus* species strain CB- TKL-1 were observed to be a pH interval of 5.0 to 6.0 with an optimum pH of 5.5. Yang *et al*., 2020 [37] investigated that CotAs from Bacillus are recognized as bacterial laccases that can withstand elevated temperatures and alkaline environments, rendering them appropriate for use in dye degradation. Ayed *et al*., 2009 [38] examined that at a lower pH, the H+ ions successfully contend with dye cations, resulting in a decline in colour removal efficiency.

Furthermore, at higher pH above this point charge, the surface of biomass gets negatively charged, which enhances the positively charged dye cations through electrostatic force of attraction. Shanmugam *et al*., 2017 [39] perceived that the catalytic activity of laccase was well maintained at pH 6.0, which is the reason for the maximum degradation. Al-Zaban *et al*., 2022 [40] observed that pH is the primary factor in the adsorption process, as it affects the charge and ionization of active groups in the cell walls of the biomaterial, including amino, carbonyl, phosphate, and carboxyl groups.

Dahri *et al*., 2013 [41] showed the removal of MV diminished slightly as the pH rose from 4 to 10. Therefore, it can be concluded that pH has no notable impact on the adsorption of MV onto CEN. Amin *et al*., 2015 42 demonstrated that the level of Reactive Black 5 adsorption is primarily influenced via the surface charge on the bentonite clay, which is affected by the solution pH.

Effect of Inoculum Size:

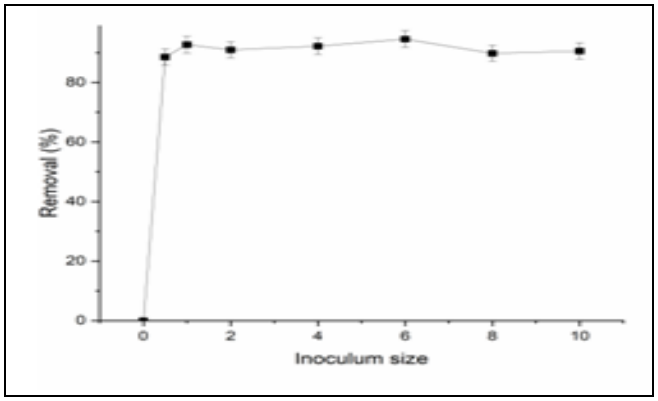

FIG. 4: VARIATION OF PERCENTAGE DEGRADATION (Y-AXIS) AGAINST INITIAL INOCULUMS SIZE (X-AXIS)

The study examined the impact of different inoculum size levels while maintaining dye concentration constant at 40 ppm. Inoculum size ranging from 0.5%, 1%, 2%, 4%, 6%, 8% and 10% (v/v). The results, displayed in the figure, indicated that the rate of degradation increased from 0.5% to 6% than further decreased. SSAU6 could degrade up to 94.58% of methyl violet within 24 hours when exposed to inoculums of size 6% (v/v). However, the degradationrate of 0.5%, 1%, 2%, 4%, 6%, 8% and 10% (v/v) is 88.56%, 92.68%, 90.95%, 92.18%, 94.58%, 89.78% and 90.56% respectively.

The observation that removal efficiency remains stable across different inoculum sizes suggests that the bacteria possess high metabolic efficiency. Regardless of the population size, they can consistently produce the necessary enzymes and metabolic pathways to degradethemethyl violet dye compound.

This suggests that their metabolic activity is robust and not limited by population size. Li *et al.*, 2014 [4] shown that the decolorization of crystal violet and methyl violet was not significantly improved by increasing the inoculum size beyond 2%. Parshetti *et al*., 2011 [43] reported that with an increase in the inoculum concentration of *A. radiobacter*, the degradation of Crystal Violet (100 mg/L) was increased.

Effect of Temperature:

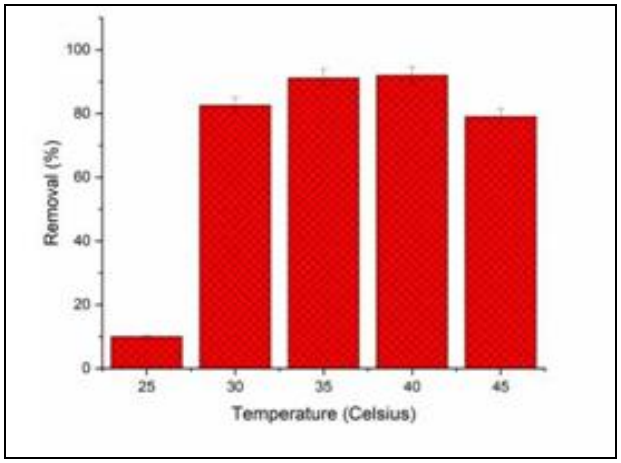

FIG. 5: VARIATION OF PERCENTAGE DEGRADATION (Y-AXIS) ON INITIAL TEMPERATURE VARIATION (X AXIS)

The effect of temperature on dye removal was measured by maintaining a dye concentration constant at 40 ppm. Temperatures varying from (25ºC, 30ºC, 35ºC, 40ºC, and 45ºC). Result revealed that it increases from 25ºC to 40ºC than it was decreased. SSAU6 could degrade up to 91.96% of methyl violet dye within 24 hours at temperature 40ºC. However, the degradation rate at 25ºC, 30ºC, 35ºC, 40 ºC and 45 ºC is 82.58%, 91.18%, 91.96% and 79.01% respectively.

The bacteria likely produce temperature-stable enzymes that are able to function efficiently across a vast range of temperatures. These enzymes, such as laccases or peroxidases, are critical for breaking down methyl violet dye compounds and remain active regardless of temperature fluctuations. This temperature stability ensures consistent dye degradation in various environments.

Li *et al*., 2014 [4] reported that temperature significantly affects the decolorization capacity of microorganisms by activating or inhibiting the enzymes responsible for decolorization.

Kumar *et al*., 2011 [11] shown that optimal decolorization of MV was demonstrated at temperatures above 35^{o}C and below 25^{o}C, a decrease in decolorization rate was observed. Yang *et al*., 2020 [37] showed that with increasing temperature, the enzyme activity of CotA decreased.

Ayed *et al*., 2009 [38] showed essentially no thermal inactivation of decolorization activity under operational temperatures, indicating that *Bacillus* sp. could acclimatize them to a wide variation of temperatures in dyeing wastewater.

Al-Zaban *et al*., 2022 [40] investigated that elevated temperatures often enhance biosorption capacity due to the increased kinetic energy and surface activity of the solute.

Dahri *et al*., 2013 [41] showed that as the temperature rises, the diffusion rate of the dye molecule increases due to the decrease in the solution's viscosity. This results in an enhancement of the sorption process as the movement of molecules from the bulk solution to the solid surface accelerates at elevated temperatures. Amin *et al*., 2015 [42] showed that it can be linked to high surface coverage, the synthesis of active and reactive sites, and expansion at higher temperatures.

Effect of Static and Shaking Condition:

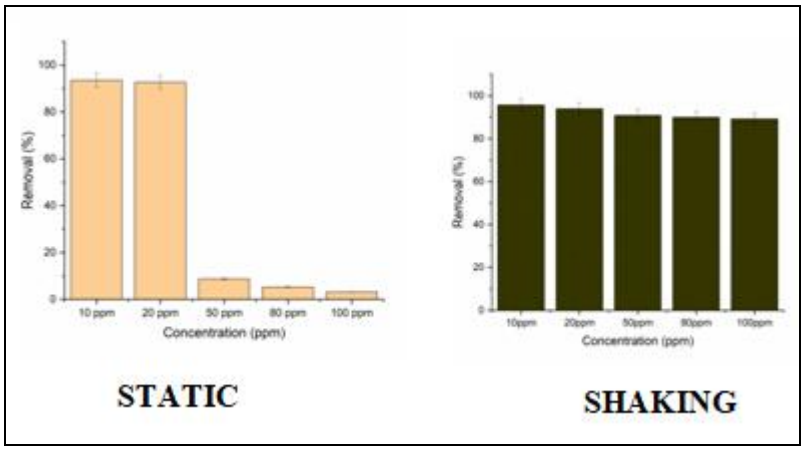

FIG. 6: VARIATION OF PERCENTAGE DEGRADATION (Y-AXIS) AGAINST STATIC AND SHAKING CONDITION

To assess the impact of static and shaking conditions on decolorization, this effect was researched while maintaining a dye concentration constant of 40 ppm, a salinity constant at 20 g/L, pH constant of 7, a temperature constant of 35ºC and an inoculum size constant of 2% (v/v) conditions, respectively. Comparatively, SSAU6 in shaking condition was more efficient in decolorizing methyl voilet dyes than in static condition.

Among them, the decolorization efficiency on methyl violet was significantly higher in shaking condition with 95.70%, 93.97%, 90.84%, 90.01% and 89.22% at 10 ppm, 20 ppm, 50 ppm, 80 ppm, and 100 ppm, as compared to 93.52%, 92.63%, 8.70%, 5.24% and 3.12% for the same concentration in static condition.

In shaking conditions, the bacteria and dye molecules can be constantly mixed, increasing the chances of interaction between the bacterial cells as well as the dye. This enhances the efficacy of dye degradation because the bacteria have more frequent contact with the methyl violet dye, facilitating faster breakdown.

Li *et al.*, 2014 [4] reported that the decolorization under shaking conditions was either partially or entirely inhibited compared to static conditions. Parshetti *et al.*, 2009 [23] indicate that the removal of color was due to biodegradation. Gao *et al.*, 2020 [44] observed that the higher decolorization under shaking conditions than in static conditions is primarily based on the oxidative reactions by key enzymes such as LiP and MnP.

Effect of Dye Concentration:

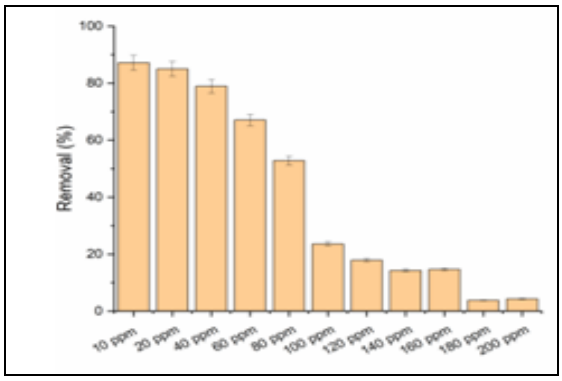

FIG. 7: VARIATION OF GRAPH SHOWS THE TRENDS OF DEGRADATION RATE (Y-AXIS) AGAINST VARIED DYE CONCENTRATION (X-AXIS)

The study examined the effect of various dye concentration levels (ranging from 10 ppm, 20 ppm, 40 ppm, 60 ppm, 80 ppm, 100 ppm, 120 ppm, 140 ppm, 160 ppm, 180 ppm, and 200 ppm). The results showed that the rate of degradation increased notably at dye concentration 10 ppm but then declined sharply. SSAU6 could degrade up to 87.16% of methyl violet within 24 hours upon exposure to a dye concentration of 10 ppm. However, the degradation rate at a concentration of 10 ppm, 20 ppm, 40 ppm, 60 ppm, 80 ppm, 100 ppm, 120 ppm, 140 ppm, 160 ppm, 180 ppm and 200 ppm is 87.16%, 85.04%, 78.90%, 67.07%, 52.84%, 23.60%, 17.91%, 14.28%, 14.67%, 3.79% and 4.29%. Bacteria can degrade lower concentrations of methyl violet, which is ideal for environmental applications, including the remediation of drinking water sources, groundwater, and natural water bodies where pollution levels are typically low.

This highlights their potential role in more sensitive ecosystems where even small amounts of dye can have harmful effects. Li *et al.*, 2014 [4] reported that an isolated bacterium could utilize crystal violet as both a carbon and energy source and that the cell biomass significantly increased with the addition of crystal violet. Adenan *et al.*, 2021 [9] at elevated dye concentrations, the toxicity of the dyes may have negatively impacted the viability and enzyme activity of live cells, resulting in a reduction in decolorization efficiency. Ayed *et al.*, 2009 [38] suggested that the decrease in decolorization efficiency might be owing to the toxic effect of dyes. Parshetti *et al.*, 2011 [43] with higher loads of dye substrate demand more time to be degraded. AL-Niaimi *et al.*, 2023 [45] shown that it provides a sufficient driving force to overcome each of the mass transfer barriers between the aqueous and solid phases. Al-Zaban *et al.*, 2022 [40] reported that when all binding sites are saturated at elevated initial dye concentrations, a greater number of dye molecules remain unbound in the solution, leading to a lower removal percentage. Dahri *et al.*, 2013 [41] showed that an increase in concentration leads to enhanced interaction between the dye molecules and CEN, thereby boosting the sorption process. Gao *et al.*, 2020 [44] suggest that the toxicity of the dye may become more significant at higher concentrations, potentially inhibiting microbial growth.

Impact of Carbon and Nitrogen Source:

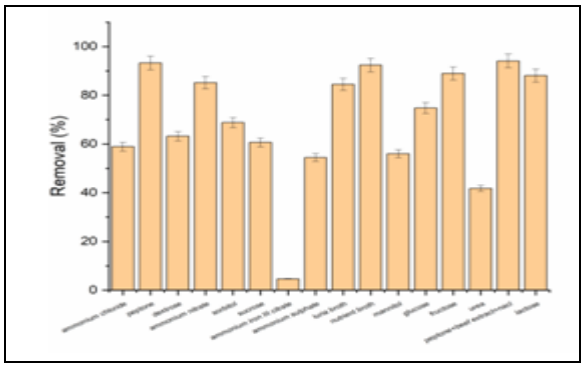

FIG. 8: VARIATION OF PERCENTAGE DEGRADATION (Y-AXIS) ON VARYING CARBON AND NITROGEN SOURCES (X-AXIS)

The effect of differing carbon sources, including lactose, fructose, glucose, sucrose, sorbitol, dextrose, mannitol, and nitrogen sources, ammonium chloride, peptone, ammonium nitrate, ammonium iron III citrate, ammonium sulfate, luria broth, nutrient broth, urea, and peptone+beef extract+Nacl (control), on reduction of methyl violet by *Enterobacter* spp. SSAU6 was studied while keeping other parameters constant. The results showed that all external carbon sources, except mannitol, had a negative impact on degradation. The highest removal efficiencies, 88.11% and 89.00%, were observed with lactose and fructose, respectively. In contrast, glucose, sucrose, sorbitol, dextrose, and mannitol led to lower removal efficiencies of 74.77%, 60.65%, 68.80%, 63.22%, and 56.02%, respectively. Regarding external nitrogen sources, all except ammonium iron (III) citrate and urea negatively impacted degradation. The highest removal efficiencies, 93.30%, 92.41%, and 94.08%, were noted with peptone, nutrient broth, and peptone+beef extract+NaCl (control), respectively. However, ammonium chloride, ammonium nitrate, ammonium iron (III) citrate, ammonium sulfate, luria broth, and urea yielded lower removal efficiencies of 58.92%, 85.21%, 4.63%, 54.46%, 84.48%, and 41.74%, respectively. The bacteria's ability to thrive and degrade methyl violet in the context of different nutrient sources indicates their adaptability to varied environmental conditions. In natural or industrial environments, nutrient levels fluctuate, and the bacteria's ability to break down the dye under changing conditions reflects their robustness and potential for large-scale bioremediation. Parshetti *et al*., 2011 [43] evaluated that the presence of various culture conditions may exert either a stimulatory or inhibitory effect on the enzymes involved in dye decolorization, leading to differences in the time required for decolorization as well as variations in the decolorization percentage.

Antibiotic Test:

FIG. 9: ANTIBIOTIC RESISTANT PROFILE OF SSAU-6 STRAIN

This section presents a summary of the outcomes of the antibiotic sensitivity test. In the analysis of antibiotic sensitivity, it was apparent that bacterial isolates showed resistance to several different antibiotics. Here, Isolate was resistant to co-trimoxazole (sulpha/trimethoprim) (COT/25 mcg)and colistin (methane sulphonate) (CL/10 mcg) with no zone of inhibitionbut susceptible to amoxicillin (AMX/10 mcg), augmentin (AMC/30 mcg), netillin (netilmicin sulphate) (NET/30 mcg), norfloxacin (NX/10 mcg), ciprofloxacin (CIP/5 mcg), gentamicin (GEN/10 mcg), ceftriaxone (CTR/10 mcg), cefotaxime (cephotaxime) (CTX/30mcg), amikacin (AK/30mcg) and furazolidone (FR/50mcg). Mahmud *et al*., 2023 [46] determined that wastewater from hospitals, pharmaceutical factories, and agricultural runoff contaminates surface and groundwater by introducing various antibiotic-resistant bacteria (MARB) and antimicrobial medicines. Abu-Sini *et al*., 2023 [47] investigated similar findings that the results of antimicrobial susceptibility testing showed that all *E. coli* isolates (13 of 13; 100%) were sensitive to gentamycin, ciprofloxacin, and levofloxacin. The sensitivity of the isolates to ceftazidime, doxycycline, tetracycline, azithromycin, and amoxicillin/clavulanic acidwas 92%, 61%, 46%, 23% and 15%, respectively. Out of 13 *E. coli* isolates, 10 were found to (77%), 6 (46%) and 1 (8%) exhibited resistance to azithromycin, amoxicillin/clavulanic acid, and ceftazidime, respectively. Nath *et al*., 2019 [48] shown that environmental pollution not only initiate heavy-metal co-selection processes, but it also elevates the tolerance level to certain antibiotics due to the co-regulation of resistance genes. Rima *et al*., 2022 [26] reveals that isolate A and isolate B exhibit varying resistance patterns. Isolate A is immune to more antibiotics than Isolate B but remains susceptible to others. Knowing which antibiotics each isolate is resistant to or susceptible to is essential for selecting effective treatments, particularly for infections caused by resistant bacteria. Alam *et al*., 2014 [49] reported that the resistance percentages reported for Cephradin (57.69%) and Rifampicin (34.61%) also suggest that *E. coli* isolates show resistance to various antibiotics. The occurrence of resistance to various drug classes could limit the options available for treatment and potentially lead to more severe or persistent infections

SEM (Scanning Electron Microscopy Observation):

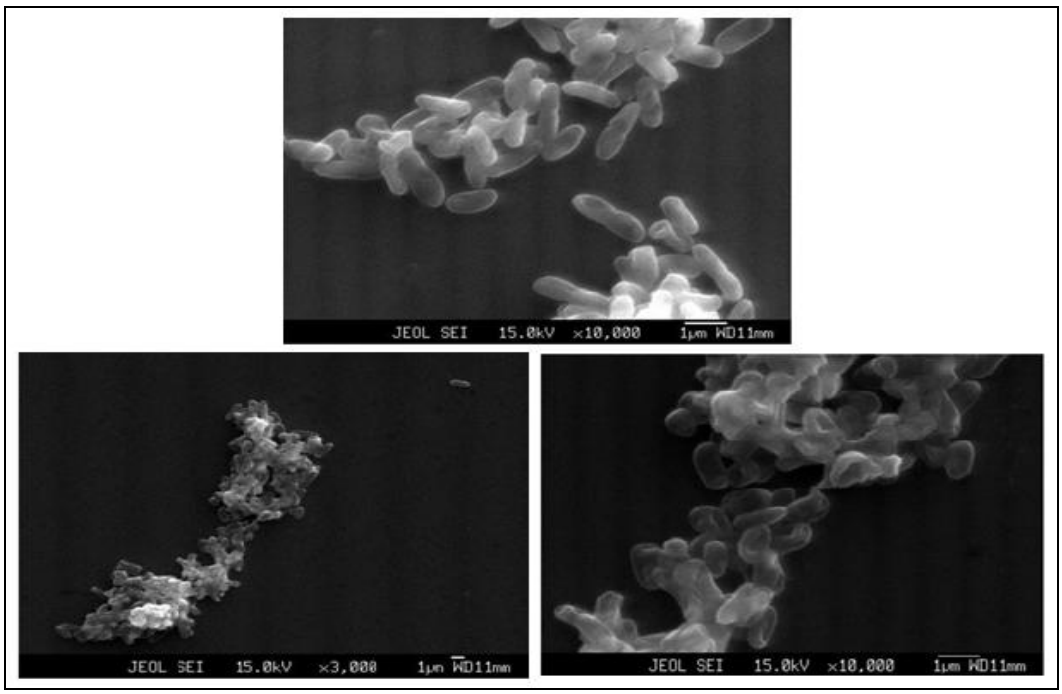

FIG. 10: SEM IMAGE OF METHYL VIOLET DYE ONTO *ENTEROBACTER*

The morphological changes in biomass before and after dye biosorption were examined through SEM analysis. The micrograph of the bacterial biomass indicated a transformation in thesurface,

characterized by pores of different shapes and sizes, resulting in a clumped morphology that offers an increased surface area for dye biosorption. The alterations observed on the biomass surface after the biosorption of MV dye revealed aggregation in various forms and sizes. This phenomenon may result from dye molecules forming a monolayer on the biosorbent's surface. This pattern of aggregation could elucidate the strong affinity of bacterial biomass for the MV dye, underscoring the effectiveness of the biosorption process. Gupta *et al*., 2023 [50] indicated that the SEM micrographs show the generation of spheres as well as the preservation of some irregular pattern, which suggests the consumption of all the dye during polymerization. However, further increasing the dye concentration level results in a greater number of spherical particles caused by the adequate dye available for the polymerization and thus converting all irregular PEDOT: peroxodisulfate to dye-induced spheres. Anbarani *et al*., 2023 [51] Showed that the FESEM image of *S. cerevisiae* before and after crystal violet removal. FESEM analysis illustrates that *S. cerevisiae* surface is inhomogeneous and rough. After the dye is removed, the surface of the yeast appears relatively smooth. This suggests that the yeast surface has become fully saturated with crystal violet dye molecules.

FTIR (Fourier Transform Infrared Spectroscopy):

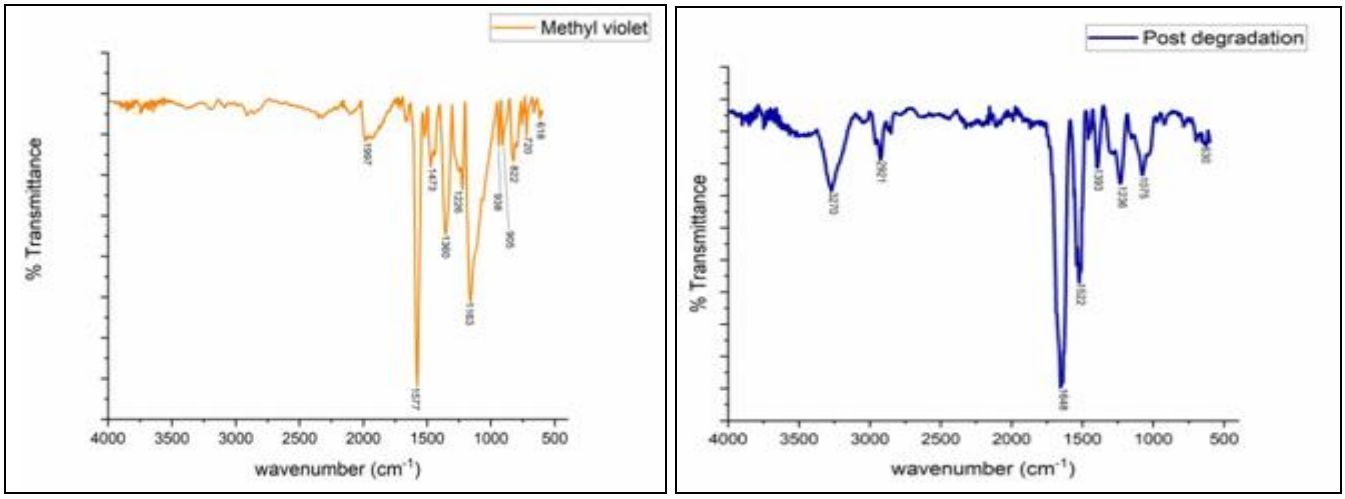

FIG. 11: FTIR IMAGE BEFORE AND AFTER METHYL VIOLET DYE ONTO *ENTEROBACTER*

For FTIR analysis, the first metabolite extraction was done from the degradation product. Centrifuge the degradation product at 5000 rpm and collect the supernatant. Place the supernatant in a hot air oven at 65°C to concentrate on the solution. Mix the concentrated supernatant with ethyl acetate in a 1:1 ratio and stir the mixture on a magnetic stirrer for 24 hours. Then, add sodium sulfate to the mixture until it becomes concentrated, followed by incubation in a hot air oven for further analysis. The FTIR spectra of the control MV as well as the sample after 24 hours of dye degradation showed a significant variation in the fingerprint region (4000-400 cm^{-1}), indicating that *Enterobacter* sp. SSAU6 facilitated the degradation of MV. In the reference MV spectrum, specific peaks within the fingerprint region correspond to monosubstituted and para-disubstituted benzene rings, with a maximum at 1, 577 cm^{-1} representing C = Cstretching in the benzenering. Additionally, a peak at 1, 226 cm^{-1} is linked to aromatic C-N stretching vibrations. The characteristic peak at 1,997 cm^{-1} indicates a free NH_2 group linked to amine antisymmetric stretching. Peaks at 1,473 cm^{-1} and 1,360 cm^{-1} reflect the C=C and OH bending sequentially, while the C-O stretch appears at 1,163 cm^{-1}, and peaks at 938 and 905 cm^{-1} represent symmetric bending of benzene ring hydrogen. In contrast, the FTIR analysis of the decolorized MV products showed peaks at 3,270 and 2,921 cm^{-1}, attributed to hydroxyl group stretching and C-H a symmetric stretching, respectively. The prominent absorption bands at 1,648 and 1,522 cm^{-1} correspond to N-H bending vibrations, while peaks at 1,393, 1,236, and 1,075 cm^{-1} are linked to sideways ring stretching, $-CH_2$ scissoring, and $=CH_2$ out-of-plane

twisting. Absorption bands below 900 cm^{-1} indicate the aromatic characteristics of the decolorized products. Gao *et al.*, 2020 [44] shown that the analysis of the decolorization mechanism indicates that the decolorization was primarily facilitated by the isolate SWUSI4, which involves both the absorption by biomass and/or the degradation by enzymes. The biosorption of dyes was attributed to the binding of dye molecules to hydroxyl, amino, phosphoryl, alkane, and ester–lipid groups, as determined by Fouriertrans form infrared (FTIR) analyses. Al-Zaban *et al*., 2022 [40] concluded that there is a shift in certain active groups, including hydroxyl, amide, carboxyl, and carbonyl, which may result from electrostatic forces and the formation of hydrogen bonds between the dye molecules and the surface of algal biomass. These functional groups could facilitate the biosorption of dye molecules onto the surface of *C. tamariscifolia*.

Kinetics Studies:

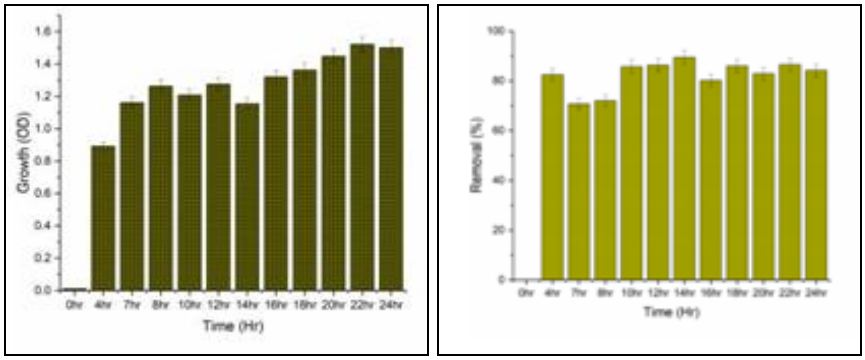

FIG. 12: GROWTH AND PERCENTAGE DEGRADATION WITH VARYING TIME

This analysis was done to know about the order of reaction, result revealed from above figure is noted at 4 hr growth is 0.892 and efficiency of removal is 82.47%, at 7 hr growth is 1.161 and efficiency of removal is 70.70%, at 8 hr growth is 1.264 and efficiency of removal was 72.04%, at 10 hr growth is 1.208 and efficiency of removal was 85.71%, at12 hr growth is 1.275 and efficiency of removal was 86.32%, at 14 hr growth is 1.154 and efficiency of removal was 89.45%, at 16 hr growth is 1.32 and efficiency of removal was 80.13%, at 18 hr growth is 1.363 and efficiency of removal was 85.99%, at 20 hr growth is 1.448 and efficiency of removal was 82.86%, at 22 hr growth is 1.521 and efficiency of removal was 86.49% and at 24 hr growth is 1.501 and efficiency of removal was 84.20%.

From the above experiment, it was concluded that higher growth is noted at 22 hr and 24 hr, whereas lower growth was noted at 4 hr. simultaneously, higher efficiency of removal was noted at 14 hr. Amin *et al*., 2015 [42] showed that the pseudo-first order and pseudo-second order reaction models were applied to investigate the adsorption kinetics of RB5 dye onto bentonite clayand also indicated that chemisorption refers to the rate-limiting step. Dahri *et al*., 2013 [41] demonstrated that chemical interactions between the functional groups on the adsorbent surface and the adsorbates are frequently part of the adsorption mechanism. Other mechanisms, including mass transport processes, bulk transport, diffusion through the liquid film surrounding the surface, and diffusion into micropores and macropores, also play a role. The availability of the adsorbent's surface, along with its physicochemical properties, surface area, physical size, and the form of the adsorbent particles, are crucial characteristics that influence both equilibrium capacity and the rate of adsorption.

CONCLUSION: Dye pollution represents a significant environmental challenge, negatively affecting ecosystem health. In the context of global sustainable development, it is crucial to adopt a waste-free strategy that is both efficient and cost-effective. There is an urgent need to develop remediation strategies that work in partnership with industries liable for dye discharges. Biological methods can play a vital role when combined with advanced methods, creating a comprehensive, integrated, and economically viable approach to improve dye remediation in wastewater.

An environmentally friendly, simple, and cost-effective strategy is essential for effectively addressing dye pollution. The use of various microorganisms, nanotechnology, microbial fuel cells, and microbial biosorbents in biological approaches offers great potential for achieving cleaner and greener environments through efficient dye remediation.

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