Isolation and Screening of Dye Decolorizing Bacteria from Marine Environment

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DECLARATION BY THE STUDENT

I hereby declare that the data presented in this Dissertation report entitled, **"Isolation and Screening of Dye Decolorizing Bacteria from Marine Environment"** is based on the results of investigations carried out by me in the Marine Microbiology at the School of Earth, Ocean and Atmospheric Sciences, Goa University under the supervision of Dr. Diviya Vaigankar and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will be not be responsible for the correctness of observations or experimental or other findings given the dissertation.

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This is to certify that the dissertation report **"Isolation and Screening of Dye Decolorizing Bacteria from Marine Environment"** is a bonafide work carried out by **Mr. Premjit Bhalchandra Fadte Gaonkar** under my supervision in partial fulfilment of the requirements for the award of the degree of **Masters of Science** in the Discipline Marine Microbiology at the School of Earth, Ocean and Atmospheric Sciences, Goa University.

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PREFACE

A wealth of diverse microorganisms can be found across the wide maritime environment, which presents countless opportunities for biotechnological applications. These microbes include bacteria that can break down dyes, which are substances that are well-known for being hazardous and persistent in the environment. To counteract dye pollution in marine environments, one of the most important steps in utilizing nature's curative capacity is the isolation and screening of such dye-decolorizing bacteria. The objective of current the study is to identify new strains of bacteria that possess the capacity to metabolize and detoxify a synthetic dye by isolating them from marine sediments, seawater, and other biological niches. By employing stringent screening procedures, such as dye decolorization assessments to detect bacterial isolates possessing strong dye-decolorizing abilities and clarify the fundamental processes associated with dye biotransformation. This lays the groundwork for an exploration into the intricate world of marine microbiology, where the vast array of microbial life intersects with the quest for environmentally sustainable solutions.

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Premjit Fadte Gaonkar

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Lists of abbreviations

%	Percent
µg	Microgram
µl	Milliliter
Conc	Concentration
DB	Direct Blue
DNA	Deoxyribonucleic acid
e.g	Exempli gratia
et al	And others
etc	Etceteras
FAD	Flavin adenine dinucleotide
FADPH	Flavin adenine dinucleotide phosphate
Fig	Figure
FMN	Flavin mononucleotide
gm	Gram
GMO	Genetically Modified Organisms
hrs	Hours
l	Liter
LiP	Lignin Peroxidase
mg	Milligram
MnP	Manganese Peroxidase
NADP	Nicotinamide adenine dinucleotide phosphate
^{o}C	Degree centigrade

OD	Optical density
pH	Negative logarithm of hydrogen ion concentration
Rpm	Rotation per minute
RR	Reactive Red
RY	Reactive Yellow
SEM	Scanning Electron Microscopy
TDC	The Dyers Council
TLC	Thin Layer Chromatography
UV	Ultraviolet
ZMA	Zobell Marine Agar
ZMB	Zobell Marine Broth

ABSTRACT

The present study investigated decolourization of Congo Red dye using a bacteria isolated from different marine habitat of Goa, India on a laboratory scale study. A total of 15 bacterial isolates were isolated from marine water and sediment samples collected by enrichment technique. These isolates were screened for their dye decolorization potential in Zobell Marine Agar medium supplemented with 0.02% Congo Red dye. Out of all, five isolates viz. DW4, PMSS1, PMCH2, RMS and RMW were selected for further investigation. Based on maximum dye decolorization in a solid and liquid medium where strain RMS showed the highest decolorization (52%) after 72 h of incubation was selected for further studies. Biological and biochemical assays, DNA extraction, and SEM (Scanning Electron Microscopy) analysis were carried out for identification of the strain. To determine its ability to biodecolorize Congo Red dye, physicochemical parameters, including different dye concentrations, pH, temperature, NaCl concentration, and growth behaviour of strain RMS were tested. The highest decolorization occurred at pH 8.0, reaching 55% after 72 h of incubation. The highest decolorization of dye (56%) occurred at 0.02% of dye, after 72 h of incubation. The optimum incubation temperature was found to be 28 °C, which reported 55% decolourization after 72 h of incubation. Whereas, maximum decolourization occurred at 2% NaCl concentration, reaching 57% after 72 h of incubation. It was observed that with increasing time increase in the decolorizing was observed (59%) after 72 h of incubation carried out at optimum conditions. The decolourized dye effluent was checked on TLC where R*f* value of the decolorized Congo Red and untreated dye was found to be different. Immobilization of cells was carried out using 2% sodium alginate and 4% calcium chloride which showed 64% decolorization of dye in 1st cycle followed by 57% by 2nd cycle carried out in a laboratory batch reactor. Lastly, the decolourized dye effluent was checked for phytotoxicity on moong seeds. When compared to the control, seeds exposed to decolourized dye effluent exhibited germination, shoot and root development which was not seen for untreated dye effluent. Therefore, the bacterial strain RMS could be an ideal candidate to be used for the treatment of dye effluent with further improvisations.

Keywords: Bacterial dye decolorization, Congo Red, Marine Environment, Immobilization, Phytotoxicity.

CHAPTER 1:
INTRODUCTION

1.1 BACKGROUND

Dyes are an important source for a variety of industries, including textile, leather, paint, food, cosmetics, and paper. There are about 25 types of dye groups based on their chromophore chemical structures. Dyes contain at least one nitrogen-nitrogen double bond (N=N), but a variety of structures exist. For instance, the azo dyes, diazo and triazodyes contain two and three N=N double bonds, respectively, but monoazo dyes only have one (Sudha et al., 2014; Benkhaya et al., 2020). Over a thousand dyes are classified as textile dyes, which are used to dye a variety of fabrics (Sponza, 2006; Abe et al., 2019). Intermediate dyes are precursors to dyes. It can be produced through various chemical reactions from raw materials such as naphthalene and benzene (Gregory, 2000; Guo et al., 2018).

1.1.1 CLASSIFICATION OF DYES

The Dyers' Council (TDC) and the American Textile Colorers Association have classified dyes according to color, structure, and application method, and have been revised every three months since 1924. Each dye is given a common name based on its color, purpose, and properties. These dyes have different absorption spectra, which are associated with electronic transitions between molecular orbitals. Dyes are classified into two types, natural dyes and synthetic dyes, depending on their origin.

1.1.1.a Natural Dye

The dyes obtained from natural sources are called natural dyes. They are mostly applied to textiles using mordants. Based on the chemical constitution, a natural dye can further be classified as indigoid dyes, alpha-hydroxynaphthoquinones, flavones, dihydropyrans, anthocyanidins, and carotenoids. Indigoid dye’s most common indigo and Tyrian purple are some of its examples (Serrano- Andrés et al., 1997). They are obtained from the leaves of a wood plant as it possesses indigo as the key component in dyeing.

Anthocyanidins, naturally occurring compounds, serve as an orange dye for cotton, and they are extracted from the leaves of *Bignonia chica*.

1.1.1.b Synthetic Dyes

Synthetic dyes are man-made petrochemicals that may consist of lead, mercury, copper, sodium chloride, toluene, chromium, and benzene. Synthetic dyes have a brighter and wider range of colors, longer color permanence, and are easier, faster, and cheaper to produce in comparison with natural dyes. There are over 10,000 different types of synthetic dyes. Synthetic dyes are categorized based on their chemical composition and the method of their application in the dyeing process. Although natural dyes are important from the chemical and historical point of view, these dyes are very expensive, need purification, and do not bind well because they lack the chemical grouping required to react with the binding sites of a fabric. Therefore, for dyeing applications, synthetic colors are utilized in place of natural dyes. Synthetic dyes are classified into three categories anionic, non-ionic acid, and azo dyes. Anionic synthetic dyes include acid and whereas non-ionic dyes are basic, dyes are also known as cationic. On the other hand, dyes can be classified based on their chemical structure into anthraquinone dyes and azo dyes (Dos Santos et al.,2007).

Azo dyes are characterized by being strong, having good all-around properties, and being less expensive. This dye has at least one azo bond (-N=N-), along with one or more aromatic structures. Azo dyes are intended to transport resistance and high photolytic stability toward major oxidizing agents. They have a wide variety of applications in the textile, food-making, and cosmetic industries (Fleischmann et al., 2015). Moreover, they have amphoteric properties due to the presence of additional carboxyl, hydroxyl, amino or sulfoxyl functional groups. Azo dyes can behave anionic (deprotonation at the acidic group), cationic (protonated at the amino group) or non-ionic depending upon the pH of

the medium. Most notable azo dyes are acid dyes, basic dyes (cationic dyes), direct dyes (substantive dyes), disperse dyes (non-ionic dyes), reactive dyes, vat dyes and sulfur dyes.

Approximately from all color additives, 50% azo dyes are extensively used as coloring substances in cosmetic, drug and food industries. This increases concerns related to health and safety. Global usage of azo dye as a food additive is being regulated (Jiang et al., 2020). Azo dye toxicity is based on benzidine and its counterpart like dimethoxy- and dimethyl benzidine. It may show mutagenic effects on monkeys, humans, dogs, and rodents which lead to diseases like cancer (Suryavathi et al., 2005; Bencheqroun et al., 2019). Dye industrial activity negatively affects human health and environmental conditions through large amounts of waste discharged into open water sources (Chung, 2016; Bencheqroun et al., 2019). The use of azo dye shows undesirable effects on soil microbial populations and affects plant growth and germination (Lellis et al., 2019). The presence of a very small amount of dye in water (<1 ppm) is highly visible, affecting the aesthetic merit, water transparency and gas solubility in lakes, rivers and other water bodies (Couto et al.,2009).

The effluents from these industries are complex, containing a wide variety of dye products such as dispersants, acids, bases, salts, detergents and oxidants. Discharge of these colored effluents into the rivers and lakes reduces dissolved oxygen concentration, thus creating anoxic conditions that are lethal to resident organisms (Wang et al.,1991). A considerable amount of wastewater is generated having strong color, a large number of suspended solids, a highly fluctuating pH, salts, heavy metals, sulphides, chlorine, temperature and COD concentration (Gurnham (1965). The disposal of untreated textile wastewater is a serious threat to the environment. It accounts for 15-20% of total wastewater in the country (Gopal B, 1994). The strong color of discharged dyes even at very small concentrations has a huge impact on the aquatic environment caused by its turbidity and high pollution strength. Additional toxic decolorization products can be

formed (Pourbabaee et al.,2006). The complexity of the dye structure (crystal ponceau 6R (502.4 g/mol molecular weight), reactive green 19, remazol red (560.5 g/mol molecular weight), Direct Blue 71 (1029.87 g/mol molecular weight) makes its decolorization difficult (Ajaz et al., 2020).

There are various methods namely physical, chemical and biological treatment (individually or in combination) have been reported to be widely used for decolorizing dyes (Lua et al., 2019; Lan et al., 2019).

1.1.2 PHYSIO-CHEMICAL DECOLORIZATION

Physio-chemical methods include, flocculation, wet oxidation, membrane separations, adsorption and precipitation are examples of physico-chemical treatment (Wang et al.,2020; Kumar et al., 2020). However, this method suffers from several drawbacks, including high demand for chemicals, elevated maintenance needs, significant costs, and the generation of substantial sludge, necessitating safe disposal measures (Ajaz et al., 2020).

1.1.3 BIOLOGICAL DYE DECOLORIZATION

Biological decolorization of pollutants is eco-friendly and shows complete mineralization of organic compounds with low sludge generation. This method has been reported as the most effective (Varjani et al., 2019; Kumar et al., 2020). Biological decolorization can be conducted under aerobic or anaerobic conditions (Khan et al., 2012; Bhatia et al., 2017). Various microorganisms such as bacteria, fungi, yeast and algae were used for dye decolorization and decolourization (Ali, 2010; Ajaz et al., 2020). Utilizing bacteria for dye decolorization offers a natural, efficient, and cost-effective solution to tackle textile dye pollution. Bacteria possess specialized enzymatic pathways that efficiently break down complex dye molecules, making them versatile for deployment in various industrial settings and contaminated environments. Moreover, microbial

decolorization is environmentally friendly, resulting in harmless byproducts and minimizing the risk of secondary pollution. Thus, exploring bacteria for dye decolorization holds promise for sustainable environmental remediation efforts.

The marine environment, encompassing oceans and coastal ecosystems, hosts a diverse array of microbes vital for nutrient cycling, carbon sequestration, and ecosystem balance. These resilient microorganisms adapt to extreme conditions and play crucial roles in maintaining marine health. They offer promising solutions for bioremediation and biotechnology.

Dyes enter the marine environment primarily through industrial discharge, urban runoff, and maritime activities. Industrial wastewater from textile manufacturing often contains dyes, which are released directly into water bodies. Urban runoff carries dyes from laundered clothes into rivers and coastal areas, while maritime operations contribute through antifouling paints. Once in the marine environment, dyes can persist, posing threats to marine life by disrupting physiological processes and ecosystems by altering light absorption properties. Marine bacteria possess specialized often referred to as dye-decolorizing enzymes or oxidoreductases, target the chemical bonds within dye molecules, breaking them down into simpler, less harmful compounds (Abadulla et al., 2000) . Additionally, marine bacteria have evolved metabolic pathways that can utilize dye molecules as a source of carbon and energy for growth and survival. This metabolic versatility allows marine bacteria to thrive in environments contaminated with dyes and actively participate in the decolorization process. Furthermore, the diverse and unique microbial communities found in marine ecosystems provide a rich source of genetic diversity, potentially harbouring novel enzymes with enhanced dye-decolorizing capabilities.

1.2 AIM AND OBJECTIVES

AIM: To isolate and screen Congo red decolorizing bacteria from marine environments.

OBJECTIVES:

1) Isolation of Congo red decolorizing bacteria from different marine habitats of Goa.

2) Characterization and optimization of growth conditions for effective dye decolorization.

3) Investigating the efficacy of immobilized cells for Congo Red dye decolorization in a laboratory-scale batch reactor system.

4) To evaluate the toxicity of bacterial-decolorized Congo Red dye effluent on seed germination.

1.3 HYPOTHESIS

Marine bacteria possess specialized enzymatic pathways or metabolic capabilities that enable the efficient decolorization of synthetic dyes. We aim to demonstrate an enhanced dye-decolorizing strategy by marine bacteria using a laboratory-scale batch reactor. Thus, suggests that marine bacteria can serve as effective agents for environmentally sustainable remediation of dye pollution.

1.4 SCOPE

The current study encompasses the isolation and characterization of Congo Red dye-decolorizing bacteria from diverse marine habitats in Goa, along with the optimization of growth conditions to enhance their efficacy in dye decolorization. Furthermore, the research investigates the feasibility of employing immobilized cells in a laboratory-scale batch reactor system for efficient dye remediation. Additionally, it examines the potential environmental impact of bacterial-treated dye effluent on plant seed germination, shedding light on the broader implications of employing bacterial decolorization for textile dye wastewater treatment.

CHAPTER 2
LITERATURE REVIEW

2.1 Microorganisms in dye decolorization

Various microorganisms such as bacteria, fungi, yeast and algae have been reported for dye decolorization and decolorization as shown in table 2.1. For the decolorization of various dyes different microbes can be used, they have different mechanisms and pathways for degradation of dyes (Cao et al., 2019; Ebrahimi et al., 2019). Azo dyes are a useful class of dyes with the highest diversity of colors. Under anaerobic conditions and with the help of azoreductase, microorganisms decolorize azo dyes and as an end product, they form colorless aromatic amines (Ali, 2010; Ajaz et al., 2020; Dong et al., 2019). Fungi and algae have been successfully to decolorize dyes. Some microorganisms are known to decolorize dyes (Table 2.1).

The microbial decolorization of textile dyes is more effective under anaerobic conditions. However, toxic aromatic amines are formed at the end of the anaerobic process, which can only be decolorized by microbes under aerobic conditions. In this context, it has been suggested to combine the anaerobic cleavage of azo dyes with an aerobic treatment system for decolorization of amines formed. In other words, a sequenced anaerobic/aerobic biological treatment of textile dye effluents by microbial consortia is suggested (Banat et al., 1996; Elisangela et al., 2009).

In 2004, Senan and Abraham, developed aerobic bacterial consortium consisting of two isolated strains BF1, BF2 of *Pseudomonas* and *Pseudomonas putida* MTCC1194 for aerobic degradation of a mixture of azo dyes. The analysis of degradation products showed that the dye was converted to low molecular weight compounds (Senan and Abraham, 2004).

In their study, Khan et al. (2015) isolated five microbial strains from textile effluents and tested their efficacy in decolorizing common dyes and showed up to 50mg/l of dye decolotization at 37°C and pH 7 ± 0.2, indicating a promising potential for color removal. In Pokharia et al.'s (2013) study, indigenous microbial strains proved adept at decolorizing dye effluents. Physico-chemical analysis of the effluent highlighted significant pollution levels. Six bacterial species, such as *Bacillus*, *Klebsiella*, *Planococcus*, and *Micrococcus*, were isolated. Optimal conditions for the two most effective species, *Planococcus* and *Bacillus*, were determined through carbon and nitrogen source optimization, pH adjustments, temperature variations, and inoculum percentages. These species exhibited the highest decolorization efficiency, notably with Coractive Blue 3R dye, during the optimization process. Yet another study using the marine bacterium *Pseudomonas* sp. ESPS40 from the Arabian Sea, India, showed a higher ability for MG degradation (86-88%) at varying NaCl concentrations, with the most increased degradation (~88%) observed at 1% NaCl (Kumar et. al., (2023).

Table 2.1: Dye decolorizing microorganisms

Type of microorganisms	Strains	Name of the dye	% of decolorization	Time of decolorization (hrs)	Ph	References
Bacteria						
	Bascillus spp.	Reactive red 198	98	36	7	Gaudie et al. (2017)
	Alcaligens spp.		90	24		Pandey et al., 2016
	Brevibacterium spp.		97	120		Franciscon et al., 2012
	Aspergillus flavus		84.96	24		Esmaeilli and kalantarikalantari, 2012

	Brevibacillus laterosporus		92	18		Kurafe et al.,2015
	Aerophilus Hydrophila	Malachite Green	80	48	7	Mahmood et al., 2016
	Bascillus sp	Methyl red			11.5	Maulin et Al., 2013
	Proteobacteria phya	Congo red				Dai et al., 2020
	Oudemansiella canarii		80	60		Iark et al., 2019
	Aliiglaciecola lipolytica	Congo red	89	34	7	Wang et Al., 2020
Fungi						
	Cunninghamella elegans	Malachite green				Cha and Doerge, 2001
	Geotrichum sp	Reactive black 5				Kuhad et al., 2004
	Shewanella sp.NTOV	Crystal voilet				Chen et al., 2008
	Aspergillus ochraceus	Reactive blue 25				Parshetti et al., 2007
Algae						
	Spirogyra rhizopus	Acid red				Ozer et al., 2006
	Cosmarium sp	Malachite green				Dhaneshver et al., 2007

Yeast						
	Saccharomyces cerevisiae MTCC463	Methyl red			9	Jadhav and Govindwar, 2007

2. 2 MECHANISM OF BIODECOLORIZATION

Enzymes are the ultimate molecules which deal with the dye compounds and bring about cleavage and successive decolorization. The initial step in decolorizing the azo dye is to cleave the electrophilic azo linkage, which immediately causes decolorization. Azoreductase brings about the cleavage of azo linkages in compounds containing azo bonds to produce aromatic amines. Many bacterial strains have been found to contain unspecific cytoplasmic enzymes that act as azoreductase.

The mammals can synthesize azoreductase (called hepatic azoreductase) in their body. In mammals the hepatic azoreductase and the bacterial azoreductase can breakdown the azo dyes to their corresponding amines. However, bacterial azoreductase is more active than hepatic azoreductase which reduces the azo dyes to mutagenic and carcinogenic amines (Puvaneswari et al., 2006). Azo reductases are of two types—membrane-bound and cytoplasmic reductases which greatly depend upon the reducing equivalents such as NADPH, NADH and FADH, to catalyze azo dye degradation reaction (Saratale et al.,2011).

The phenoloxidases namely lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase has a great potential to decolorize many aromatic compounds. These enzymes are involved in the decolorization of lignin, which are complex polyaromatic polymer. The enzymatic treatment methods positively affect the environment as they pose a low chance of biological contamination. Enzymes of both bacterial as well as fungal origin such as

lignin peroxidase, laccase, and manganese peroxidase can metabolize xenobiotic compounds (Xu et al.,2013). Enzymes like horseradish peroxidase have been immobilized and utilized for the treatment of effluents in textile mills and paper industries (Peralta-Zamora et al.,1998). Laccases (benzenediol: oxidoreductase; EC 1.10.3.2) belong to the class oxidoreductase- a multicopper oxidase family, which can oxidise phenols, polyphenols and aniline by one-electron abstraction. The first prokaryotic laccase has been reported by *Azospirillum lipoferum* (Kalme et al.,2009). Laccase basically catalyzes azo dyes decolorization by non-specific free radicals without the mutagenetic and toxic aromatic amines formation. Laccase belongs to the multicopper oxidases group which has low substrate specificity and is highly capable of degrading the spectrum of xenobiotic compounds and aromatic as well as non-aromatic substrates (Parmanik and Chaudhuri, 2018). These catalysts have good bioremediation potential at the same time*Pseudomonas syringae* and *Pedomicrobium* spp. have shown laccase-like activity (Telke et al.,2011). Reports are available for purified laccase enzyme extracted from *Bacillus* spp. and *Pseudomonas desmolyticum* that can decolorize several textile dyes efficiently (Kalme et al.,2009; Telke et al.,2011). The fungal strain *Podoscypha eleganscan* was reported to decolorize five azo dyes (Congo Red, Direct Blue 15, Orange G, Rose Bengal, and Direct Yellow) efficiently (Parmanik and Chaudhuri, 2018). Similarly, fungal strains of *Aspergillus oryzae, Trametes versicolor,* and *Paraconiothyrium* variable also could decolorize azo dyes through the laccase enzyme production (Forootanfar et al., 2012).

Tannase (Tannin acylhydrolyase, EC 3.1.1.20) is a ligninolytic enzyme that acts on carboxylic ester bonds. It catalysis the hydrolysis of central ester bonds between two aromatic rings of digallate. It is a key enzyme in the decolorization of gallotannins which is hydrolysable tannin. Depending upon the reducing equivalents utilized, azo reductases have been categorized into FMN-dependent, FMN-independent, NADPH-dependent,

NADPH-independent and NADH-DCIP-dependent azo reductases. Other enzymes such as laccase, peroxidase, tyrosinase, NADH-DCIP reductase and MG reductase also complement the process of degradation (Telke et al., 2015). Some bacteria completely mineralizes the decolorized dye metabolites by utilizing them as the sole carbon or nitrogen source, thus capable of making products from glucose (Lima et al.,2014). These membrane-bound enzymes utilize the metabolic products of certain cellular substrates as the redox mediators to act as an electron shuttle (Fig. 2.1). Under aerobic conditions, presence of oxygen leads to the reduction of the redox mediators instead of the azo dye molecules because the oxygen molecules compete with the dye molecules to react with the redox mediators. Conversely, soluble cytoplasmic azoreductases are the potent decolorizers of non-sulfonated azo dyes capable of entering the cell through the membrane, by employing different kinds of degradation mechanisms (Chako and Subramaniam,2011).

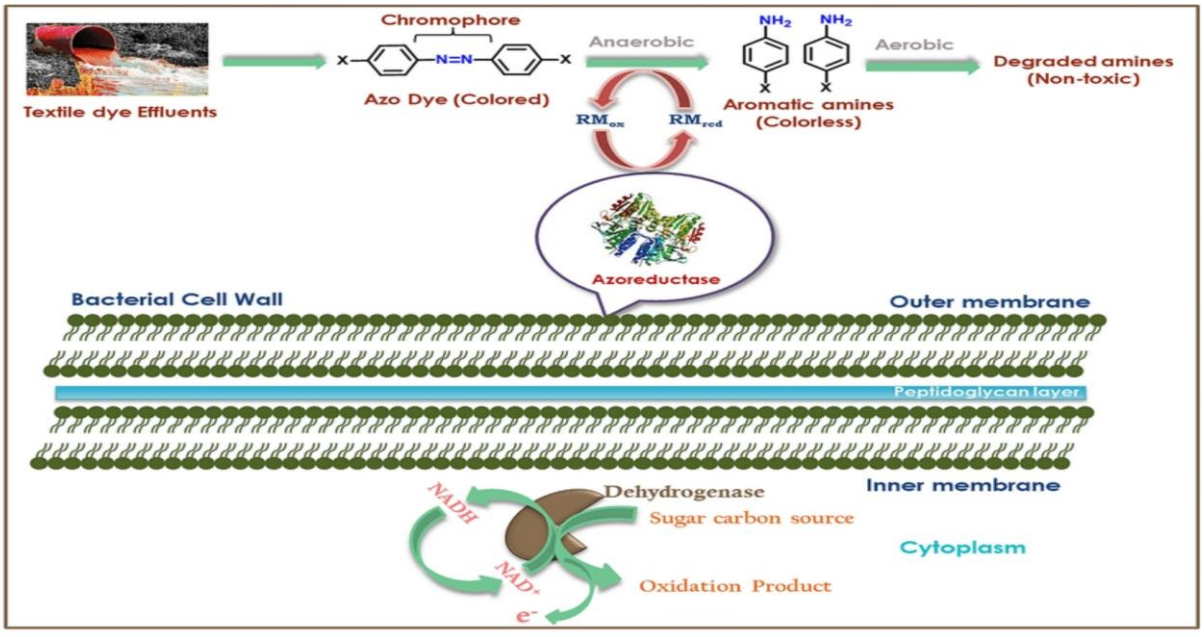

Fig 2.1: Mechanism of azo dye degradation by bacterial azo reductase enzyme.

2.3 FACTORS AFFECTING BIODECOLORIZATION OF DYES

It has been reported that metals, salts and other compounds make the decolorization of dyes more difficult and it is toxic for bacterial growth too (Ghosh et al., 2020). Factors like temperature, pH, dissolved oxygen, nutrients, dissolved organic matter, metals and organic pollutants influence water quality (Al-Amrani et al., 2014). The factors affecting dye decolorization are mainly divided into two categories. i) Environmental factors, ii) Nutritional factors

2.3.1 Environmental factors

2.3.1.a pH

pH is important factor for growth of bacteria and also an essential characteristic for effluent treatment (Varjani and Upasani, 2017b). pH can be acidic, alkaline or neutral based on the type of dyes and salts used. The rate of dye decolorization in dye-containing effluent may change through its pH. The problem can be solved by (a) adjusting pH of effluent to support the growth of dye-decolorizing bacteria or (b) selecting the microbial sp. which can grow at effluent pH (Al-Amrani et al., 2014). Basutkar and Shivannavar (2019), reported maximum dye decolorization at pH range of 8–10 by using *Lysinibacillus boronitolerans* CMGS-2. 98% degradation of malachite green was achieved RuO2–TiO2 and Pt coated Ti mesh electrodes at pH 4.5 (Singh et al., 2016).

2.3.1.b Temperature

Water temperature affects activities prevailing in water such as mineralization, diffusion, and chemical process which increase pH of water (Delpla et al., 2009; Varjani and Upasani, 2019b). Extreme temperatures can kill bacteria/affect the growth if bacteria present in waste water (Al-Amrani et al., 2014; Varjani and Upasani, 2017b). A faster rate of decolorization of dye can be achieved by giving bacterial culture an optimum temperature which is generally reported as 30–40 °C for most bacteria.

Das and Mishra (2017) used a bacterial consortium of *Bacillus pumilus* HKG212 and *Zobellella taiwanensis* AT 1–3 for the decolorization of reactive green 19 and reported the highest decolorization at 32.04 °C. However, few thermophilic bacteria are reported for drcolorization of azo dye at high temperatures. Gursahani and Gupta (2011), reported 75% degradation of effluent at 60 °C by using *Anoxybacillus rupiensis*. It has been reported that the decolorization rate decreases as temperature increases (Imran et al., 2015).

2.3.1.c Oxygen and agitation

Different microorganisms require different conditions such as aerobic condition, anaerobic and semi-anaerobic. Oxygenation can be improved by shaking. It is supposed that reductive enzyme activities can be increased under anaerobic conditions. However, for aerobic dye degradation oxidative enzymes play an important role which requires the presence of oxygen (Khan et al., 2012).

2.3.2 Nutritional factors

2.3.2.a Soluble salts

Wastewater from the dye industry contains high electric conductivity due to the use of high salt concentration in the dying process which can be detected using a conductivity meter. To increase ionic strength and development of dye fixation on fabrics salts like Na_2SO_4, NaCl and $NaNO_3$ are usually added in the dye bath. Hence, with the release of dye pollutants, salts are also released in industrial wastewater. Dyes containing high salt concentration may decrease the biodegradation rate by reducing biological movement (Basutkar and Shivannavar, 2019).

2.3.2.b Carbon and nitrogen supplements

nutrient supplements for quick degradation of pollutants (Varjani and Upasani, 2019a). Organic sources like peptone, yeast extract or a combination of carbohydrates and complex organic sources have been reported to obtain high and quick dye decolotization rates by both pure cultures and mixed cultures. Dye decolorization efficiency can be increased by the addition of glucose. Glucose has been reported as the most effective and easily available carbon source for microbial metabolism of dyes or dye intermediates (Khan et al., 2012).

2.3.2.c Dye concentration and dye structure

structure influences degradation/decolorization of dye. Low dye concentration may not have been identified by enzymes which are secreted from dye-decolorizing bacteria. On the other hand, high dye concentration is toxic to bacteria and also affects the decolorization of dye by blocking enzyme active sites. Likewise, low molecular weight and simple structure containing dyes are easy to decolorize. Whereas, high molecular weight and complex structure-containing dyes have low decolorization rates (Li et al., 2019). Increased dye concentration decreases dye decolorization and/or degradation (Liu et al., 2016).

Chapter 3:

MATHODOLOGY

3.1 Sample collection

Surface water and sediment were collected in sterile polycarbonate bottles and zip-lock bags, respectively, from different marine and estuarine ecosystems in Goa, India. Various physiological measures were recorded, including temperature, pH, latitude, and longitude.

3.2 Enrichment of dye decoloriation marine bacteria

Enrichment of water and sediment samples was carried out by inoculating (1 ml/ 1 gm) of sample in 100 ml sterile Zobell marine broth (ZMB) supplemented with 0.02% Congo red dye in a 250 ml Erlenmeyer flask. These were incubated at room temperature (RT) 28 °C at 120 rpm on an orbital shaker for 48-72 h and observed for growth.

3.3 Isolation dye decolourizing bacterial isolates

The enriched suspension (100 µl) was diluted and spread-plated on Zobell marine agar (ZMA) supplemented with 0.02% dye and incubated at RT (28 °C) for 72 h. After incubation potential dye-decolorizing bacterial colonies were selected, purified by repetitive quadrant streaking on ZMA plates and used for subsequent studies.

3.4 Screening of dye decolourizing bacterial isolates

3.4.1 Dye decolourizing on solid medium

ZMA media containing dye with increasing concentrations of congo red (0.02, 0.04, 0.06, 0.08, 0.2, 0.4, 0.6, and 0.8 %) were prepared. The selected 15 axenic bacterial colonies were spot-inoculated on all plates using sterile toothpicks. The plates were then incubated

at RT at 28 °C. A zone of clearance around the colonies was observed indicating dye decolorization.

3.4.2 Dye decolorization assay using potential isolates in liquid media

ZMB media containing 0.02% dye was prepared in sterile conical flasks. Selected five cultures namely DW4, RMW, RMS, PMSS1, and PMCHW2 were inoculated using sterile nichrome loops and incubated for 24, 48, and 72 h. After incubation, initial scanning of the culture supernatant was carried out using a UV-Vis spectrometer (200-700 nm). After every 24 h, (2 ml) sample was transferred into the microcentrifuge tubes and centrifuged at 4000 rpm for 10 minutes. The supernatant obtained was subjected to scanning using a UV-Vis spectrophotometer (SHIMAZDU UV-1787). Absorbance at 490 nm for congo red was monitored at 24, 48 and 72 h. Subsequently, the % dye decolourization was calculated using the following formula: (kumar et al.,2022).

Percent dye decolorization = (A°- A^) / A° × 100

Where, A°= initial absorbance

A^ = final absorbance

3.5 Growth and maintenance conditions

The selected colonies were sub-cultured on ZMA slants and ZMA plates containing 0.02% dye by streak plate technique. The plates and tubes were incubated at RT (28°C) for 24-48 h to obtain a pure culture of the colony. Then the tubes were kept in the refrigerator at 4 °C for further needs.

3.6 Biological characterisation of selected dye-decolorizing bacteria

Colony characterisation viz. margin, size, shape, type of colony, nature of colony (mucous, rough, smooth, transparent etc), elevation, opacity, consistency along with Gram staining, motility, fermentation of sugars along various other biochemical tests were performed for identification of potential bacteria (Ezhilarasu, 2016).

3.7 Biochemical characterisation

3.7.1 Gram staining

A thin smear of the purified bacterial strain RMS was applied onto a clean, grease-free slide. It was then air-dried and heat-fixed. Subsequently, the slide was flooded with crystal violet for 1 minute and washed with distilled water for 2 seconds. The slide was flooded with iodine mordant for 1 min, followed by washing with running distilled water for 2 seconds. The smear was blotted dry with absorbent paper. Subsequently, it was immersed in 95% ethanol for 30 seconds. The slide was stained with safranin and washed with water. Finally, the slides were allowed to air dry completely before examination under a microscope to detect the presence of Gram-positive and Gram-negative bacteria. Gram-positive cells appeared purple, while Gram-negative cells displayed a pink or red colouration.

3.7.2 Methyl Red (MR) Test

To detect acid production, the isolate was inoculated into sterile MR broth and incubated at 37 °C for 24 h. After the incubation, 2-3 drops of methyl red reagent were added, and

the colour change in the medium was observed. Methyl red turned red in acidic conditions, indicating a positive result and yellow colour indicated a negative one.

3.7.3 Voges-Proskauer (VP) Test

To detect the production of non-acidic end products by the test organisms, the isolate was inoculated into VP broth and then incubated at 37 °C for 24 h. After incubation 2-3 drops of Barrit's reagent were added, and the colour change was observed. A positive result was indicated by pink or red colour, while yellow or brown indicated a negative.

3.7.4 Citrate Utilization Test

To detect the ability of citrate utilization by the test organisms, Simmon Citrate Agar (SCA) slants were prepared. Subsequently, the isolate was streaked on the agar slants and incubated for 18 - 24 h at 37 °C. After the incubation, the colour change in the medium was observed. A transition from green to blue indicated a positive result, signifying that the organism could utilize citrate as a carbon source. Conversely, the absence of any colour change indicated a negative result.

3.7.5 Motility test

To assess the motility of the organisms, motility agar was prepared in test tubes. The selected isolate was stabbed and the tubes were then incubated at 37°C for 24 h. A growth pattern radiating outward from the stab line indicated a positive result for motility. Equally, if the growth was only present along the stab line, it was considered a negative result.

3.7.6 Fermentation of sugars

The isolate was tested for the utilization of five different sugars viz. glucose, maltose, sucrose, and lactose. A loopful of culture suspension was inoculated into 5 ml of sterile

peptone water containing 1% sugar and 1% v/v Andrade's indicator. The tubes were incubated at 37 °C for 24 h with Durham's tubes. Colour change from red to yellow and bubble formation in the tubes were recorded.

3.7.7 Catalase test

A smear of the bacterial isolate was prepared on a glass slide and hydrogen peroxide (2-3 drops) was added to the smear. The appearance of bubbles within 1 minute after the addition of hydrogen peroxide indicated the presence of catalase.

3.7.8 Oxidase test

- Sterile oxidase discs were taken onto a slide using sterile forceps. Subsequently, the culture was spotted on the discs with the help of a sterile loop. The formation of a purple colour indicated the presence of the oxidase.

3.7.9 Hugh-Leifsons test

The RMS culture was inoculated into two tubes of Hugh-Leifson's media. One tube was covered with paraffin oil to create anaerobic conditions. The tubes were then incubated at 37°C for 24 hours. The formation of a yellow color indicated a positive result for breakdown of sugars.

3.7.10 Triple Sugar Iron (TSI) Test

To detect the ability of the test organisms to ferment Glucose, Lactose, Sucrose and H_2S production. The isolate was stabbed on the TSI medium. The inoculated tubes were incubated at 37 °C for 24 h. The formation of red indicated the fermentation of sugars.

3.7.11 Phenylalanine Deaminase Test

To test phenylalanine deamination ability of an organism.

Phenylalanine Deaminase agar was prepared by autoclaving at 121 °C for 15 min. The isolates were stabbed onto the medium and tubes were incubated at 37 °C for 24 h. After incubation few drops of 10% solution of ferric chloride were added. The development of a green colour indicated a positive result, while the absence of a green colour was regarded as negative.

3.8 Molecular characterisation of selected dye-decolorizing bacteria

Bacterial DNA of strain RMS was extracted by using DNeasy Blood and Tissue kit. Briefly, 24 h old grown cells were centrifuged for 10 minutes at 2000 rpm, discarding the supernatant. The pellet obtained was resuspended in 0.2 ml phosphate-buffered saline (PBS), followed by adding 0.02 ml proteinase K. Subsequently, 0.2 ml (Buffer AL) was added to the tube and mixed thoroughly by vortexing. The sample was then incubated at 60 °C for 10 minutes. Next, 0.2 ml ethanol (90-100%) was added and mixed by vortexing. The resulting mixture was pipetted into a DNeasy spin column in a 2 ml collection tube and centrifuged at 4000 rpm for 2 minutes. The flow-through and collection tubes were then discarded. Subsequently, the spin column was placed onto a new 2 ml collection tube, and 0.5 ml Buffer AW1 was added. The tube was centrifuged at 4000 rpm for 2 minutes, and the above step was repeated. Following these steps, the spin column was transferred into a new 2 ml microcentrifuge tube. DNA was eluted by adding 0.2 ml Buffer AE to the center of the spin column membrane and incubating for 1 minute at 37°C. The eluted DNA was then centrifuged for 2 minutes at 4000 rpm. Lastly, the DNA samples were run on an agarose gel electrophoresis and observations were recorded.

3.8.1 Agarose gel electrophoresis

For agarose gel electrophoresis gel was prepared by dissolving agarose powder (0.8%) in a TAE buffer solution (40ml). Simultaneously, ethidium bromide (mg/ml) was prepared by dissolving 0.05 g in 5 ml of distilled water and vortexing. Electrophoresis assembly was cleaned using methanol and a clean gel tray was placed on the levelled surface and the comb was inserted. Following this agarose gel mixture was heated in a microwave until it was completely dissolved. Around 5-6 µl of Ethidium bromide solution was transferred to agarose and mixed carefully. The agarose mixture was poured into the gel casting tray and allowed to solidify completely for 30 minutes. The DNA sample mixed with bromophenol blue and was loaded onto wells and electrophoresed for 20-30 min. Once the electrophoresis was completed, the gel was removed carefully from the tray and visualised using a UV transilluminator.

3.9 Scanning electron microscopy (SEM) analysis of bacterial strain RMS

Isolate RMS was grown in ZMB with (0.02%) and without Congo red and incubated at 28^{o}C temperature for 48 h. After incubation, a smear of bacterial strain grown in broth was made on the coverslip. This was then immersed in a 2% Glutaraldehyde solution overnight at RT (28 °C). Subsequently, the fixed samples were subjected to dehydration with varying concentrations of acetone treatments (20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100%) for 10 min each. The coverslip was then air dried, and subjected to a sputter coater with thin gold film using spi-module sputter and viewed under SEM (ZEISS EVO 18) (Kumar et Al.,2022).

3.10 Optimization of media parameters for efficient dye decolorization

3.10.1 pH

ZMB (25 ml) was prepared in a 50 ml conical flask. The pH of the media in different flasks was adjusted to (6, 7, 8, 9, 10, 11, and 12) using NaOH and HCl with the help of a pH meter (EUTECH-pH700). The media was autoclaved at 121 °C, followed by the addition (0.02%) of congo red. After inoculation, the flasks were incubated at RT (28 °C) on an orbital shaker at 120 rpm. At 0, 24, 48 and 72 h growth scans of the culture supernatant were obtained using a UV-Vis spectrophotometer (200-700 nm) and absorbance at 490 nm was recorded.

3.10.2 Varying concentration of dye

A 25 ml ZMB was prepared in a 50 ml conical flask, and the pH was adjusted to 8. The media was autoclaved at 121 °C. Different concentrations viz. 0.02, 0.04, 0.06, 0.08, 0.2, 0.4, 0.6 and 0.8 % of the dye were added to the media respectively. After inoculation, the flask was incubated at RT (28 °C) on an orbital shaker at 120 rpm. % dye decolorization was calculated following the protocol mentioned above (2.4.1).

3.10.3 Temperature

A 25 ml ZMB media with pH 8 was prepared in a 50 ml conical flask. The media was autoclaved at 121 °C. after which 0.02% dye was added and culture (RMS) was inoculated. The flask was incubated at different temperatures (viz. 28, 32°C and 37°C) on an orbital shaker at 120 rpm. % dye decolorization for 24, 48 and 72 h was calculated.

3.10.4 NaCl

A 25 ml ZMB media with 2, 3, 4 and 5 % NaCl was prepared separately. These were then autoclaved at 121 °C following which 0.02% Congo red dye was added. After inoculation, the flask was incubated at RT (28 °C) on an orbital shaker at 120 rpm. % dye decolorization for 24, 48 and 72 h was calculated.

3.10.5 Growth behaviour of strain RMS

ZMB media (100 ml) was prepared and the pH was adjusted to 8. Then media was autoclaved at 121 °C. After the addition of congo red (0.02%) to the media it was inoculated with strain RMS and % dye decolorization for 24, 48 and 72 h and growth behaviour was studied after every 4 hrs of time intervals.

3.11 Immobilization of strain RMS for congo red dye decolorization

Mid-log phase old culture of strain RMS pre-grown in ZMB was added to 2% sodium alginate solution in equal amounts. The slurry containing Na-alginate and cells was gently mixed and was added dropwise to 100 ml of 4% $CaCl_2$ solution through a clean syringe (Fig.3.1). The 10 cm distance was maintained between the tip of the syringe and the surface of the $CaCl_2$ solution to obtain spherical beads of 1 mm size. Immobilized cells containing beads were left to settle for 30 min at RT (30°C), strained and washed twice with 3 M NaCl before being used for further experiments (Gaonkar et al., 2018).

3.11.1 Use of immobilized cells for treatment using a batch reactor

Immobilized cell beads were added into the ZMB medium with a 0.02% concentration of dye. The flasks were then aerated at 120 rpm, at RT (28°C) for 72 h. After an hour of standing, the beads were separated from the mixture and centrifuged at 4000 rpm for 10 mins and readings were obtained using a UV-Vis spectrophotometer. The beads were used for continuous decolorization of dye for the next cycles (Fig.3.2).

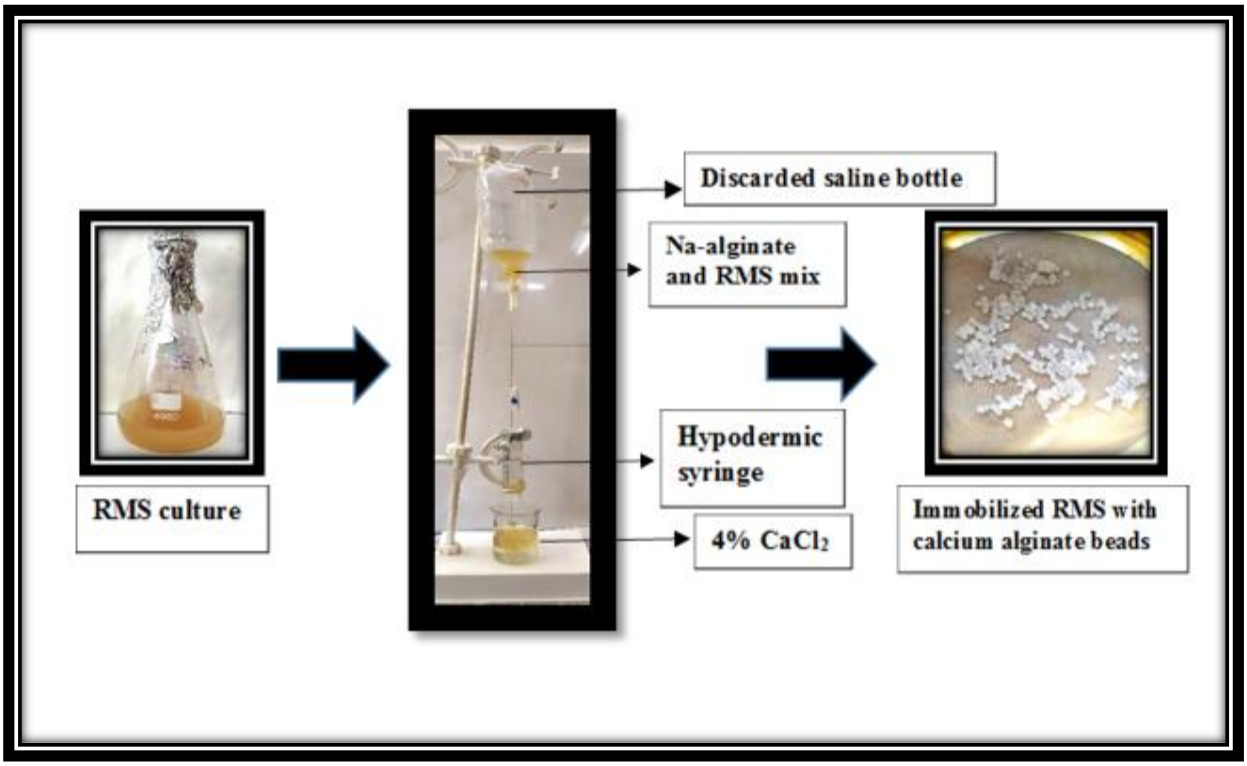

Fig 3.1: Schematic representation for immobilising RMS within sodium alginate beads using a hypodermic syringe clamped stand and discarded saline bottle.

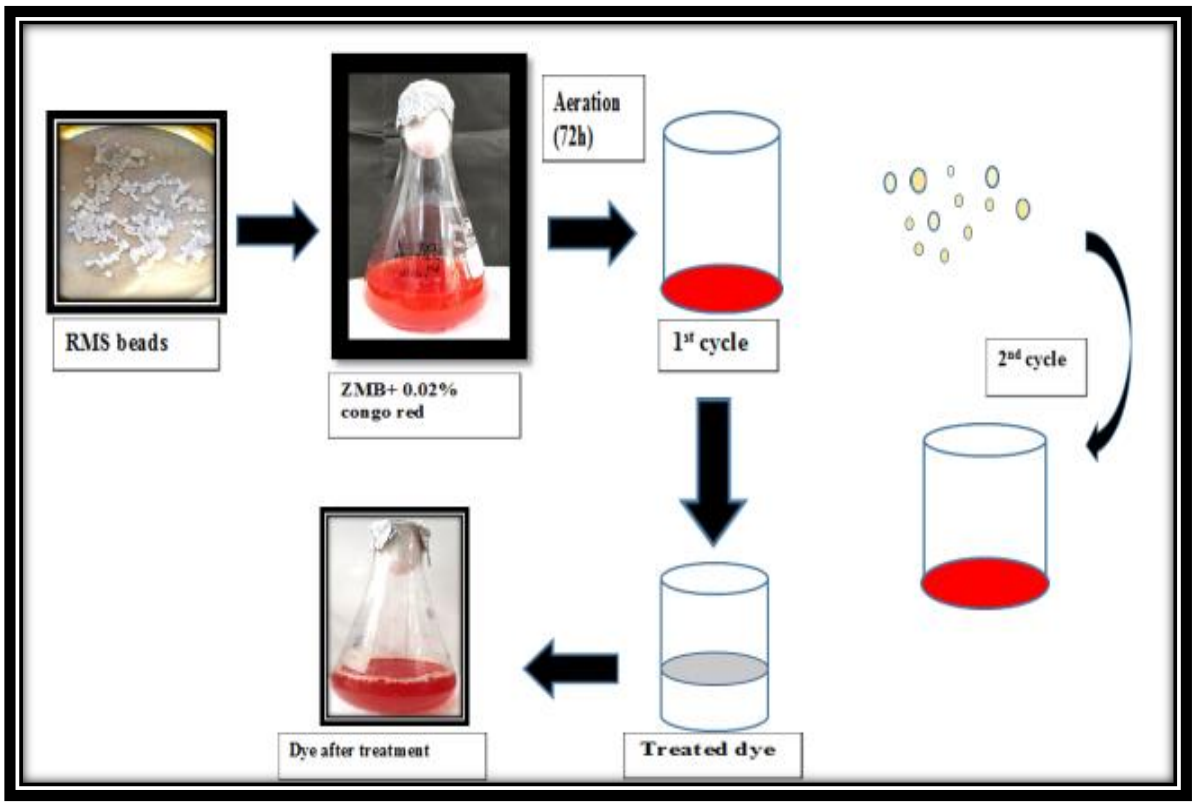

Fig 3.2: Schematic representation of batch model reactor for treatment of dye.

3.12 Characterization of biodecolorized product

3.12.1 Thin layer chromatography (TLC)

The decolorized dye product was extracted by centrifugation and the supernatant was extracted with an equal volume of ethyl acetate and without RMS congo red was used as a control and this was utilized to analyse using TLC. The developing solvent systems used were ethyl acetate: methanol (2:1). The plate was immersed and allowed to run in the solvent system for 30 mins and observed for any visible bands. Additionally, the plate was also exposed to UV light to check for fluorescent decolorized products (Nair et al., 2017).

3.12 Effect of biodecolorized product on plant growth

Mung seeds (Vigna radiate) were surface sterilized by using 0.1% sodium hypochloride. Following each cycle, the supernatant was collected, and seeds were soaked in it overnight. Sterile plates, each containing sterile filter paper, were prepared, and the seeds were placed onto the petri plates. The plates were sprayed with sterile distilled water regularly. An appropriate control group of seeds soaked in untreated dye samples was maintained. Then plates were exposed to every shadow light for 8 h and dark for 16 h daily. After 7 days of incubation the shoot, root length and % of germination was calculated by using formula (Desai, 2017).

Germination % = No. of seeds germinated / Total no. of seeds × 100

CHAPTER 4:

RESULTS AND DISCUSSION

4.1 sample collection

Samples were collected from marine and estuarine environments of Goa, India (with respective codes, Fig.4.1) and were checked for various physiological parameters (Table 4.1). The temperature for all the samples ranged from 27-33 ℃. The pH for all samples ranged from 7-7.9.

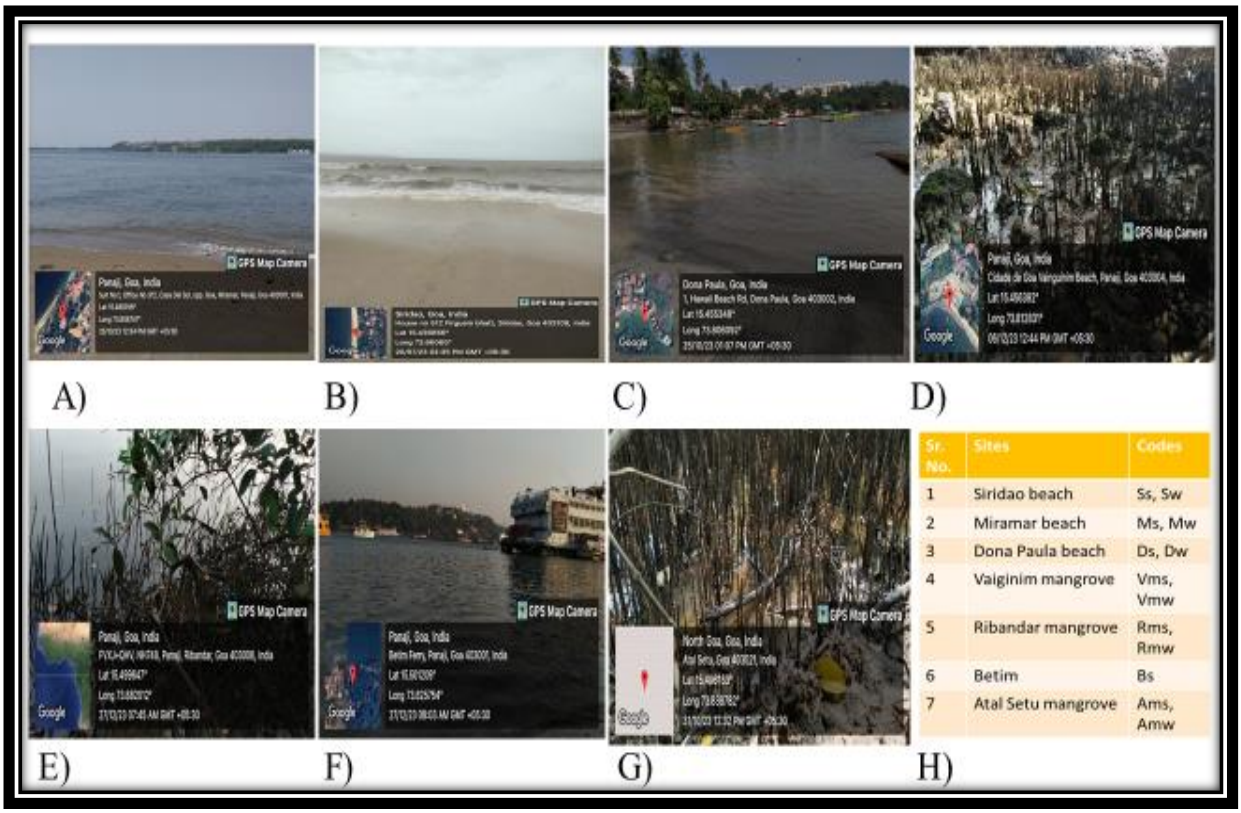

Fig 4.1: Sampling sites used for isolation of potential marine bacterial isolates: A) Siridao; B) Miramar; C) Dona Paula; D) Vaiginim; E) Ribandar; F) Betim; G) Atal Setu; H) Codes used against the respective sites.

Table 4.1: Physicochemical parameters of various samples used.

Sr. No.	Samples	Place	Temperature (^{o}C)	pH	Latitude	Longitude
1.	Water	Siridao beach	27	7	15^{o} 43’68.56” N	73^{o} 86’06.5” E
	Sediment		27	7		
2.	Water	Miramar beach	32	7.5	15^{o} 48’59.16” N	73^{o} 80’87.47” E
	Sediment		32	7.5		
3.	Water	Dona Paula beach	33	7.5	15^{o} 45’53.48” N	73^{o} 80’60.92” E
	Sediment		33	7.5		
4.	Water	Vaiginim mangrove	31	7.9	15^{o} 45’62.65” N	73^{o} 81’37.8” E
	Sediment		31	7.9		
5.	Water	Ribandar mangrove	28	7.6	15^{o} 49’96.47” N	73^{o} 88’20.12” E
	Sediment		28	7.6		
6.	Water	Betim	30	7.3	16^{o} 50’11.8” N	73^{o} 82’57.65” E
7.	Water	Atal Setu Mangrove	32	7	15^{o} 49’61.83” N	73^{o} 83’87.82” E
	Sediment		32	7		

4.2 Enrichment of dye decolorization marine bacteria

Growth was observed in seawater and sediment enriched flasks containing ZMB and dye whereas Growth was not observed in the control (Fig.4.2).

Fig 4.2: Enrichment flask after 48 h of incubation: A) Seawater sample; B) Sediment sample; C) control.

4.3 Isolation of dye decolorizing bacteria

After dilution platting of the enriched samples on ZMA containing congo red distinct colonies were obtained after 24 h (Fig.4.3). Morphologically 15 dissimilar bacterial isolates were considered for further studies.

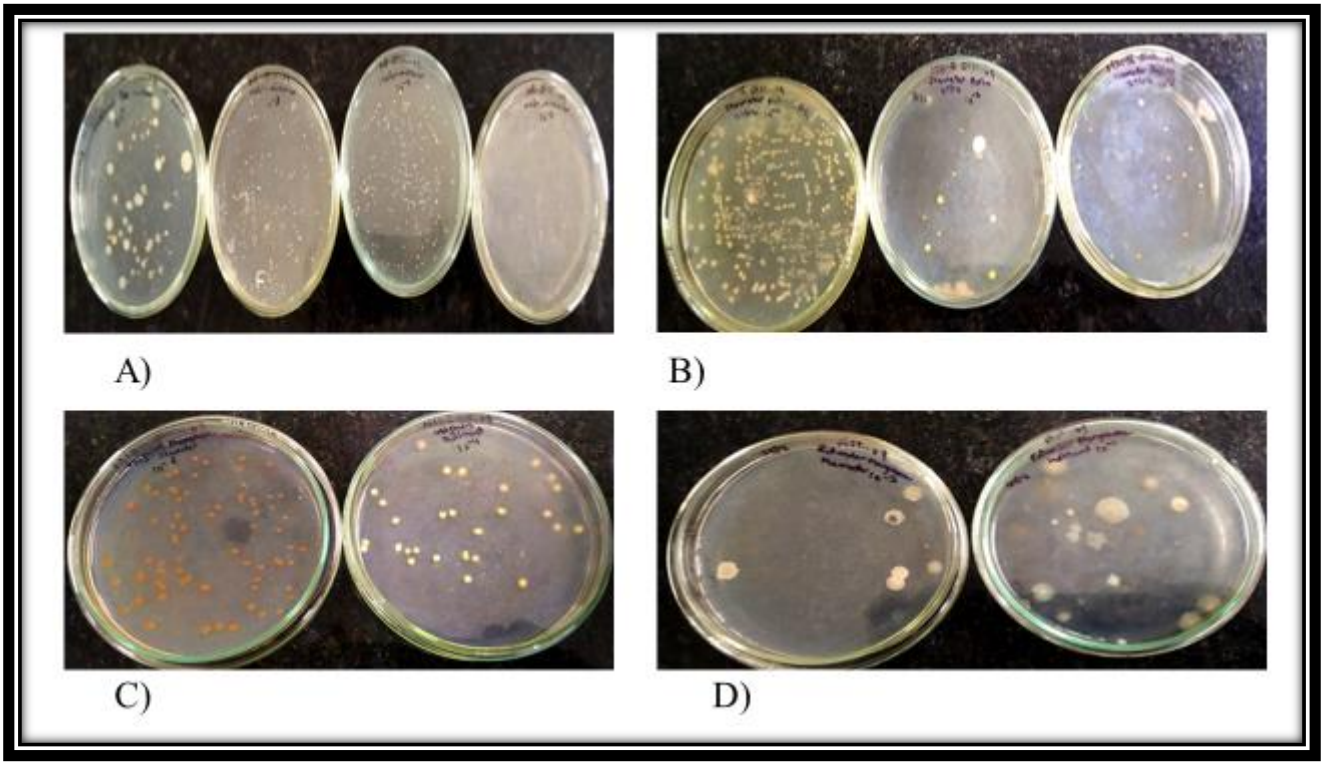

Fig 4.3: ZMA plates showing isolates from A) Miramar samples; B) Betim seawater samples; C) Vaiginim Mangroove; D) Ribandar Mangroove.

4.4 screening of dye decolorizing bacterial isolates

Among all fifteen isolates, the bacterial strains RMS and DW4 exhibited the highest zone of clearance, measuring up to 5 mm followed by RMW, PMCH2, and PMSS1 upto 2 mm (Fig.4.4). Subsequent cultivation of the selected isolates in ZMB containing 0.02% Congo red revealed a gradual decrease in red color intensity in the test flask after every 24 h of growth, indicating dye decolorization. This phenomenon was absent in the control flask (Fig.4.5). UV-Vis spectrophotometric analysis further strengthened these findings, showing a prominent peak at 490 nm in both control and test flasks at 0 hours of incubation which was diminished after every 24 hours, confirming dye decolorization (Fig. 4.6).

Among all the isolates, the culture RMS demonstrated the highest dye-decolorizing activity (52%), followed by strains PMCHW2, DW4, RMW, and PMSS1 exhibiting 47%, 41%,

38%, and 34%, respectively (Fig.4.7). Consequently, the isolate RMS, sourced from the Ribandar mangroves, was chosen for further investigation.

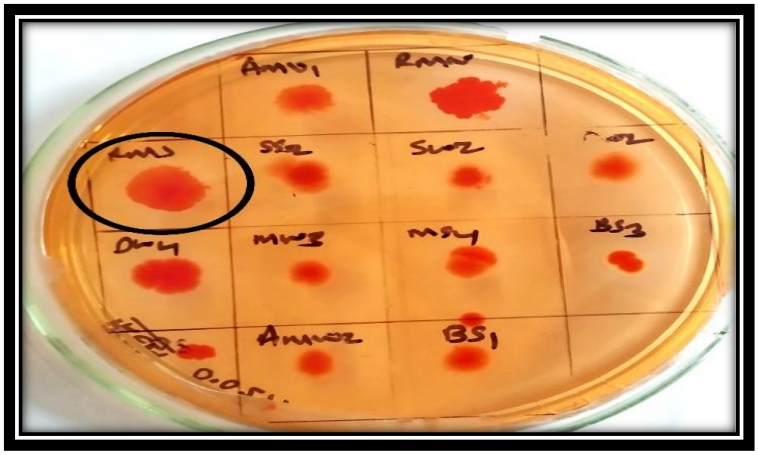

Fig 4.4: Screening of potential dye-decolorizing bacterial strains showing zone of clearance on ZMA containing Congo red (0.02%).

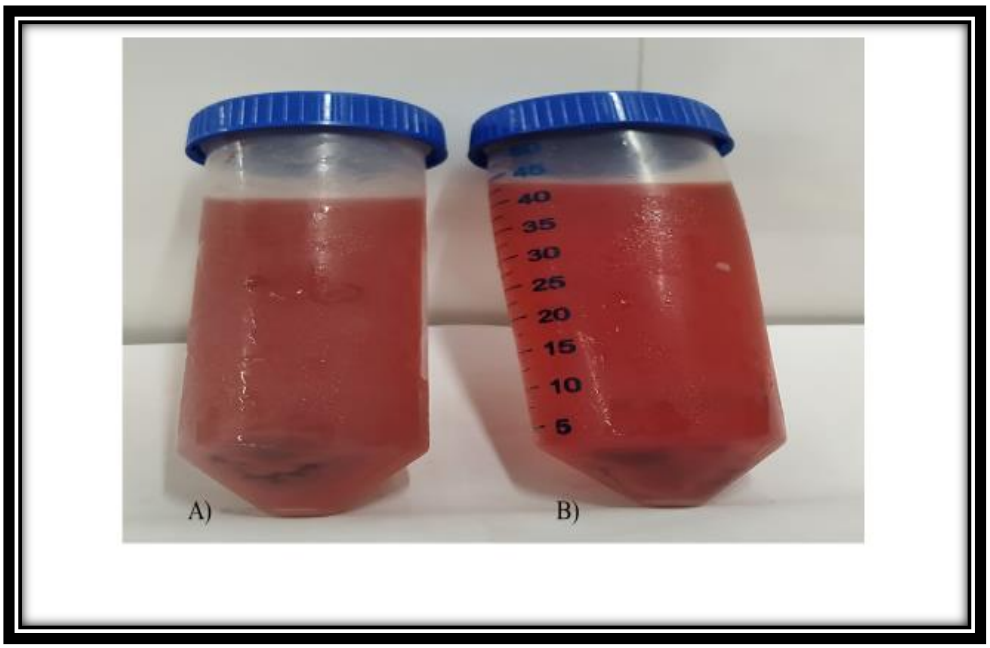

Fig 4.5: Screening for potential dye decolorization using selected bacterial Isolates; A) RMS: B) DW4.

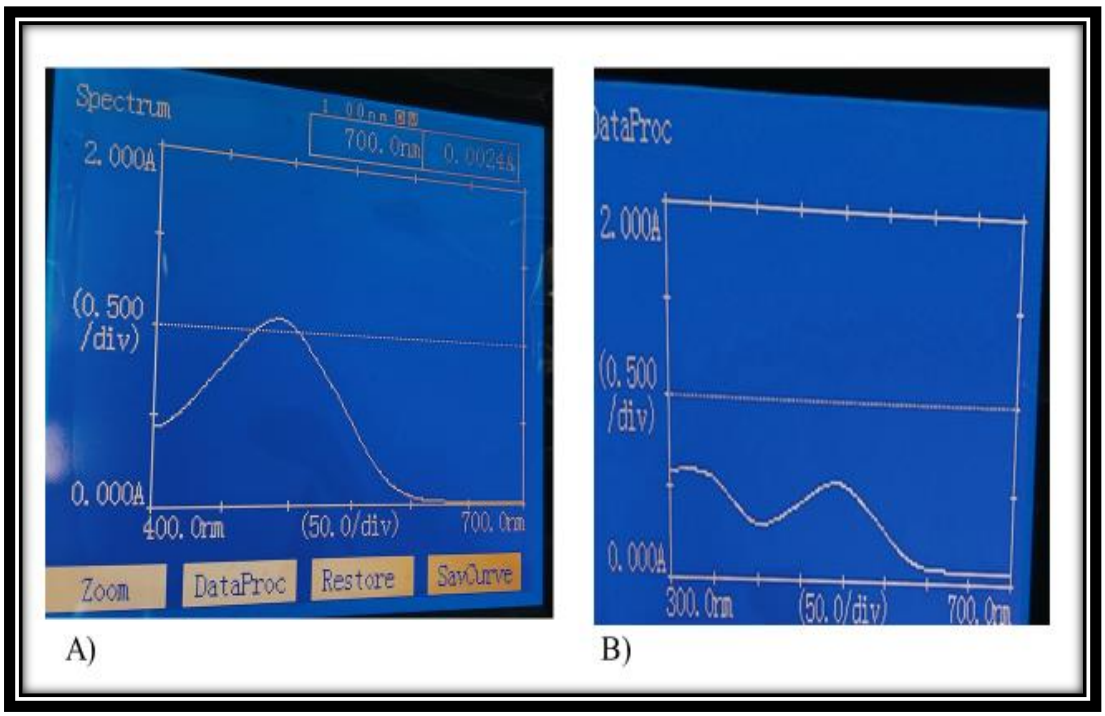

Fig 4.6: UV-Vis spectrophotometric analysis of RMS culture at 0 and 72 h of incubation.

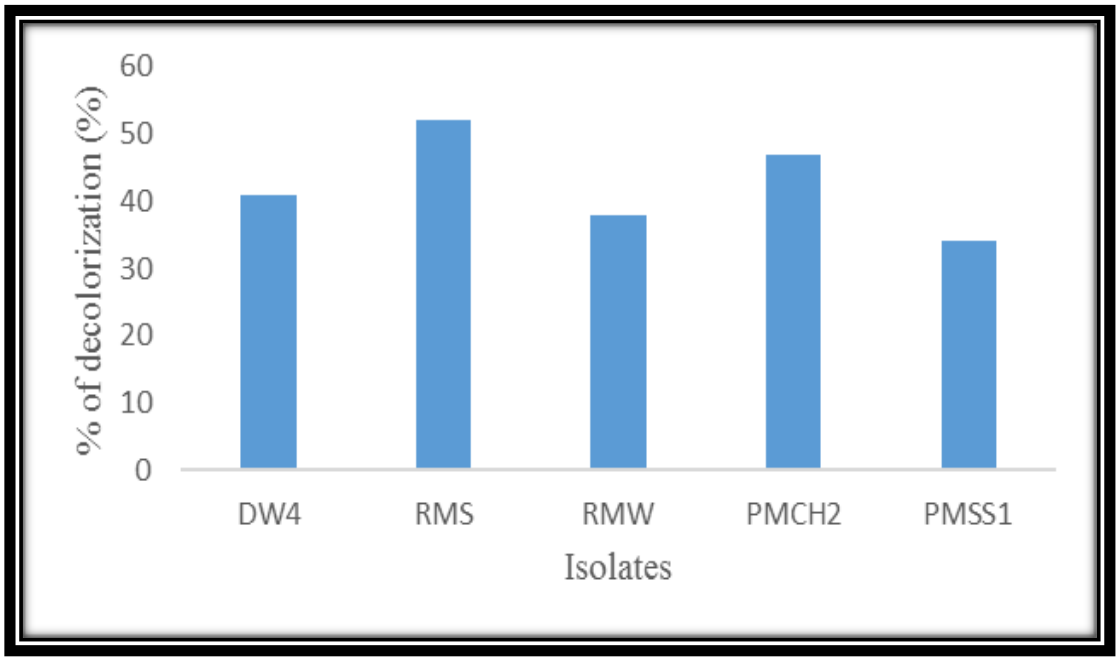

Fig 4.7: Percent dye decolorization of selected isolates in ZMB with (0.02%).

4.5 Growth and maintenance conditions

Bacterial strain RMS and other selected cultures were subcultured by parallel streaking method on ZMA plate containing 0.02% concentration of dye (Fig.4.8).

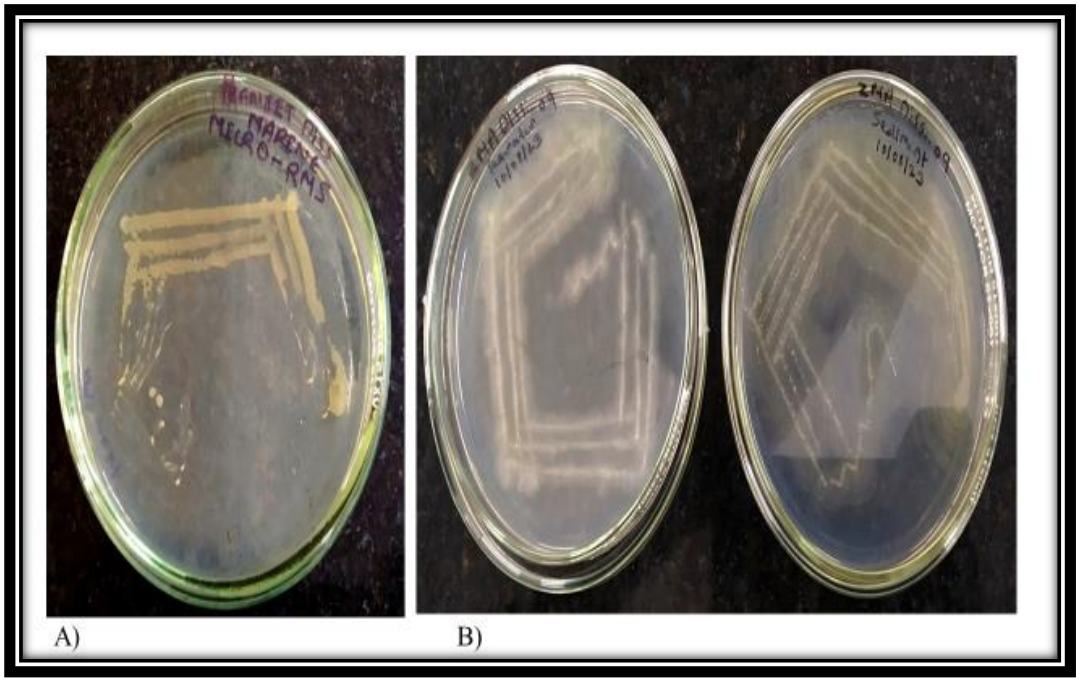

Fig 4.8: Selected dye decolorizing isolates on ZMA with 0.02% Congo red.

4.6 Biochemical characterisation of selected dye decolorizing bacteria

Bacterial strain RMS was found to be Gram-negative rods (Fig.4.9, Table 4.2). It showed positive for Methyl red, Oxidase, fermentation of glucose, Hugh-Leifsons tests whereas Voges-Proskauer, Citrate utilization, Motility, Fermentations of sugars (maltose, sucrose, lactose), Catalase, Triple sugar iron, Phenylalanine deaminase tests shows negative results shown in (Fig.4.10) and (Table 4.3).

Table 4.2: Morphological characterisation of culture RMS.

Types	RMS
Size	2mm
Shape	circular
Colour	Orange
Temperature	Room temperature
Elevation	Flat
Margin	Even
Opacity	Opaque
Consistency	Smooth
Gram	Negative
character	Rods

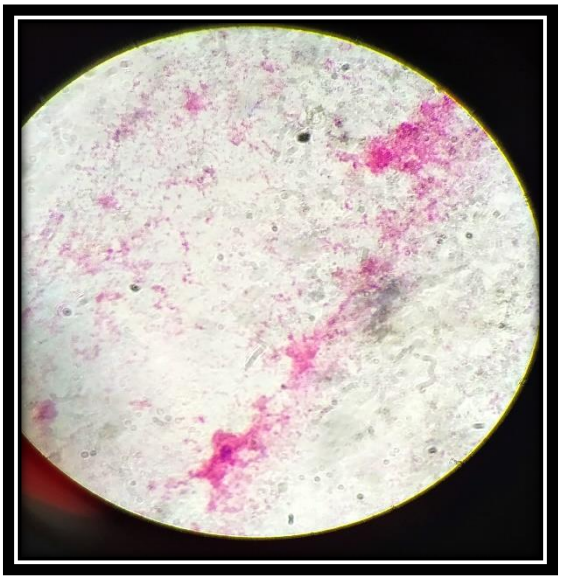

Fig 4.9: Gram staining of RMS showing Negative rods

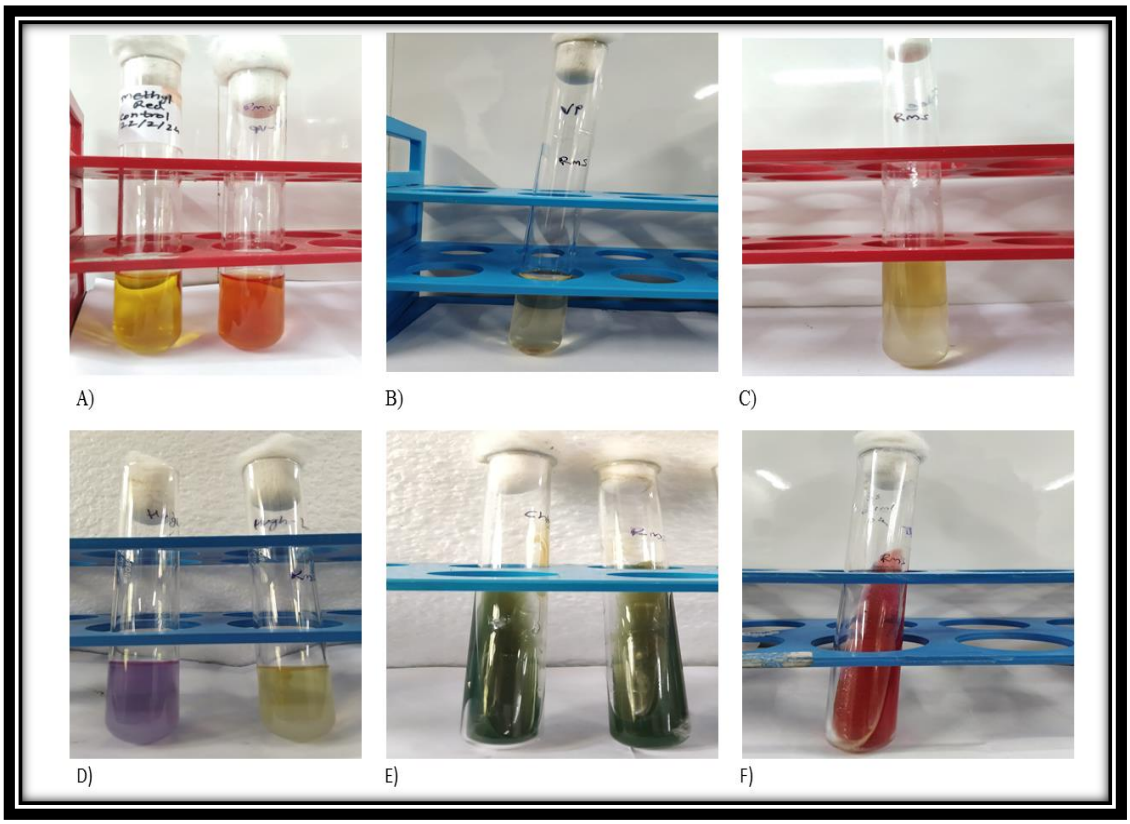

Fig 4.10: Biochemical tests: A) Methyl Red test; B) Voges-Proskauer test; C) Indole test; D) Hugh-Leifsons test; E) Citrate test; F) Triple sugar test.

Table 4.3: Biochemical characteristics of culture RMS.

Name of tests	Result
Methyl red	+
voges-Proskauer	-
Citrate utilisation	-
Motility	-
Fermentation of sugars: Glucose Maltose Sucrose Lactose	 + - - -
Catalase	-
Oxidase	+
Hugh-Leifsons	+
Triple Sugar Iron	-
Phenylalanine Deaminase	-

+: Positive -: Negative

4.7 Molecular characterisation of selected dye decolorizing bacteria

DNA was isolated from the isolates, and the purity was evaluated on 0.8% agarose gel. A single band of high-molecular-weight genomic DNA of strain RMS without RNA contamination was observed (Fig.4.11).

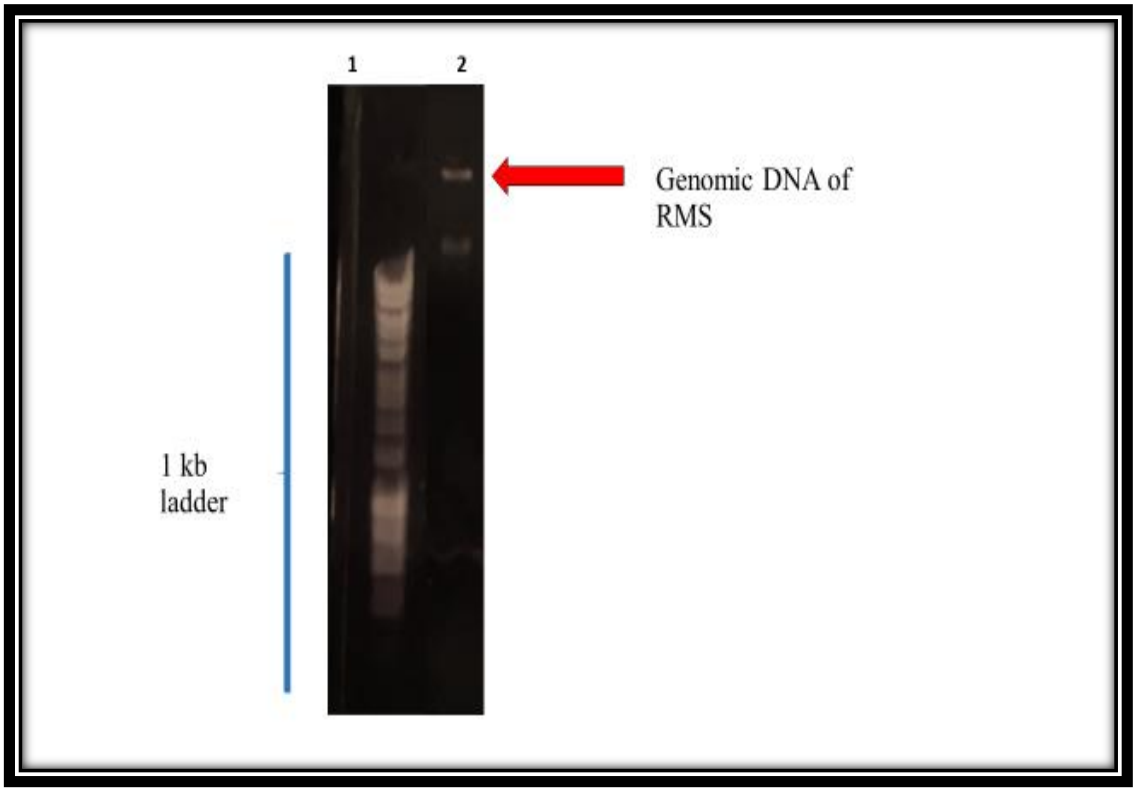

Fig 4.11: Genomic DNA profile RMS

Lane 1: 1 Kb ladder,

Lane 2: RMS DNA

4.8 Scanning Electron Microscopy (SEM)

In the control group, the culture RMS exhibited individual rods with smooth surfaces and uniform shapes. The size of the isolate was found to be ~1-2 µm. Conversely, in the presence of the dye, culture RMS displayed an altered appearance, characterized by aggregate cells also chain formation was also prominent (Fig.4.12).

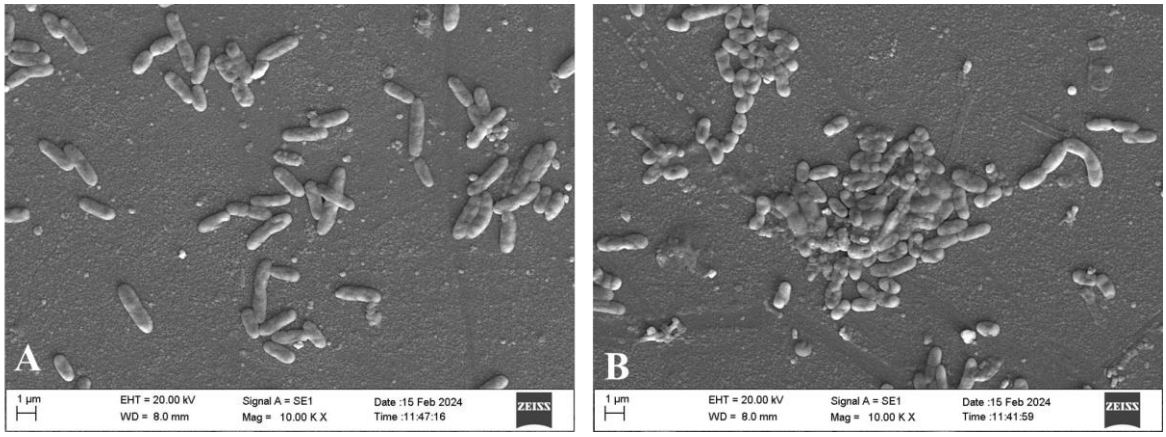

Fig 4.12: SEM analysis; A) Strain RMS without dye as control; B) Culture RMS with decolorized dye.

4.9 Optimization of culture conditions for maximum dye decolorization

pH and temperature serve as crucial physical parameters impacting dye decolorization, as they directly influence both cell growth and enzymatic activity (Sivashanmugam and Jayaraman, 2013).

4.9.1 pH

The RMS strain exhibited significant dye decolorization across a broad pH range (6,7, 8, 9, 10, 11, and 12; Fig.4.13). UV-Vis spectrophotometric analysis were estimated at 0 and 72 h of incubation (Fig.4.14). The optimal pH for dye decolorization by the RMS strain was identified to be pH 8.0, resulting in up to 55% decolorization efficiency (Fig.4.15).

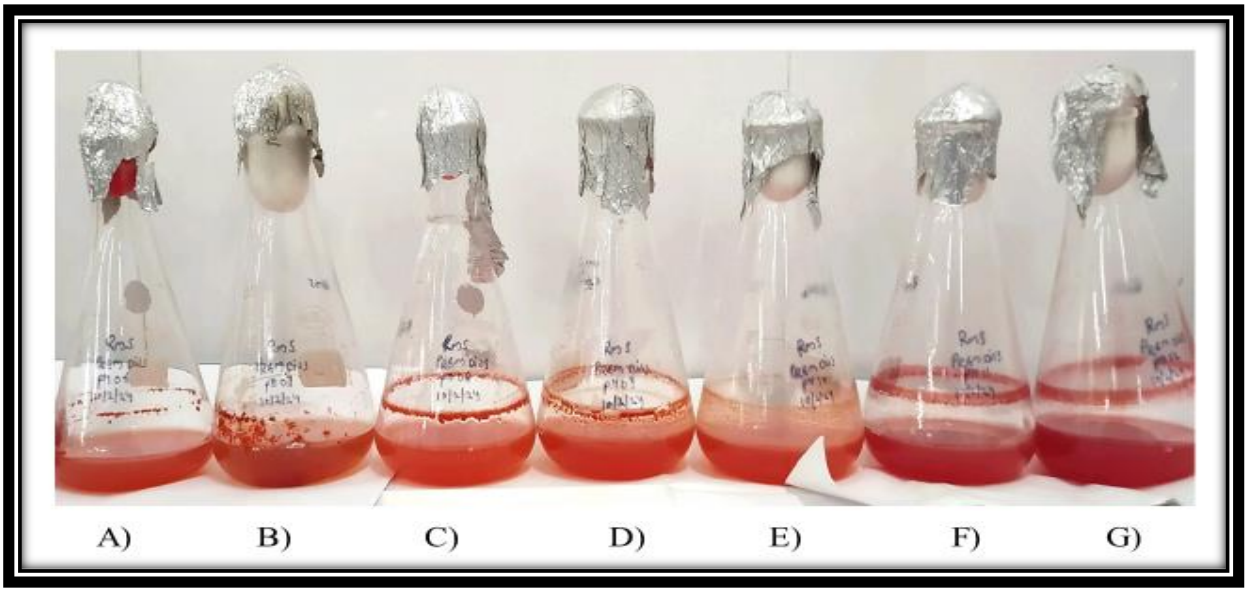

Fig 4.13: Flasks after 72 h of incubation: A) pH 06; B) pH 07; C) pH 08; D) pH 09; E) pH 10; F) pH 11; G) pH 12.

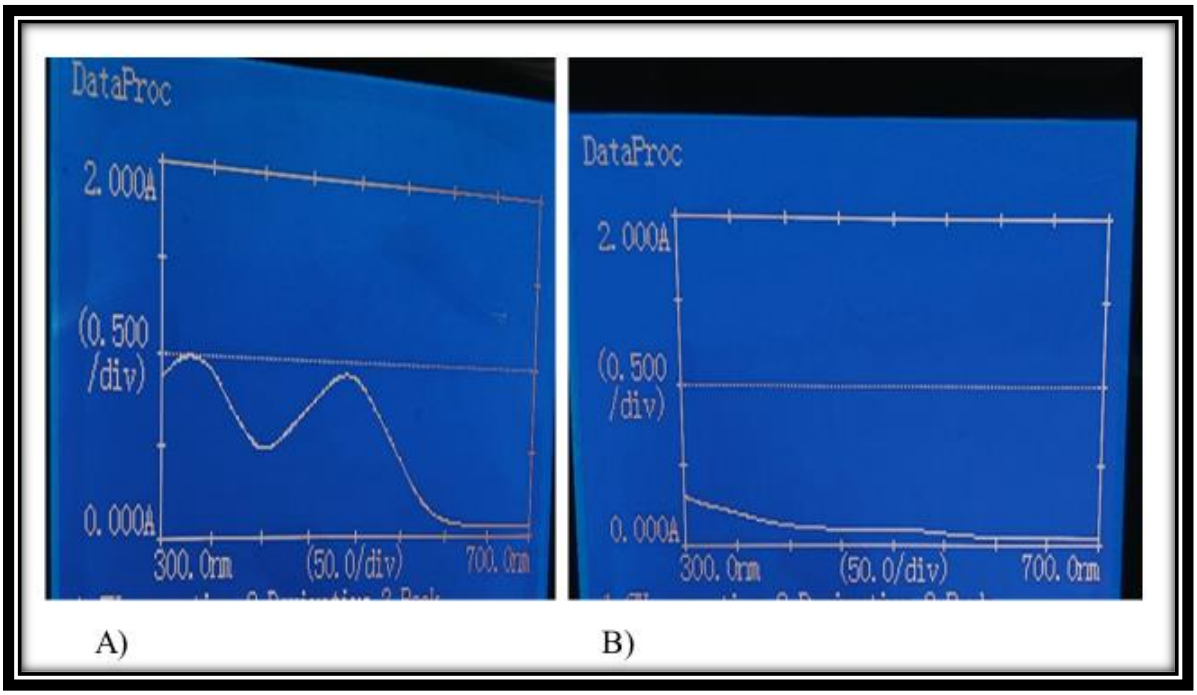

Fig 4.14: UV-Vis spectrophotometric analysis of culture RMS at pH 8: A) 0 h; B) After 72 h incubation.

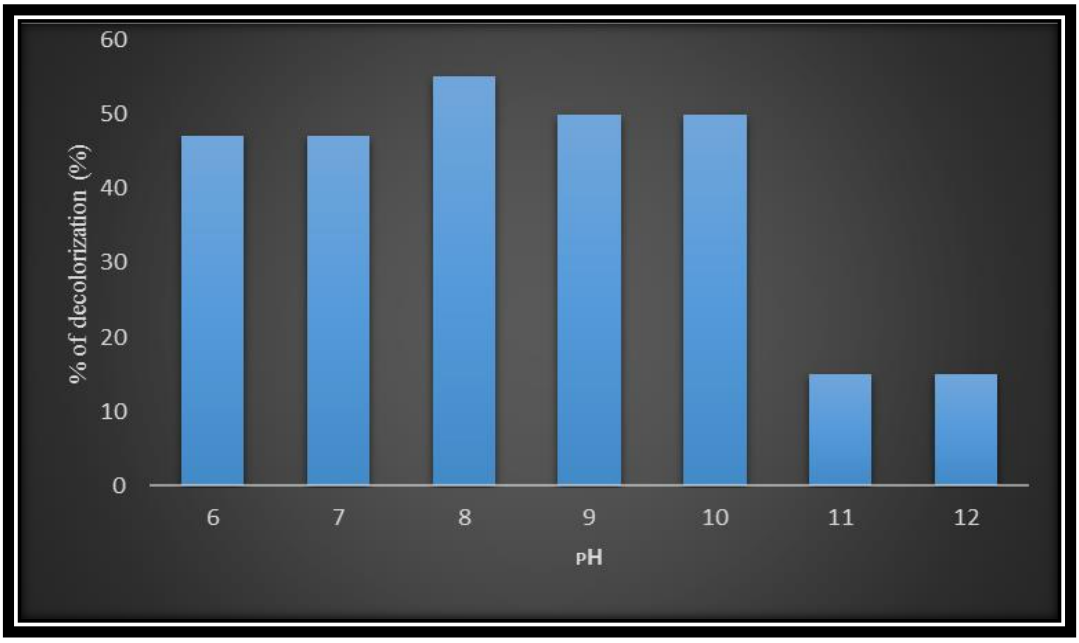

Fig 4.15: Percent decolorization of dye by isolate RMS at varying pH.

4.9.2 Different dye concentration

Decolorization with different dye concentrations ranging from 0.02-0.8% was studied using UV-Vis spectrophotometric analysis (fig.4.16 & 4.17). Isolate RMS showed maximum (56%) decolorization at a concentration 0.02% of the dye (Fig. 4.18).

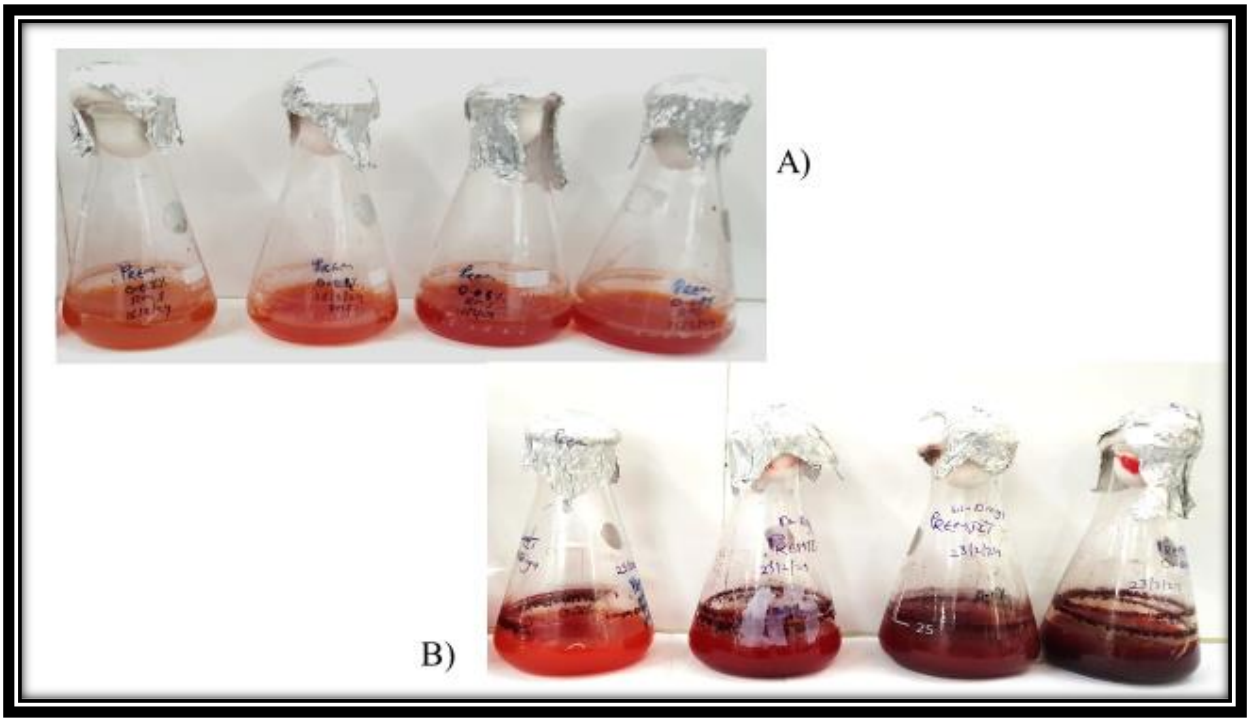

Fig 4.16: Flasks after 72 h of incubation: A) 0.02-0.08% concentration of dye; B) 0.2-0.8% concentration of dye.

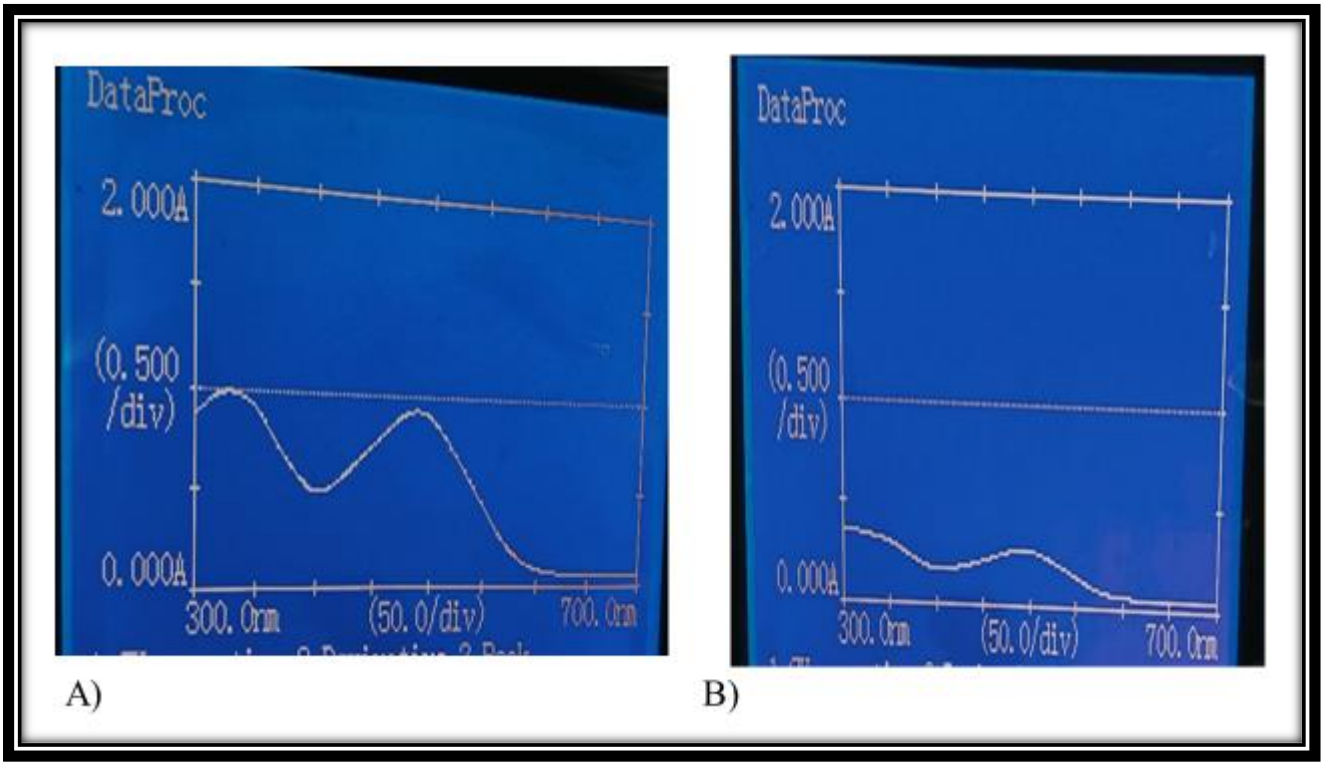

Fig 4.17: UV-Vis spectrophotometric analysis of culture RMS at 0.02% Dye: A) 0h; B) after 72 h incubation.

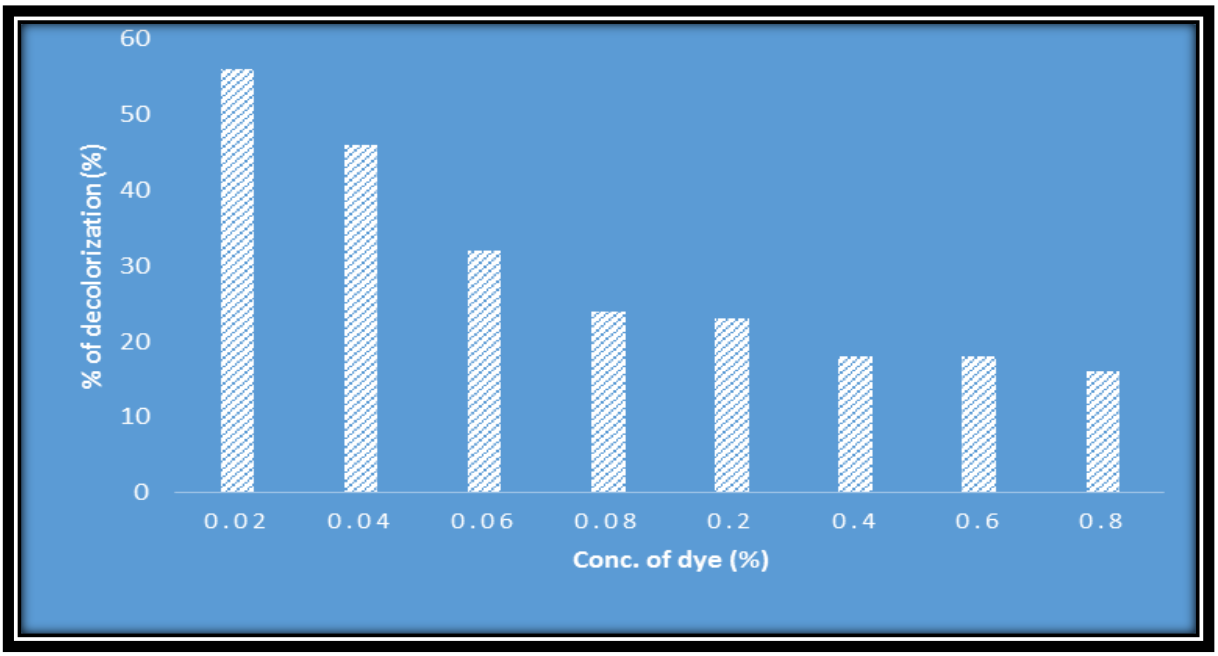

Fig 4.18: Percent decolorization of dye by isolate RMS at varying concentration of dye.

4.9.3 Temperature

The isolate RMS could decolorize dye when grown at temperatures viz. 28, 30 and 37 ℃ (Fig.4.19). UV-Vis spectrophotometric analysis were carried at 0 and 72 h of incubation (Fig..4.20). However, the optimum temperature recorded was 28 °C showing the highest % decolorization (55%) (Fig. 4.21).

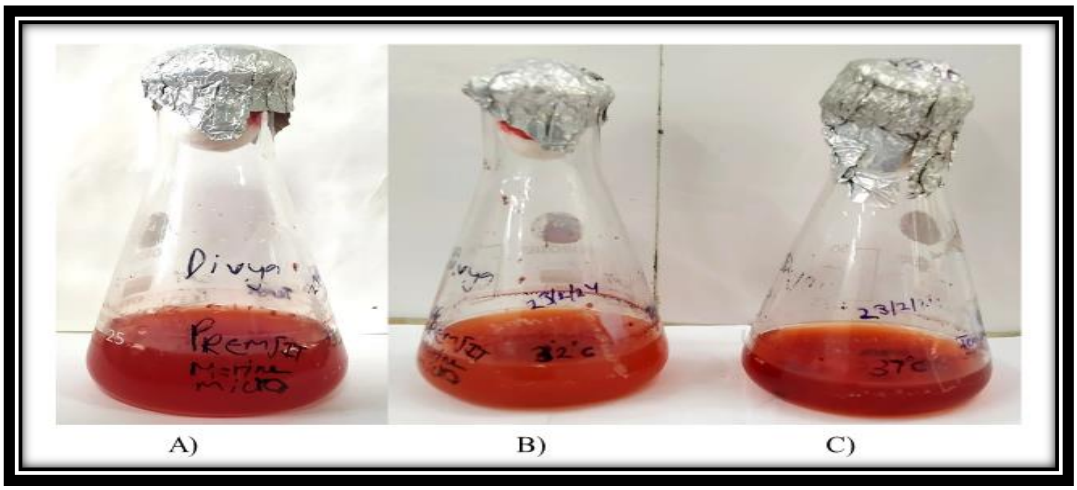

Fig 4.19: Flasks after 72h 0f incubation: A) 28 ; B) 32; C) 37 ºC.

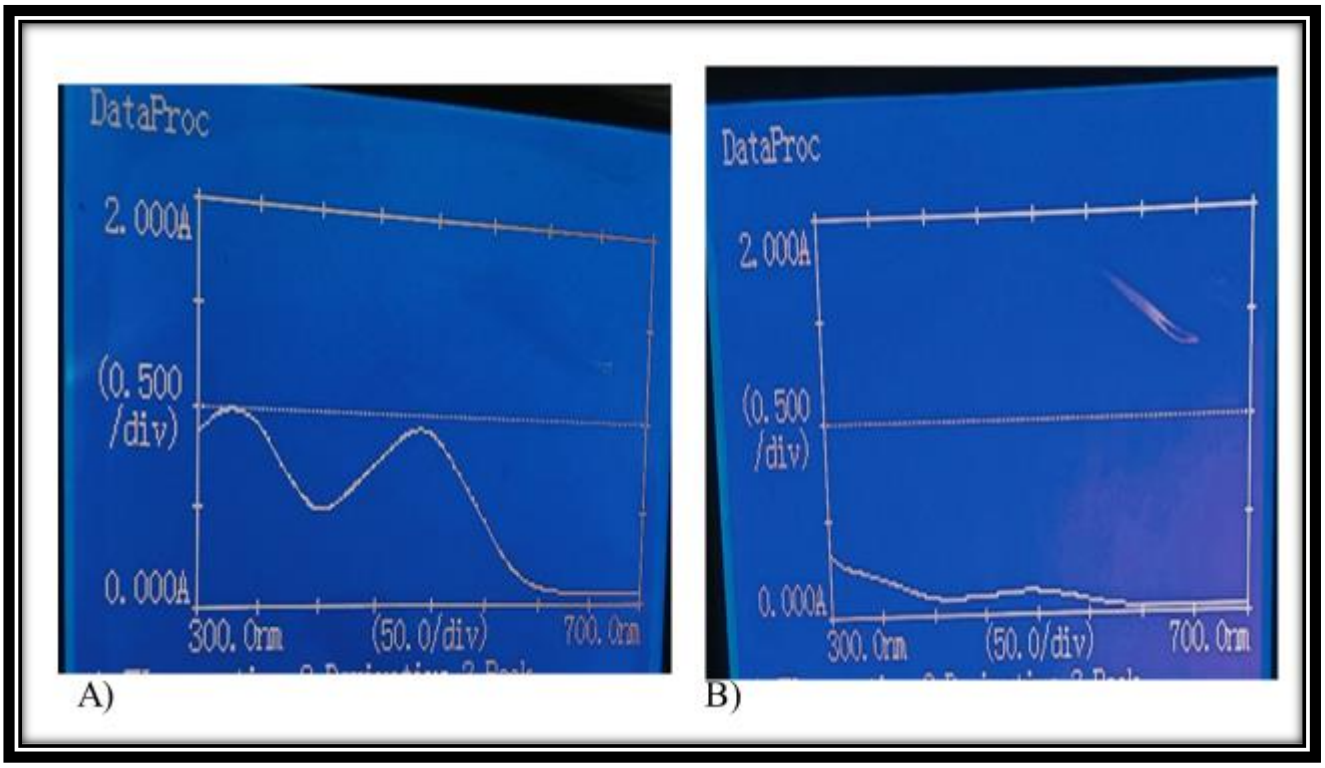

Fig 4.20: UV-Vis spectrophotometric analysis of culture RMS at temperature 28 ºC: A) 0h; B) after 72 h incubation.

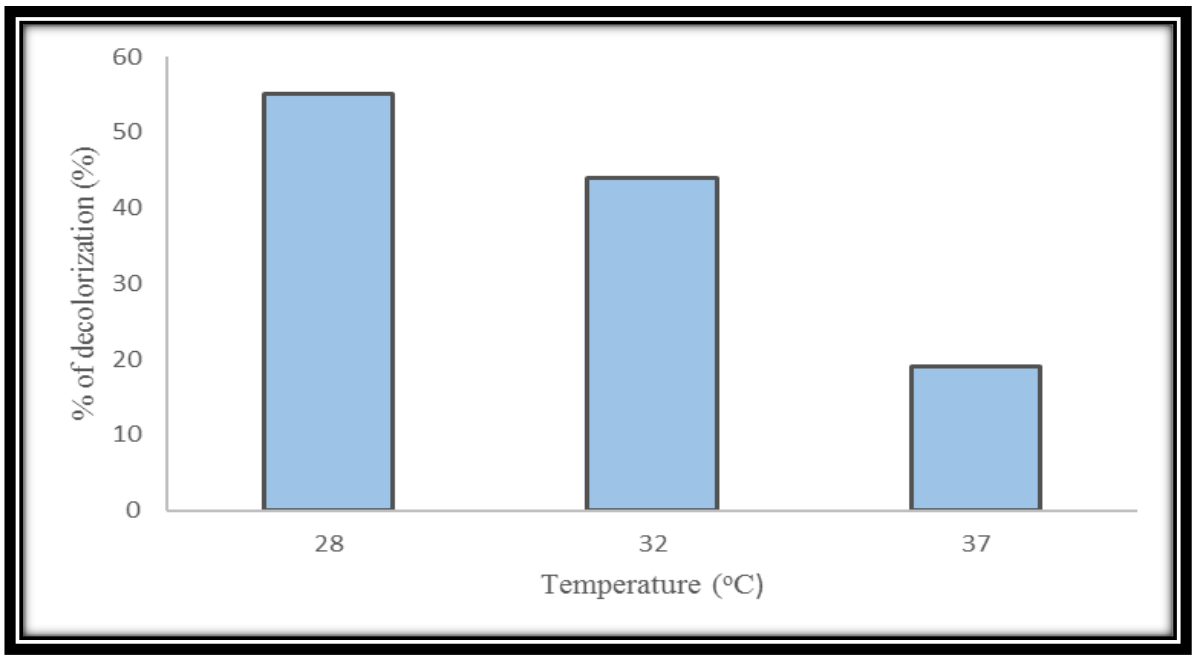

Fig 4.21: Percent decolorization of dye by isolate RMS at varying temperature.

4.9.4 NaCl concentration

Different NaCl concentration studied varying at (1.9%, 2%, 3%, 4%, 5%) (Fig.4.22). A maximum decolourization (57%) was observed at 1.9% NaCl concentration and UV-Vis spectrophotometric analysis were studied (Fig.4.23). Followed by 40%, 19% decolorization of dye was observed at 2.0%, 3.0%, 4.0%, and 5.0% NaCl respectively (Fig.4.24).

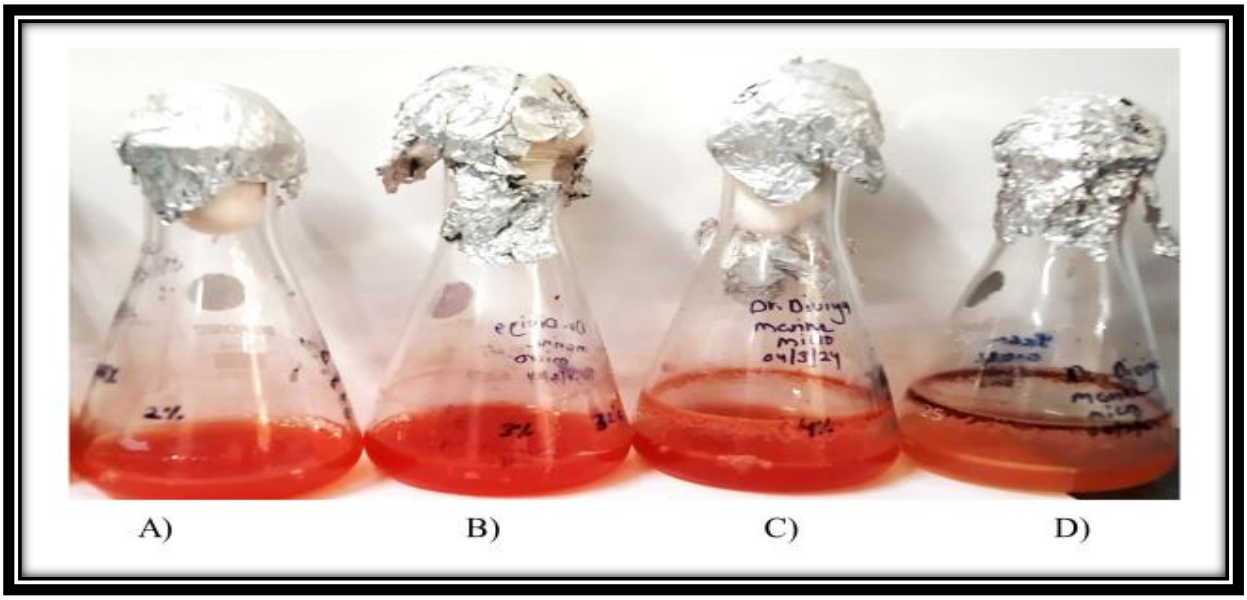

Fig 4.22: Flasks after 72 h of incubation: A) 2%; B) 3%; C) 4%; D) 5% NaCl.

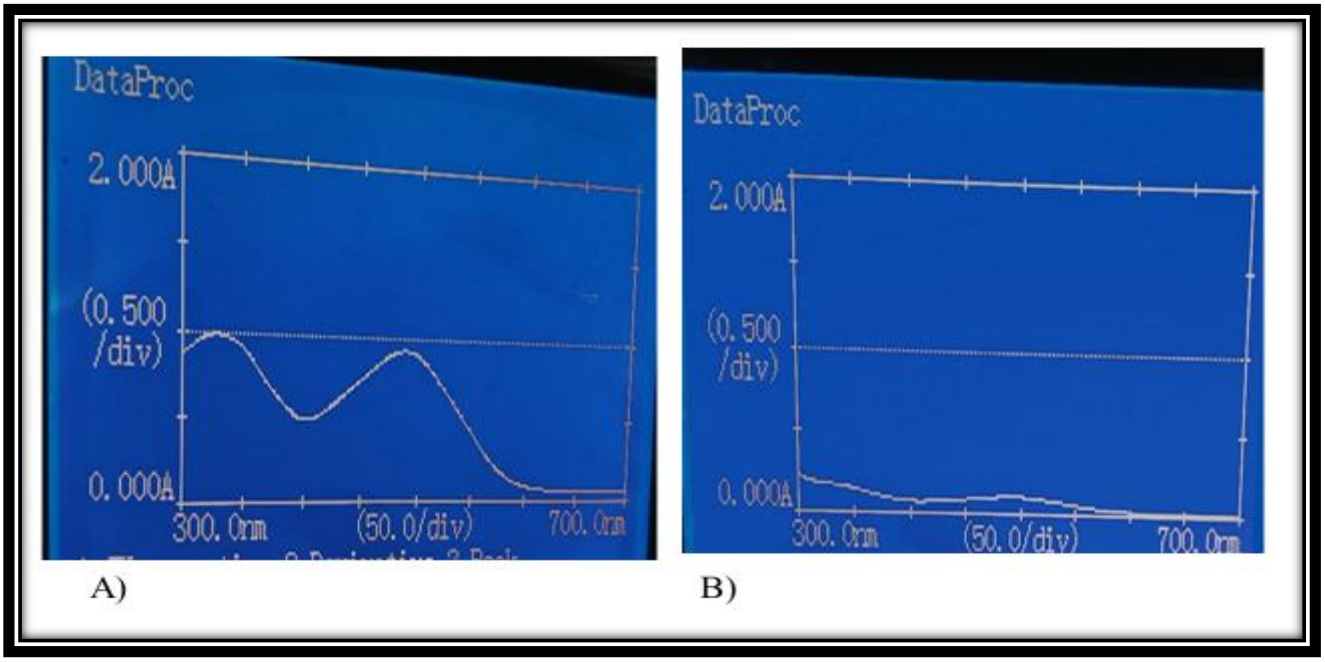

Fig 4.23: UV-Vis spectrophotometric analysis of culture RMS at 1.9% NaCl

concentration: A) 0h; B) after 72 h incubation.

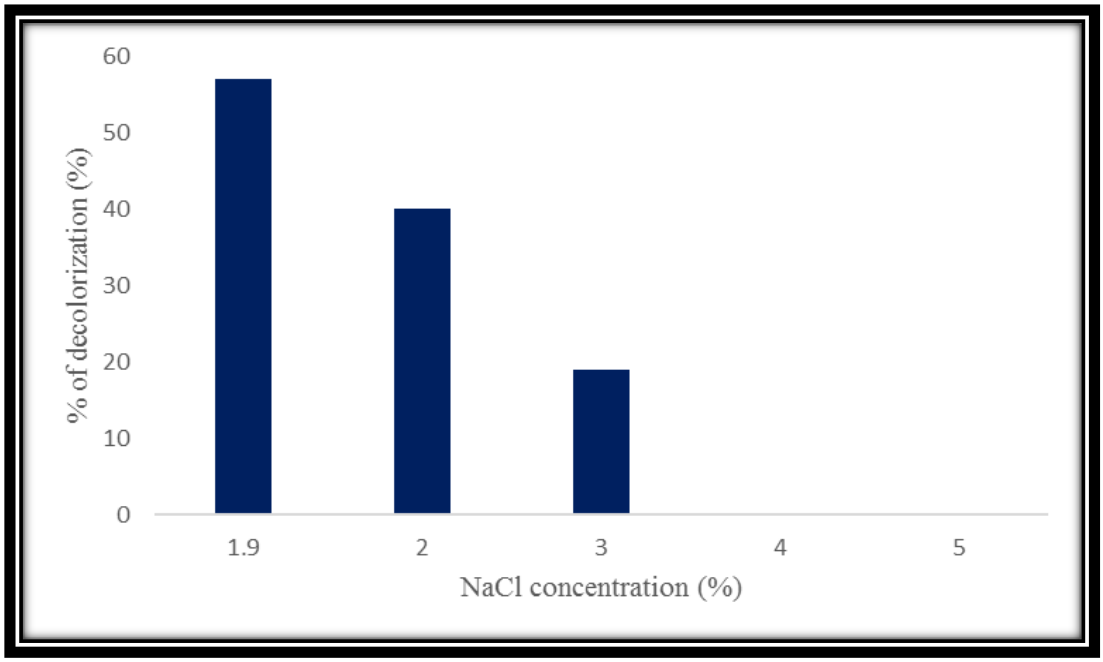

Fig 4.24: Percent decolorization of dye by isolate RMS at varying NaCl concentration.

4.9.5 Growth behaviour of culture RMS

Under the optimized conditions of pH, dye, NaCl and temperature (8, 0.02%, 1.9% and 28 ℃) (Fig. 4.25). UV-Vis spectrophotometric analysis and growth curve were captured for the % dye decolorization was found to be maximum at 72 h of incubation where 59% was reported (Fig. 4.26, 4.27 & 4.28).

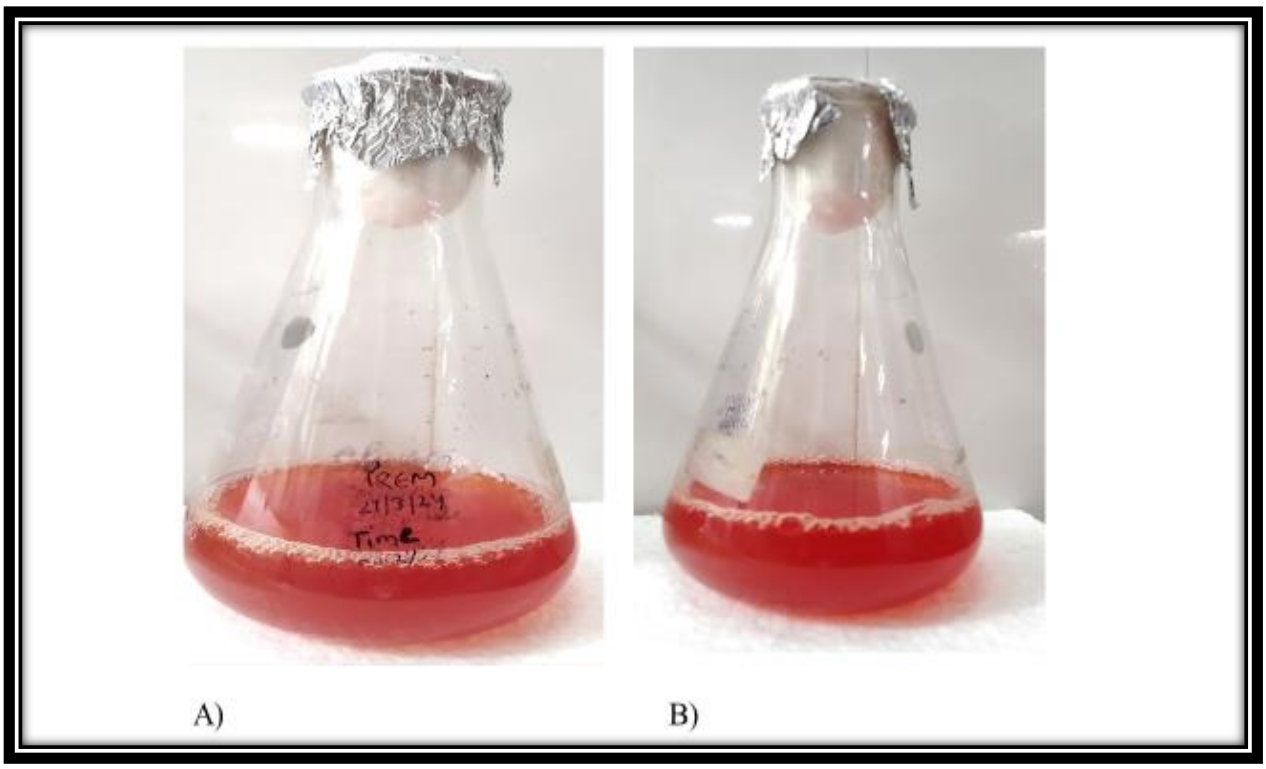

Fig 4.25: Flasks after 72 h of incubation to check growth behaviour:

A) Experimental flask; B) Control flask.

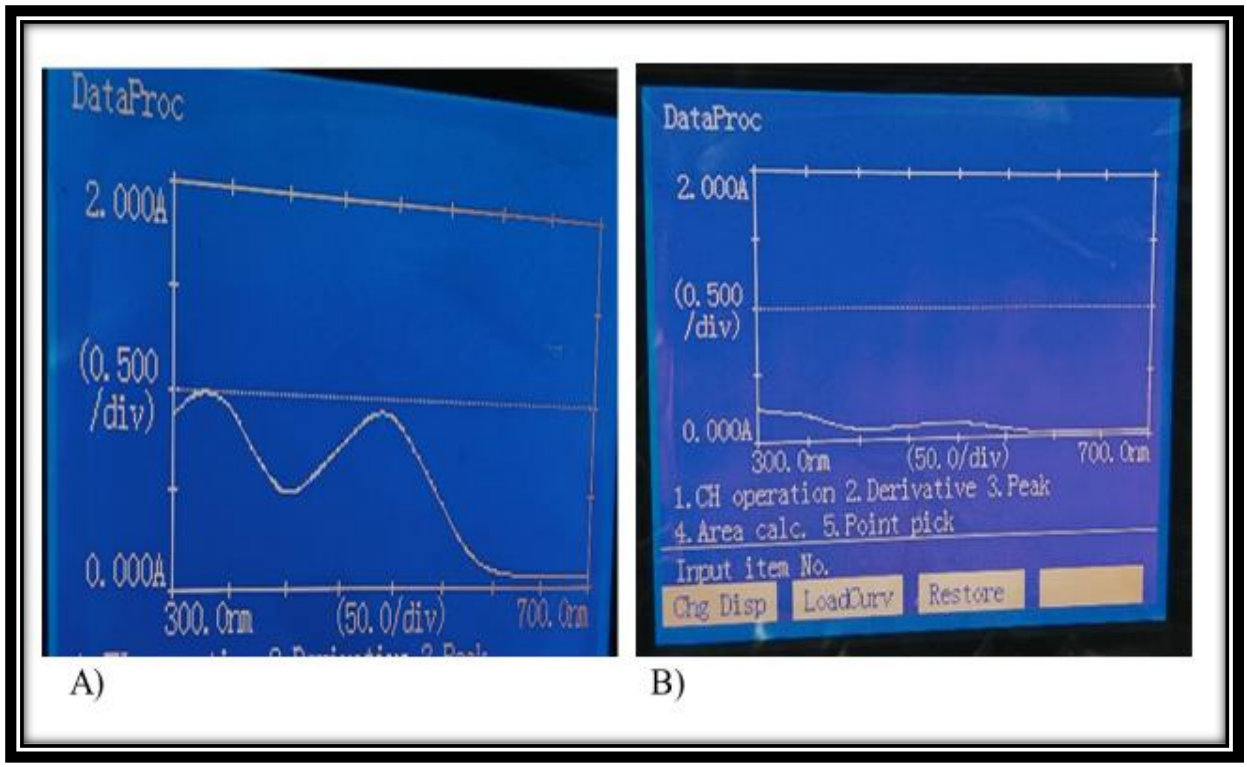

Fig 4.26: UV-Vis spectrophotometric analysis for growth behaviour of culture RMS: A) 0h; B) after 72 h incubation.

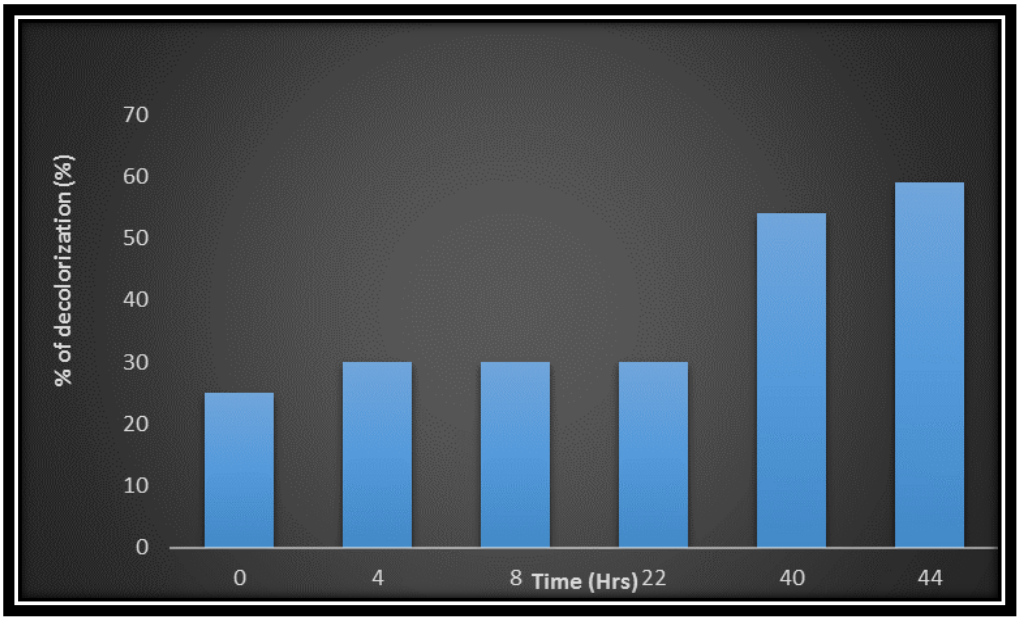

Fig 4.27: Percent decolorization of dye by isolate RMS after 72 h of incubation.

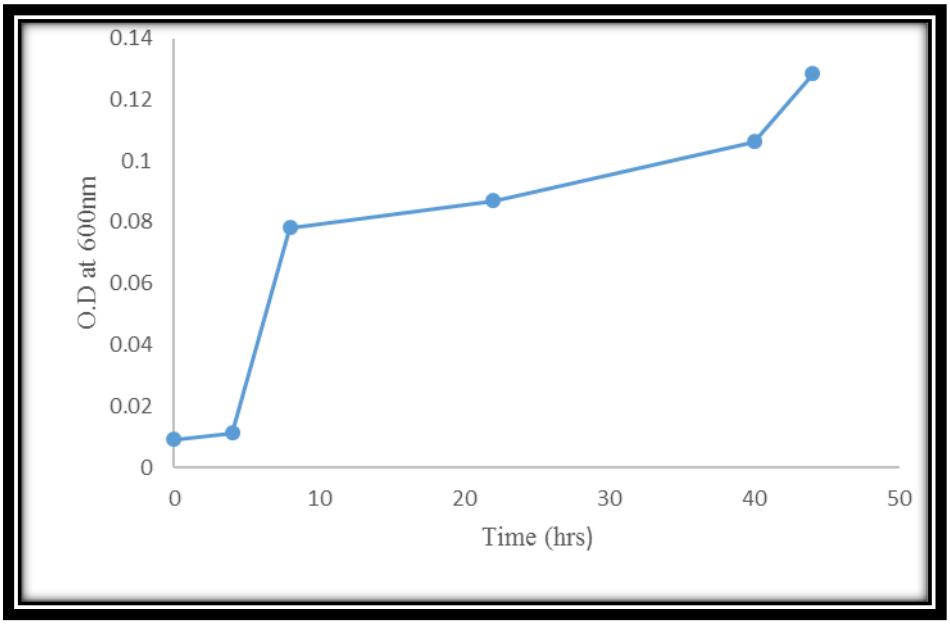

Fig 4.28: Growth curve of isolate RMS after 72 h of incubation.

4.10 Immobilization of strain RMS for Congo red dye decolorization

The immobilized beads of strain RMS obtained by using 2% sodium alginate and 4% calcium chloride when studied for dye decolorization reported 64% decolorization after 1 cycle (Fig.4.29). UV-Vis spectrophotometric analysis for 1st and 2nd cycle were obtained (Fig. 4.30 & 4.31). However, when the same beads were used for 2nd cycle a % decolorization of 57% was obtained (Fig. 4.32).

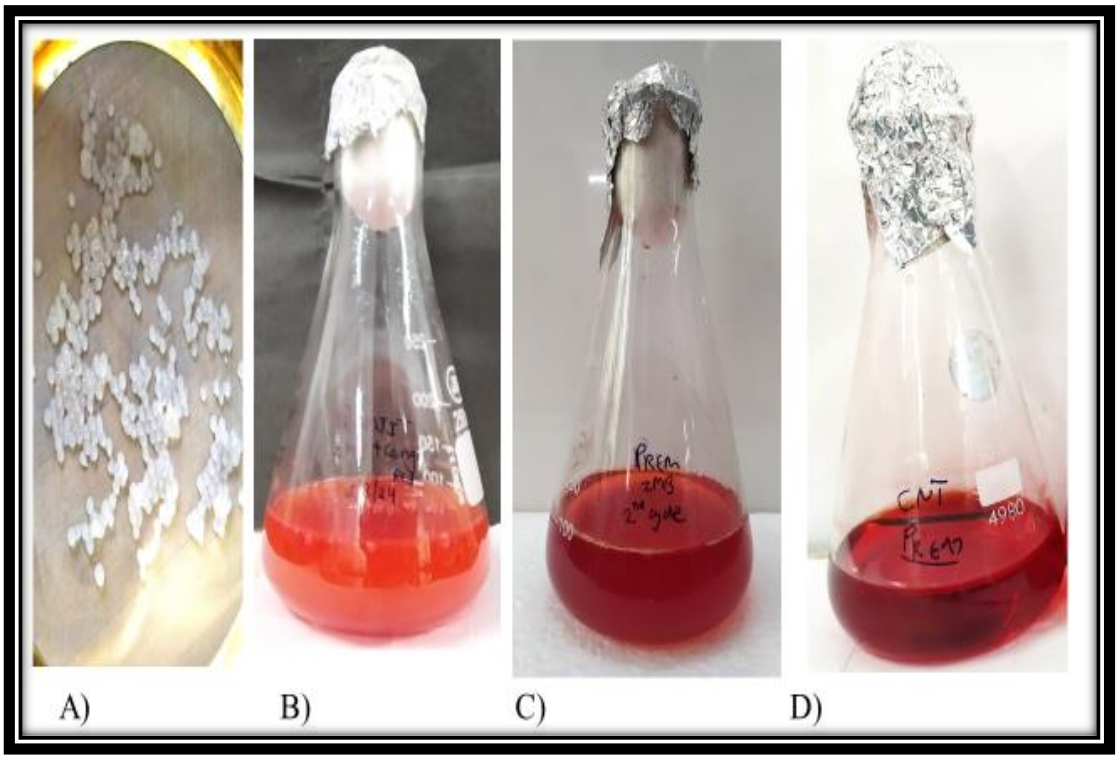

Fig 4.29: Immobilization of strain RMS; A) Strain RMS beads; B) Flask after 72 h of incubation of 1st cycle; C) Flask after 72 h of incubation of 2nd cycle.

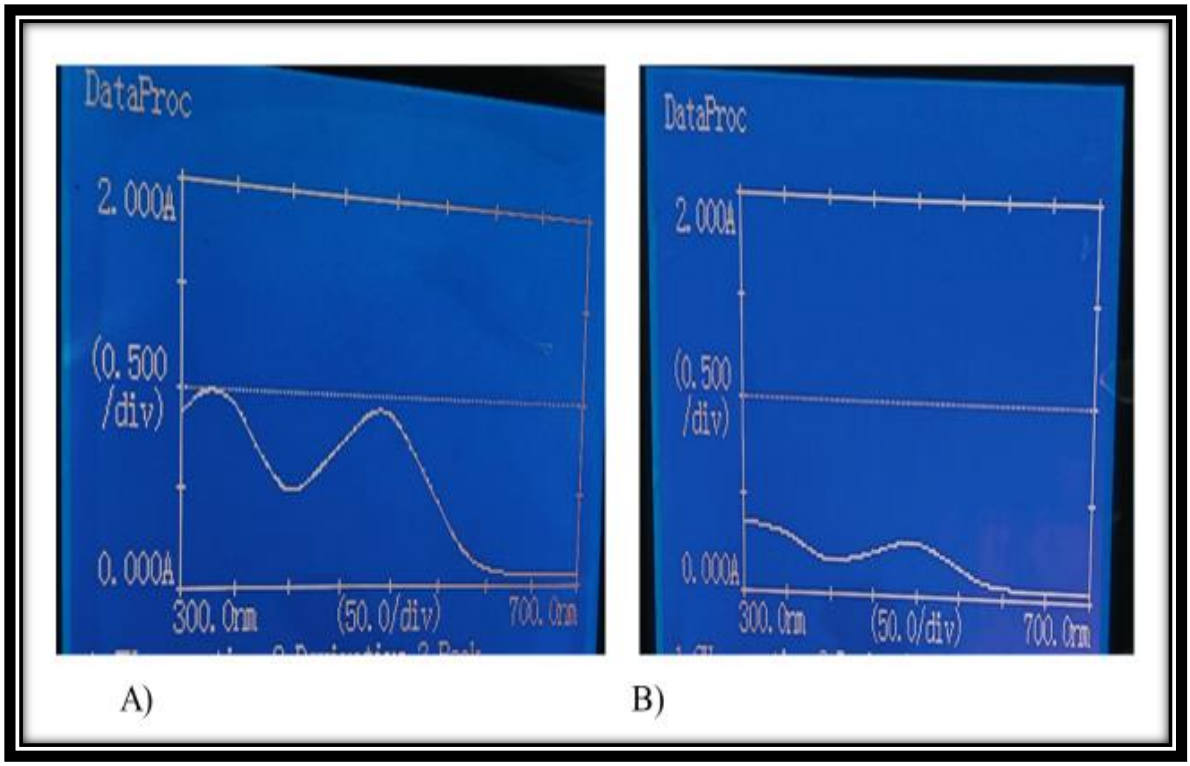

Fig 4.30: UV-Vis spectrophotometric analysis of culture RMS beads of cycle 1: A) 0h; B) after 72 h incubation.

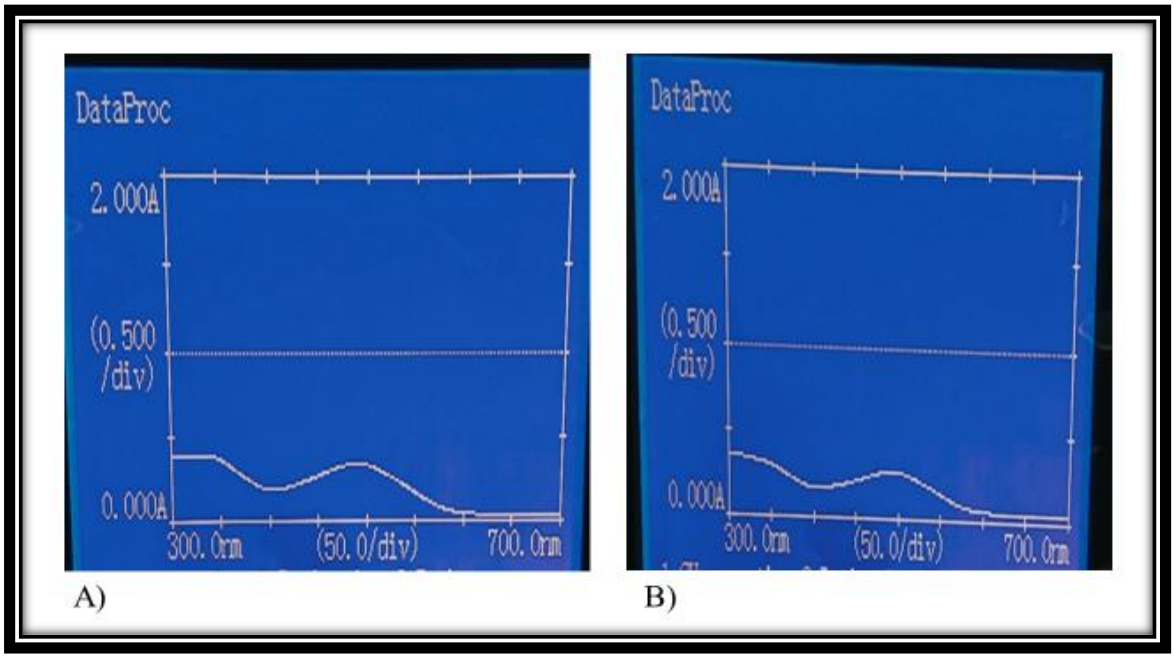

Fig 4.31: UV-Vis spectrophotometric analysis of culture RMS beads of cycle 2: A) 0h; B) after 72 h incubation.

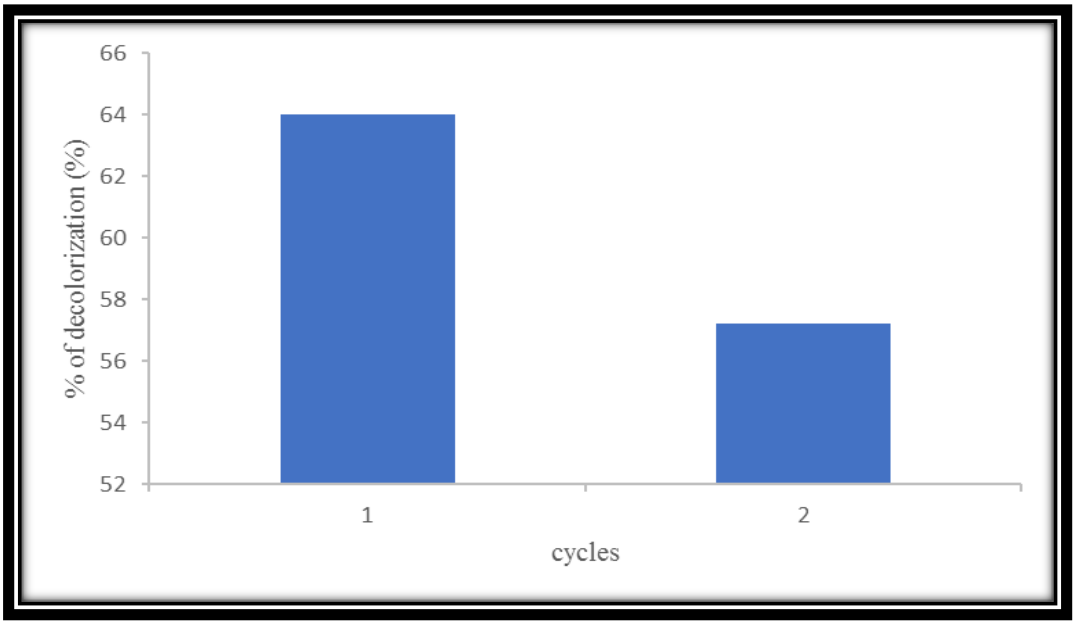

Fig. 4.32: Percent decolorization of dye by strain RMS beads of cycle 1 and cycle 2.

4.11 Characterisation of biodecolorized product

4.11.1 TLC

TLC analysis revealed a Rf of 0.8 for control (untreated dye 0.02%). Whereas the Rf value obtain for treated sample was 0.6. (Fig.4.33).

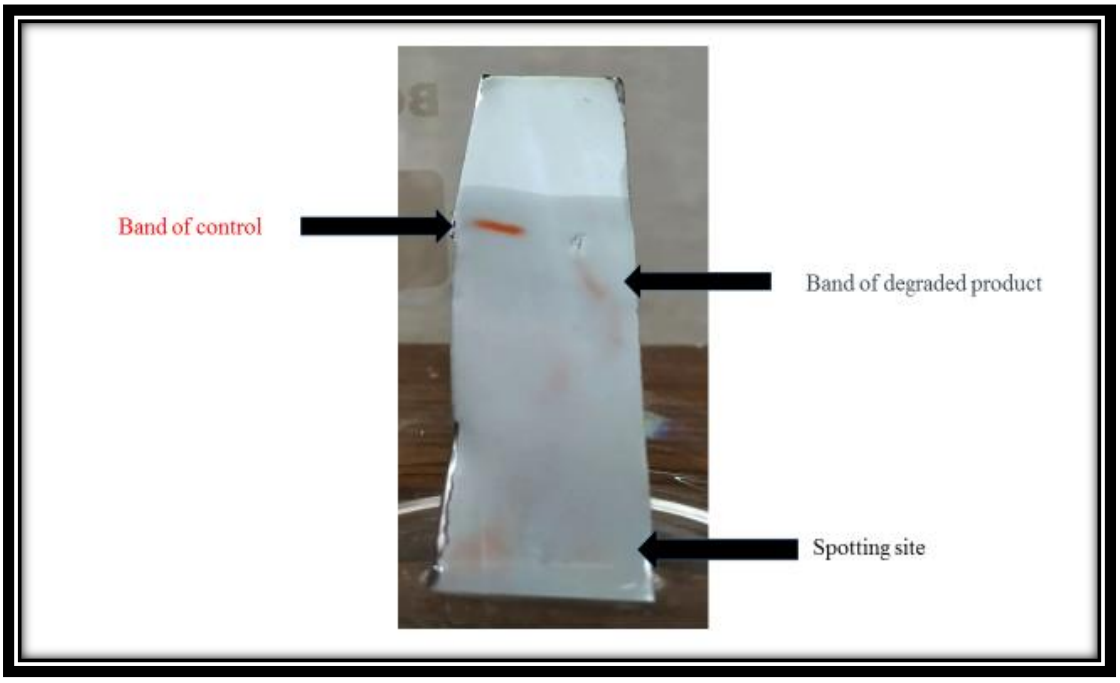

Fig 4.33: TLC analysis of the decolorized dye product.

4.12 Effect of biodecolorized product on plant growth

The toxicity analysis decolorized dye product on the germination of Moong seeds (*Vigna radiata*) revealed that the decolorized dye effluent did not hampered the seed germination. For instance, seed germination in distilled water, 1st cycle and 2nd cycle treated effluent showed 100%, 80% and 30%, respectively. However seeds soaked in untreated dye effluent did not show any germination (Fig.4.34). Vigor index calculated for distilled water, 1st and 2nd cycle effluent and untreated effluent were found to be 1186, 96 and 4.8, respectively (Fig. 4.35). The shoot length of the moong seed in water was found to be 4.81cm while in the treated sample (1st cycle) was 0.28cm and for the 2nd cycle was 0.16cm. However, no growth was observed in untreated dye. Similarly, root length measured was 7.05cm in water, and 0.92 cm in 1st cycle, and no growth was observed in 2nd cycle and untreated dye (Fig. 4.36).

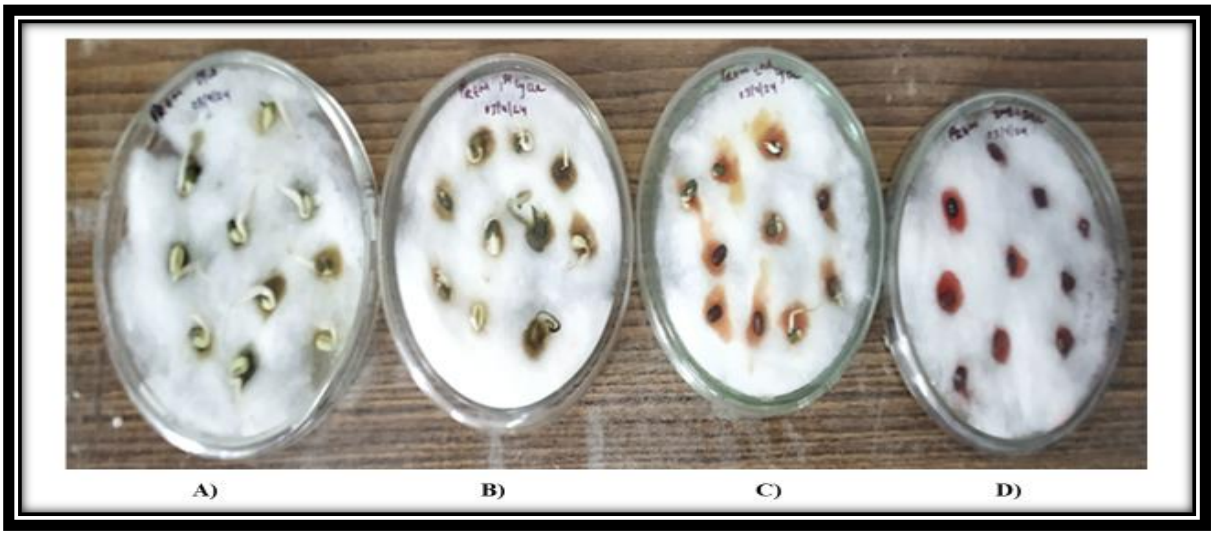

Fig 4.34: Germination of Moong seeds with A) Distilled water; B) 1st cycle of RMS beads; C) 2nd cycle of RMS beads; D) Untreated dye.

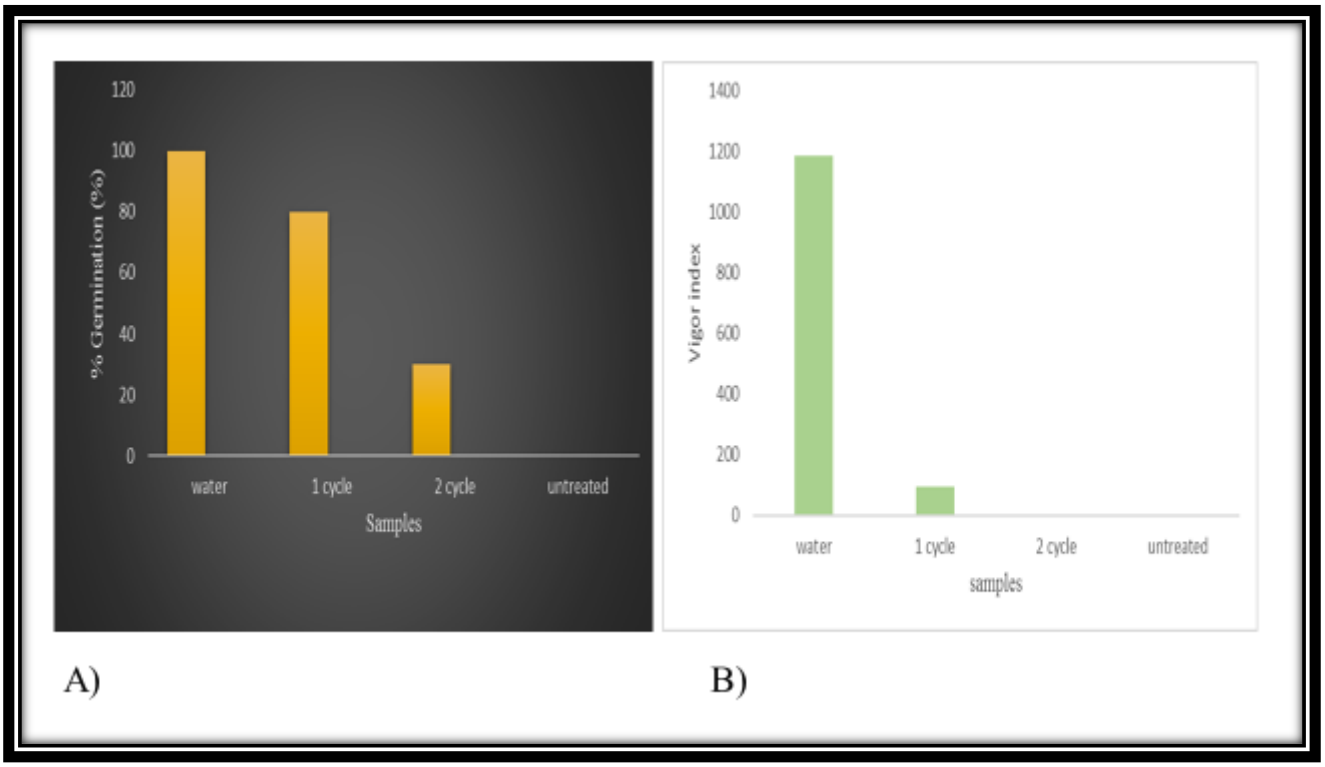

Fig 4.35: A) Percent germination and B) vigor index of under various treatment after 7 days of germination.

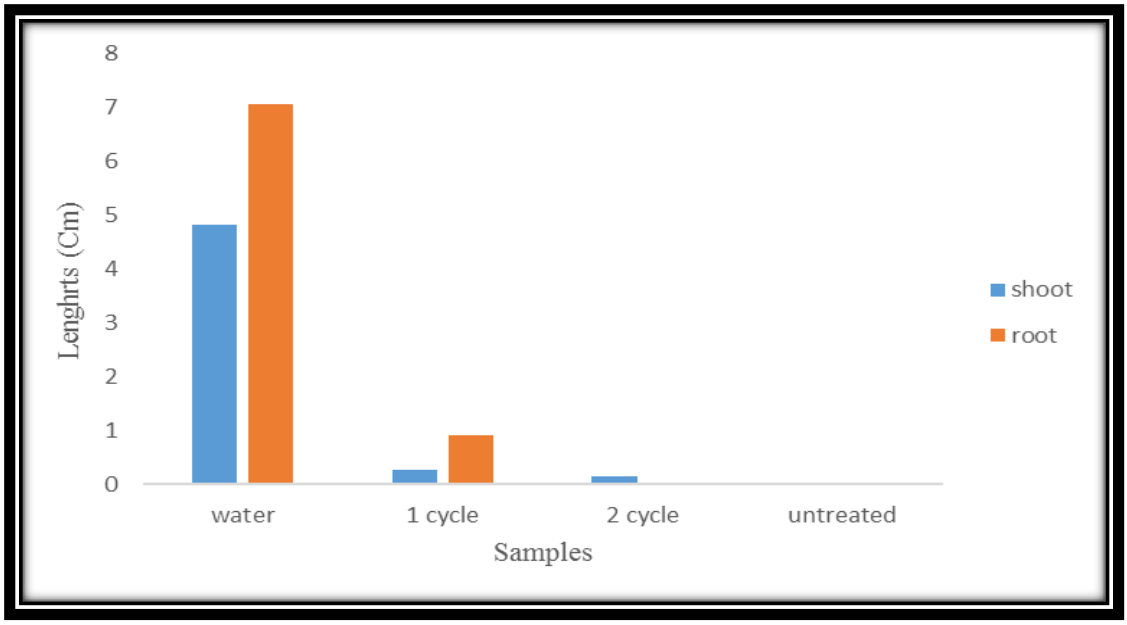

Fig 4.36: Shoot and Root lengths under various treatments after 7 days of germination.

Discussion

Dye industrial effluents represent a significant source of environmental toxicity, adversely impacting water quality and exerting harmful effects on microflora and aquatic ecosystems. The marine environment, known for its extreme conditions, harbours a diverse microbial population. These marine microbes are renowned for their unique mechanisms for tolerating extreme pH levels, high salt concentrations, and various harmful compounds including dyes (Varjani et al.,2015).

Samples were collected from various sites in Goa to isolate and screen dye-decolorizing bacteria. Mandovi and Zuary rivers are crucial waterways in North Goa and South Goa, but unfortunately, they face significant pollution challenges due to numerous industrial establishments along their banks. These industries often discharge their effluents directly into the rivers, leading to contamination and environmental decolorization. The unchecked release of industrial effluents into the river poses a major threat to aquatic life, biodiversity, and public health. The pollutants discharged by these industries can include heavy metals, chemicals, and other harmful substances, which can accumulate in the water and sediment, affecting the river's ecosystem. In Goa, there are numerous industries which are involved in the manufacture or use of these dyes in their production process viz. paints, printing and dyeing processes. Consequently, these environments offer ample opportunities for the presence of dye-decolorizing bacteria. Additionally, marine bacteria have a unique set of metabolisms which help them to thrive in these extreme conditions.

The enrichment technique is crucial for isolating and cultivating target microorganisms from complex environmental samples, such as those collected from different sites for the screening of dye-decolorizing bacteria. Enrichment involves providing favourable growth conditions that selectively promote the growth of the desired microorganisms while inhibiting the growth of others. From the morphologically distinct

colonies obtained on ZMA containing Congo red, fifteen isolates were selected for further evaluation. Among these isolates, five bacterial strains—DW4, RMS, RMW, PMCHW2, and PMSS1 demonstrated a prominent zone of decolourization of Congo red dye on ZMA, indicating their potential for dye decolorization.

When grown in ZMB containing congo red, bacterial strain RMS showed the highest 52% of decolorization as compared to other strains. Shah, (2014) reported that two of the six bacterial isolates, *Planococcus sp. ETL1* and *Bacillus sp.* (ETL3), were highly effective dye decolorizers. They reported that *Bacillus sp.* (S3) could decolorize at a rate of 68.09%, while *Planococccus* sp. ETL1 could decolorize up to 69.02%.

The genomic DNA was extracted from isolate RMS and sumitted for PCR amplification of 16 S rRNA gene and sequencing for identification. An effective tool for examining the morphology and surface properties of bacteria engaged in dye decolorization is SEM analysis. It gives the ability to view bacterial structures with details at high magnification, giving information about their size, shape, and surface characteristics. Cells grown in congo red (0.02%) containing ZMB showed aggregation and clumping which was not seen in control. This change in morphology suggests a response to the dye and indicates potential adaptations for decolorization. For optimization parameters, the highest removal efficiency and optimal pH for the removal of Congo red by the isolate RMS was determined to be pH 8.0. In biodecolorization processes, pH plays a crucial role as it can affect the solubility of the dye in solution and the enzymatic activity of microorganisms (Sahasrabudhe et al., 2014). Several studies on the removal of dyes using microorganisms, particularly bacteria, have indicated that a pH in the neutral or slightly alkaline range is optimal for removal (Junnarkar et al., 2006; Krishnan et al., 2017; Kurade et al., 2015). Consistent findings have been reported in studies on the biodecolorization of Reactive

Yellow 180, Reactive Red 180, Reactive Red 198, and Red RR by *Lysinibacillus* sp., where optimal decolorization occurred at pH 8 (Muruganantham et al., 2016).

The bacterial strain exhibited significant potential in decolorizing high concentrations of Congo red dye, showcasing efficacy even at a concentration as high as 0.8%. Specifically, the selected isolate was reported to decolorize (56%) at 0.02% of the dye. However, with an increase in dye concentration, a decline in the rate of decolourization was observed. This observation corroborates previous research findings indicating a negative correlation between microbial decolorization efficiency and higher dye concentrations, attributed to hindered bacterial growth (Chang et al., 2004). In the case of different NaCl concentrations, it was observed that the maximum decolourization was found at a 1.9% concentration of NaCl.

In a related study, *Pseudomonas aeruginosa* CR-25 was employed to decolorize Congo red dye under different NaCl concentrations (0%, 0.5%, 2%, 4%, 5%, and 6%). Results showed decolorization rates of 66, 65 and 63 % at NaCl concentrations of 0.5, 2 and 4 %, respectively, following 48 h of incubation (Joe et al., 2018). Moreover, Shang et al. (2019) noted a decrease in the decolorization of malachite green by *Klebsiella aerogenes* S27 at elevated NaCl concentrations. High salt concentrations cause plasmolysis in bacterial cells, affecting osmotic balance, and enzyme activity which slows down bacterial growth and as a result, limits the ability of bacteria to decolorize dye (Cui et al., 2014).

In the time course analysis, it was observed that removal efficiency increased with time, reaching 59% after 72 hours of incubation. Comparable findings have been documented in studies investigating the biodecolorization of various dyes such as Novacron Ruby, Novacron Brilliant Blue, Novacron Yellow, Novacron Super Black, Novacron Navy, Novacron Brilliant Yellow, Novacron Turquoise, and Novacron Blue DK by *Enterococcus faecium*, *Bacillus pumilus*, and *Bacillus thuringiensis* respectively (Bunjir sultana, 2017).

The present study demonstrated that the RMS strain exhibited remarkable efficacy in dye decolorization. After optimization increase of 7% dye decoloriztion was observed in comparison to the former %.

To check for decolorized dye product in the supernatant, TLC was performed however the bands were not observed in UV light may be due to a lower concentration of the decolorized product. (Kaur et al.,2023) reported that the complete decolorization of each dye by TLC analysis as the chromatographs for each of the culture JAS1-treated and -untreated dye solutions were compared with the water-dissolved solution of the original dye and lanes developed from spots made with processed supernatants hailing from dye decolorization trials in the absence and presence of JAS1 in-solution respective to each of the three dyes: Methyl Orange, Congo Red , and Reactive Black-*5*.

Immobilization of RMS cells in Na alginate beads was performed and decolorization efficiency in a batch reactor was estimated. It has been reported that immobilizing bacteria enhances the density of bacteria inside the bioreactor, which will speed up the bioreactor's rate of decolorization According to Chan et al., (2005). The substance permeates the gel surface in a bioreactor holding immobilized bacteria, enabling the bacteria to break down the material within the gel bead.

After immobilization of the RMS strain the dye decolorization percentage observed in 1st cycle was 64% while 2nd cycle was recorded to reduce 57% dye. Similar observations were reported by Prabhakar (2013), where two bacterial strains were evaluated for their ability to decolorize Reactive Black-81, Reactive Red-111, and Reactive Yellow-44 dyes when immobilized. It was found that both strains exhibited a significant improvement in decolorization percentage after immobilization compared to their free-cell counterparts.

Phytotoxicity investigations unveiled a significant impact on plant growth, particularly in seeds primed with untreated dye effluents, where germination was absent. This contrasted

sharply with seeds primed with treated dye, which showed no such inhibition of germination. Shoot and root lengths were observed in both controls (treated with distilled water) and treated dye samples. For instance, the percent germination of distilled water in Moong seeds was 100% while 1st cycle and 2nd cycle, treated effluent showed 80 and 30 % germination respectively. Similar findings were reported by Gudmalwar and Kamble (2012), who reported no germination inhibition in *Vigna radiata* and *Sorghum vulgare* seeds when treated with metabolites after complete decolourization of Reactive Red 4E8Y5 dye by *Providencia* spp. and *Bacillus*. These results underscore the potential benefits of utilizing treated dye effluents in mitigating phytotoxic effects on plant growth and development.

Conclusion:

In conclusion, this study demonstrated the biodecolorization of Congo Red dye by the bacterial strain RMS. Through optimization experiments, the optimal conditions for decolorization were identified, including a dye concentration of 0.02% and pH 8. Additionally, incubation at 28 °C with a 1.9% NaCl concentration yielded the highest degree of decolorization (59%) after 72 h of incubation. Furthermore, the use of immobilized beads showed enhanced dye decolorization of 64% in comparison to free cells. Phytotoxicity tests revealed that treated dye effluent did not inhibit the plant growth compared to untreated dye, indicating a reduction in toxicity. Overall, these findings highlight the potential of strain RMS to effectively decolorize Congo Red dye, rendering it less toxic and environmentally benign.

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APPENDIX

Appendix I: Media composition

1. Zobell Marine Agar Himedia

Ingredients	gm/L
Peptone	5
Yeast extract	1
Ferric citrate	0.100
Sodium chloride	19.45
Magnesium chloride	8.80
Sodium sulphate	3.240
Calcium chloride	1.800
Potassium chloride	0.550
Sodium bicarbonate	0.160
Potassium bromide	0.080
Strontium chloride	0.034
Boric acid	0.022
Sodium silicate	0.004
Sodium fluorate	0.0024
Ammonium nitrate	0.0016
Disodium phosphate	0.008
Distilled water	1000 mL
Agar	12
pH	7.5-7.7

2. Zobell Marine Broth Himedia

Ingredients	gm/L
Peptone	5
Yeast extract	1
Ferric citrate	0.100
Sodium chloride	19.45
Magnesium chloride	8.80
Sodium sulphate	3.240
Calcium chloride	1.800
Potassium chloride	0.550

Sodium bicarbonate	0.160
Potassium bromide	0.080
Strontium chloride	0.034
Boric acid	0.022
Sodium silicate	0.004
Sodium fluorate	0.0024
Ammonium nitrate	0.0016
Disodium phosphate	0.008
Distilled water	1000 mL
pH	7.5-7.7

3. saline

Ingredients	**gm/L**
NaCl	0.85
Distilled water	1000 mL

4. Nutrient agar Himedia

Ingredients	**gm/L**
Peptone	5
Yeast extract	3
Sodium chloride	5
Agar	15
Distilled water	1000mL
pH	6.8-7.2

Appendix II: Preparation of dye solution

Conc. of dye	Volume of dye stock	Volume of media
0.02%	0.5 mL	25mL
0.04%	1 mL	25mL
0.06%	1.5 mL	25mL
0.08%	2 mL	25mL
0.2%	5 mL	25mL
0.4%	10 mL	25 mL
0.6%	15 mL	25 mL
0.8%	20 mL	25 mL

Appendix III: Gram staining reagents

1. Crystal violet Himedia

Ingredients	**Quantity**
Ammonium oxalate	8 gm
Crystal violet	10 gm
Alcohol	100 mL
Distilled water	900 mL

2. Gram's iodine

Ingredients	**Quantity**
Iodine	1 gm
Potassium iodide	2 gm
Distilled water	300 mL

3. Decolorizer

Ingredients	Quantity
Ethanol	95 mL
Distilled water	5 mL

4. safranin

Ingredients	Quantity
Safranin powder	20 mg
Distilled water	20 mL

Appendix IV: Biochemical media

1. Sugar fermentation media

Ingredient	gm/mL
Peptone	10
NaCl	5
Distilled water	1
Phenol red	50 ml of 0.2%

Glucose	(10% solution)
Maltose	(10% solution)
Sucrose	(10% solution)
Lactose	(10% solution)

2. Hugh-Leifsons agar media

Ingredient	gm/mL
Peptone	2
NaCl	5
K_2HPO_4	0.3
Bromophenol Blue (1% aqueous)	3
Agar	4
Glucose	10 %
Distilled water	1000 mL
pH	7.1

3. Triple Sugar Iron agar media

Ingredient	gm/mL
Peptone	20
Tryptone	10
Beef extract	3
Yeast extract	3
Lactose	10
Sucrose	10
Dextrose	1
Sodium chloride	5
Ferrous sulphate	0.2
Sodium sulphate	0.3
Phenol red	0.024
Agar	15
Distilled water	1000 mL
pH	7.4

4. Phenylalanine deaminase agar media

Ingredient	gm/mL
Yeast extract	3
DL phenylalanine	2
Na_2HPO_4	1
NaCl	5
Agar	20
Distilled water	1000 mL
pH	7.4

Appendix V: Agarose Gel Electrophoresis

1. 0.8% agarose

Weigh 0.8g and dissolve in 100 mL of 1X TAE buffer to prepare 0.8% agarose. Melt the solution in microwave oven until clear, transparent solution is obtained. Add ethidium bromide to a final concentration of 0.5 µg/mL and cast the gel.

2. Ethidium bromide

Add 1.0 g of ethidium bromide to 100 mL of deionized water. Stir on magnetic stirrer for several hours to ensure that the dye has dissolve. Transfer the solution to amber coloured bottle and store at room temperature.

Appendix VI: Preparation of SEM reagents

1. Glutaraldehyde (2%)

Ingredient	**gm/mL**
Glutaraldehyde	2
Distilled water	100 mL

2. Ethanol

Conc. of ethanol	**Volume of ethanol**	**Volume of distilled water**
20%	10 mL	50 mL
30%	15 mL	50 mL
40%	20 mL	50 mL
50%	25 mL	50 mL
60%	30 mL	50 mL
70%	35 mL	50 mL
80%	40 mL	50 mL
90%	45 mL	50 mL
100%	50 mL	-