Development of Sustainable Integrated Fluidized Bed Bioreactor and Microbial Fuel Cell System Using Agro-Biowaste Derived Products for Congo red Dye Degradation

Submitted in partial fulfillment of the requirements for the award of the degree of

DOCTOR OF PHILOSOPHY

IN

CHEMICAL ENGINEERING

By

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CERTIFICATE

This is to certify that the thesis entitled "Development of sustainable integrated fluidized bed bioreactor and microbial fuel cell system using agro-biowaste derived products for congo red dye degradation" being submitted by **SHALINI** for the award of the degree of Doctor of Philosophy (Ph.D.) in Chemical Engineering to the National Institute of Technology, Warangal, India is a record of the bonafide research work carried out by her under my supervision. The thesis has fulfilled the requirements according to the regulations of this Institute and in my opinion has reached the standards for submission. The results embodied in the thesis have not been submitted to any other University or Institute for the award of any degree or diploma.

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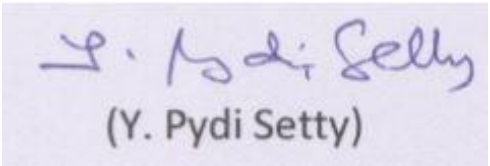

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DECLARATION

This is to certify that the work presented in the thesis entitled **"Development of sustainable integrated fluidized bed bioreactor and microbial fuel cell system using agro-biowaste derived products for congo red dye degradation"** is a bonafide work done by me under the supervision of Dr. Y. Pydi Setty and was not submitted elsewhere for award of any degree.

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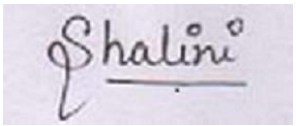

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ABSTRACT

The discharge of textile dyes into the environment without treatment is the significant problem in the context of environment, and human health. In particular, Congo red dye is predominantly used in textile industries among other azo dyes, the environmental challenges pertained to the release of Congo red dye is envisioned to develop a novel approach for biodegradation. Bioreactor and Microbial fuel cell based integrated approach is one of the ideal ways to achieve the Congo red dye degradation, which is the hypothesis of the thesis work. For this purpose, the objectives of this thesis work are divided into four, and at each objective level, it was focused to improve the efficiency of treatment and subsequently the outcomes of each objective are considered to arrive with the novel anaerobic-aerobic synergistic system i.e., microbial fuel cell-bioreactor coupled system for effective dye degradation. The objectives of the present study are: (i) To study the free cell and immobilized cell assisted decolorization of CR dye, (ii) Immobilized cell mediated CR dye decolorization in a multistage restricted fluidized bed bioreactor (MRFBBR), (iii) Microbial Fuel Cell (MFC) assisted CR dye decolorization using biowaste derived anode material, and (iv) Integrated MFC-MRFBBR system for CR dye degradation using corncob agro residue as co-substrate.

Pure culture *Bacillus subtilis* was chosen to accomplish all the objectives of thesis work. To improve dye removal efficiency through enhanced biological reaction with dye and higher colonization of microbes, it is essential to adopt immobilization technique for establishing structural support to the microbial growth and for interaction using matrix. Thus, polyurethane foam (PUF) cubes (size: 1 cm^3) were used for immobilization of *B. subtilis* throughout the thesis work. Bioreactors used for scaling up for the textile water treatment has motivated to use fluidized bed bioreactor, wherein the floating behavior of foam is limiting the active surface area availability for microbial colonization and interaction with dye. The active surface area for microbial growth and biological interaction are important factors to be considered for immobilization-mediated decolorization, even in the case of bioreactor-based dye degradation approach. In the present study, the active surface area can be improved in the reactor in two ways, (i) by avoiding the accumulation of PUF on the top surface of reaction volume and (ii) by providing free moving foams in larger number. To achieve this, existing fluidized bed bioreactor (FBBR) was modified into MRFBBR by introducing a porous stainless-steel box (PSSB), which is autoclavable, custom made, and cost-effective. The integration of anaerobic and aerobic system is the potential approach to treat the aromatic amines or other intermediate

complex compounds effectively, which are available after the treatment of anaerobic or aerobic alone. Biowaste derived electrode and agriculture residue as co-substrate are the key concepts considered in this thesis work to provide cost effective, efficient, universally acceptable integrated system for Congo red dye degradation.

UV-vis spectrophotometer, Fourier transform infrared spectrophotometry (FTIR), Gas chromatography-Mass spectrometry (GC-MS), Scanning electron microscope (SEM) were used in this study. The percentage of decolorization, mineralization analysis, morphological analysis using SEM, metabolites profile using GC-MS, FTIR for dye decolorization confirmation were used as methodologies in the present investigations.

The effect of parameters (dye concentration, pH, and temperature) on dye decolorization using suspended cells was studied. The corresponding cell mass (OD600) along with the decolorization profile, was considered to understand the effect of cell mass. SEM results revealed the porous structure of PUF and colonization of bacteria on PUF. The maximum decolorization of 92% was achieved by immobilization method within 6 h, whereas suspended cell assisted decolorization showed 82% within 12 h. The characteristic azo peaks have not been found in FTIR samples of immobilized decolorization. The results confirmed that immobilization of *B. subtilis* is an efficient method for CR decolorization compared to the suspended cells.

Dye decolorization studies were conducted in both FBBR and MRFBBR to give a note of significance of MRFBBR for improved decolorization. The enhanced CR dye removal was observed in MRFBBR. Further, optimization study for MRFBBR approach showing the maximum CR dye removal (92%), was obtained at a dye concentration of 100 mg/L, PUF weight- 5 g, pH-8, and glucose concentration- 4 g/L respectively.

Biowaste derived electrodes intended to improve the surface area, roughness, hydrophilicity, play pivotal role in the enhanced biofilm formation, in turn effects the MFC performance on treatment of dye contained wastewater. In the present study, Ficus relogiosa leaves (FRL) biowaste was used to develop the bioelectrode. Here, CR dye decolorization was performed using MFC, wherein the effect of dye decolorization with respect to dye concentration, glucose concentration, and hydraulic retention time (HRT) was studied. The surface roughness, and distribution of carbon powder on carbon cloth was confirmed using SEM analysis. Also, *B. subtilis* adhesion and chain like structure formation on the electrode confirmed the biofilm

formation on the electrode. The polarization curve was obtained to assess the MFC performance. The maximum power density of 70.50 mW/m^2 and current density of 251.79 mA/m^2 at 2224 Ω was achieved. The maximum decolorization, COD reduction of 80.95±2.08%, 73.96±1.76% were obtained respectively after complete treatment of MFC for 54 h.

At last, the integrated MFC-MRFBBR was demonstrated for complete degradation of CR dye. Reducing sugars (RS) obtained after pretreatment of Corncob (agriculture residue) was used in this study to develop a proof of concept to demonstrate the feasibility of developing cost-effective, commercially scalable strategy for the removal of dye effluents. The anaerobic and aerobic treatments were employed in MFC and MRFBBR respectively, wherein *Bacillus subtilis* pure culture was used as inoculum. Initially, the optimization of RS concentration (g/L) and feeding time (h) for improved decolorization of CR dye (200 mg/L) in MFC was conducted and these optimal conditions were used in the integrated system. The presence of aromatic amines and toxic intermediates after MFC treatment was noticed. Further, degradation of aromatic amines and formation of simple, non-toxic intermediates after treatment in the integrated system was confirmed by GC-MS, FTIR and UV-Vis analysis. The non-toxic nature of these metabolites was demonstrated with the phytotoxicity studies using *Vigna radiate* seeds.

Overall, the results of the studies have confirmed that MFC-MRFBBR integrated system is very effective for the Congo red dye degradation. The experimental results and remarkable evidences concluded that this anaerobic-aerobic system using the developed MFC-MRFBBR system is a promising strategy for dye degradation, and importantly this approach is cost-effective, scalable, and eco-friendly. The outcomes of the study could be directed for further research to upgrade this system for other azo dye degradation, and even this system may be adopted for wastewater treatment.

1. Introduction

1.1 Historical Note on Dyes/Brief History on Dyes

Dye is a substance which is applied to materials like paper, leather, wood, and textiles etc. for coloring. The dyes are aimed ideally to provide coloring to the material without any alteration due to washing or exposure to light and heat. Dyes are usually chemically bonded with the substrates, whereas pigments are not chemically bonded with the substrates and both have coloring property. Owing to the increased demand for aesthetics and social reasons, the color has significantly attracted the attention of humankind. Dyes have the coloring nature because of their innate characteristic features that include i) ability to absorb the light in visible spectrum range, ii) possessing the color bearing group (chromophore), iii) exhibit the electron resonance, and iv) having chemical structure with single and double bonds (Greenhalgh 1977). The color may lose even if the dye does not follow any single attribute mentioned above. Besides having chromophores, color helpers such as amino, carboxylic, hydroxyl groups and sulfonic acid also present in most of the dyes, called as *auxochromes,* contribute for the shifting of colorant and induce the solubility nature.

The mankind started using dyes for clothes and body decoration from the dawn of civilization. The scientific evidence witnessed that the dyes for fabric dying were used in China from long way back 2600 BC. In ancient days, the natural dyes mixed with oil and water were used for coloring the skin, jewelry, and clothes. The natural dyes extracted from plant and animal sources have been used for fabric dying and other purposes until 1990s. The dye obtained from madder plant have been found to be used in dying the clothes used for wrapping the Egyptian mummies. The dyes were used in the war for spraying on the soldiers to deceive in the war, historical evidence recorded that red color dye extracted from madder plant was sprinkled on the soldiers of Alexander and found that this dye constitutes alizarin (Bafana et al. 2011). The use of dyes in cosmetics, paper printing, pharmaceutical, food, textile, photography and various industries are limited to the availability of natural dyes. The scientific intervention started to develop the synthetic dyes with use of chemistry knowledge, the silk dyed with bright yellow color was reported first time through synthetic route by Woulfe in 1771, which was the result of picric acid formation with the chemical reaction of nitric acid with indigo. William Henry Perkin synthesized mauve from coal-tar chemicals and synthetic dye industry was launched at London in 1856. The era of synthetic dye started with first world synthetic dye and Otto Witt

invented other synthetic dye like azo dye in 1870 named as London yellow. Initially, the world's leading dye producer and supplier was from England. Later, Germany has become the leading supplier of synthetic dyes in the early 1990s and more than 85% of global requirement was fulfilled by Germany, in which other European countries (France, England, and Switzerland) had a share of 10%. Several reasons hampered the US industrial dye production, which include the civil war, non-availability of coal-tar, and favored tariff regulations for importing dyes (Bafana et al. 2011). Germany has focused on making explosives during World War I in 1914 and shut the dye production which, resulted in higher price raise. The increased demand for dyes has attracted the attention of many countries for establishment of dye industries (Morris and Travi 1992).

Dye industry market slowly turned towards Asia owing to the cheaper production cost. India and China have scaled up the dye industrial sector and are listed in the huge dye producing countries. However, the dye industry has got boosted after independence in India, established several industries which include Atul Products Ltd, Indian Dyestuffs, Atic Industries, Cibatui. The textile industries are the predominant users and accounted for higher consumption of dyes in India. Current statistics on the dyes and pigments global market and increasing demand with respective to application is summarized below as per the reports of grand view research ("Dyes And Pigments Market Size | Industry Report, 2020-2027"). The rapid increased demand for dyes in textiles, plastics, paints, construction, and coatings accounted for the global market size of 33.2 billion USD in 2019 and expected to be increased 5% more by 2027. The dye and pigment manufacturing industries have been utilizing the advanced treatment approaches for the removal of toxic and hazardous elements and aiming to increase the manufacturing venture to meet the global demand. Construction growth is being enormously increasing across the globe and substantially accounting for raised demand for dyes and pigments. In particular, the raise in population across the countries like US, India, China, Indonesia, UAE and Saudi Arabia augmented for the development of necessary infrastructure, which accounts for higher growth in construction sector. The increased demand for construction sector resulted in escalating the demand for dyes and pigments. However, the environmental concerns associated with the discharge of these dyes and pigments has initiated for stringent environmental protection policies all over the world and had huge impact on the dye industries.

1.2 Dye types

Dyes are classified into two types: natural and synthetic dyes. In the following sections, detailed note on natural and synthetic dyes is given. Importantly, the azo dyes, market growth, and environmental challenges etc. are discussed in this section.

1.2.1 Natural dyes

Natural dye coloration is known from ancient period. It is being widely used for handicraft, painting and handloom textiles. Natural dyes are ecofriendly dyes extracted or derived from plants, invertebrates or minerals. The main source of natural dyes are vegetables and plant sources like roots, berries, bark, leaves, and wood. Fungi is also the biological source for natural dyes. Natural dyes are non-toxic and non-allergic dyes since they are extracted from natural resources. In comparison to synthetic dyes, natural dyes produce uncommon, soothing and soft shades (Samanta and Agarwal 2009).

1.2.2 Synthetic dyes

Synthetic dyes are manufactured using organic molecules via chemical route. Owing to their feasibility in developing various kinds of dyes and huge production, use of synthetic dyes has been increasing across the world. In particular, textile industries are mostly preferred because of the need of dyes in large quantity and dye fixing is effective. Hereafter, various synthetic dyes have been discussed as given below.

1.2.2.1 Acid dyes

Acid dyes are water soluble with complex chemistry and consist of sulfo or carboxyl group. These dyes consist of non-caustic acids, has affinity for atmospheric fibers but lack dyeing affinity for cellulosic fibers. Acid dyes are used for coloring protein fibers i.e. wool, angora and silk. It is also useful in dying of milk protein fibers namely silk Latte and Soya protein. Acid dyes are categorized into three types such as; Equalizing/ leveling acid dyes, milling acid dyes, super milling acid dyes (Sekar 2011).

1.2.2.2 Basic (cationic) dyes

Basic dye is cationic stain, which reacts with the negatively charged material. Aniline dye is a cationic dye, which consist of amino groups / alkyl amino groups (as their auxochromes). These dyes are insoluble in water due to their basic nature and can be achieved by converting base into salt. Basic dyes also own great tinctorial strength, wide shade range, brightness and are relatively economical. Examples of basic dyes are; Methylene blue, Crystal violet, Basic

fuchsin safranin. Basic dyes are extensively used for dyeing of: Jute, Cut flowers, dried flower, Coir, Acrylic fibers (Uday et al. 2016).

1.2.2.3 Direct (substantive) dyes

Direct dyes are anionic dyes basically used for dyeing cellulosic fibrous materials. Sodium chloride (NaCl) or Sodium sulfate (Na_2SO_4) act as an electrolyte in aqueous dye bath solution in dying process. These dyes are also used as pH indicator and in biological stain process. It is a water-soluble dye due to its anionic nature and shows good affinity towards various fibers like; Cotton, Viscose, Silk, Jute, Linen. Direct dyes are categorized into anionic and cationic type of dyes (Sekar 2011).

1.2.2.4 Disperse dyes

Disperse dye is one kind of organic substances which is free of ionizing group. Disperse dyes are insoluble or partially soluble in water and are used for dying synthetic textile materials (nylon, orlon, polyesters and cellulose acetate). Use of dying agents allows for aqueous dispersion, and their insoluble aqueous dispersion derived the name as disperse dyes. It is mainly used for dying polyester yarn of fabric. Disperse dyes sometimes cause allergic problem than other textile dyes (Uday et al. 2016).

1.2.2.5 Sulfur dyes

Sulfur dye contains disulphide (S-S) linkage in their chemical structure. It is a water insoluble dye used in dying cotton, cellulose and regenerated cellulose. Insoluble sulfur is mainly used in rubber industry. Sodium sulfide (Na_2S) is used as a reducing agent, in order to make insoluble sulfur dye soluble. Na_2S is toxic in nature due to presence of sulfur, and it can be eliminated by using glucose ($C_6H_{12}O_6$) as reducing agent; to make it sulfur free (Gregory 1990).

1.2.2.6 Pigment dyes

Pigment dyes are inorganic coloring materials that are insoluble in water medium. Binding material (acrylic polymer with nonionic and cationic nature) is required for coloring process, whereas, they do not have direct affinity towards textile materials. Pigment dyeing is not a color dyeing process. Here, pigment sticks on the fabrics with the help of binder. In pigment dyeing no actual chemical reaction takes place between the dye and the fabric (Uday et al. 2016).

1.2.2.7 Mordant dyes

Mordant dyes are attached on the textile fibers with the help of mordant, because they do not have affinity towards the textile fibers. Alum, tin, iron, copper and inorganic chromium, are used as mordants. Mordants are used to improve the bond between the dye and fabrics.

1.2.2.8 Vat dyes

Vat dyes are water insoluble dyes, which are converted into soluble form by reducing agent (sodium hydrosulphite with NaOH) treatment in alkali and then are reconverted into their soluble form by oxidation. Inkodye/ indigo is a type of vat dye that uses light rather than oxygen to fix the dye, it changes from yellow to green in the bath and then blue as the air hits it. Vat dyeing process takes place in wooden vessel (Chattopadhyay 2011; Uday et al. 2016).

1.2.2.9 Reactive dyes

Reactive group in the dye establish covalent bonding between the dye and cellulosic fiber. These organic dyes chemically react with cellulose and form a covalent bond between the dye and fiber and hence are called as reactive dyes. A chromophore contains a reactive substituent in the dye, which allows chemical reaction directly on the substrate surface. Cellulosic fibers are essential for coloration and even these dyes can be used for the coloration of wool and nylon. However, either alkaline or acidic conditions should be maintained for fixing dyes on the fabric. For example, weakly acidic conditions are favorable for dying nylon. The hydrolysis of dyes takes place owing to their innate ability of reactive dyes to react with water and it causes the low utilization degree compared to other dyes. The presence of one functional group that is a reactive group in the dye resulted for low dye fixation. To improve the dyestuff efficiency in terms of coloration and fixation, bi or multi-functional groups contained reactive dyes have emerged (Uday et al. 2016).

1.2.2.10 Macromolecular dyes

Macromolecular dyes are simply composed of a group of compounds possessing the colored polymers. The inherent properties of the compounds that contribute for coloration and the application of these dyes are dependent on the molar mass. These dyes are being used as polymers and are also used for coloring the yarn, or fabric (Guthrie et al. 2002).

1.2.2.11 Metal Complex dyes

Metal complex dyes are also known as premetallised dyes. These dyes are classified into two groups, 1:1 metal complexes and 1:2 metal complexes. Mostly, 1:1 metal complex dyes are the

monoazo structured dyes which consists of carboxyl, hydroxyl, or amino groups that are responsible for generating the strong bond complexes with transition metal ions. Chromium, nickel, and cobalt were predominantly used as transition metal ions. These dyes are suitable for coloration of protein fibers. The other class of 1:2 metal complex dyes are suitable for coloration of polyamide fibers. In particular, pH regulators, levelling agents and electrolytes also play a significant role in dying process along with the composition of metal complex dyes (Gregory 1990; Chattopadhyay 2011).

1.2.2.12 Naphthol dyes

Napthols are the insoluble phenols that come under the class of insoluble azo dyes. These dyes are applied to the fiber with combination of diazotized base at low temperature. The alkaline environment is essential for dye solubility and especially these dyes are mostly cotton substantive. The alkaline pH of 12-14 is maintained in textile dying and these dyes in the market are available in micro pulverized form. Various methods have been employed for fixing napthol dyes on fibers including open check, circulating liquor, jigger, padding and tub dip (Gregory 1990).

1.2.2.13 Anthraquinone dyes

Second most important class of dyes is anthraquinone dyes, after azo dye. They have advantages like brightness and good fastness properties whereas disadvantages are expensiveness and are tinctorically weak. They are being used extensively because they provides combination of red and blue shades. Anthraquinone dyes are under attack from other types of dyes like heterocyclic azo dyes and chromogens of benzodifuranones (Sekar 2011).

1.2.2.14 Azo dyes

These dyes are synthetic organic dyes that contain azo group -N=N-. The number of azo groups in azo dyes varies from one to four, where two radicals are attached to azo group of which one or both are aromatic. Mostly, 50% of the commercial dyes are azo dyes. Azo dyes are thoroughly colored on textiles and can provide pack of rainbow colors to industries. It covers almost 60-70% of all dyes in textile and food industries (Gregory 1990).

1.3 Commercial Production of Azo Dyes and their extensive use in textile industry

R–N=N-R′ is the chemical description of azo compounds. Here, –N=N- represents azo group, and R or R′ can be either aryl or alkyl compounds. The structure of azo dye consists of amine

or a phenolic group coupled with diazotized amine and azo linkages (one or more) where, aromatic amines are used as precursor. Around 3,000 azo dyes are available, owing to their vast applications in various areas like printing inks, paints, varnish, lacquer, and wood stains. Colorants applied in the synthetic and natural textile fibers, plastics, leather, hair dyes, waxes, and petroleum also come under the category of azo dyes (Chung 2016).

Worldwide, azo dyes account for more than 50% in production and it is the largest and most versatile dye among all dyes. Apparently, 7×10^5 tons of azo dyes are produced all over the world and more than 2,000 different azo dyes are constantly being used in the present era. At present India contributes around 6% of the global market with more than 15% annual growth rate (CAGR) in the last decades. Ever increasing demand for dyes in the textile, ink and paint industry is proportionately expanding azo dye market in India. As per the Techsci report on azo dye market in India it is anticipated that 7500 metric tons of azo dyes are required for various industries by 2025. Azo dye is used as a key ingredient in dyestuff and mostly as a raw material in combination with other dyes to modulate the dye properties for enhanced dye applications in textile, paper, ink-printing and plastic industries. The infrastructure development in India and across the globe has geared up opening new avenues for azo dye market to meet the demand chain by industries. In particular, the textile industry is the topmost industry using azo dyes compared with paper, ink-printing, foodstuffs, and plastics industries and this will be estimated to continue till 2025 ("India Azo Dyes Market Study 2025 - Brochure").

1.3.1 Application of azo dyes in industries

- Azo dyes are used in dyeing textile fibers, particularly cotton but also silk, wool, viscose and synthetic fibers.
- They are abundantly available and are of low cost.
- They provide clear, bright and strong colors on textiles.
- They have extensive use in various industries for coloration; like paper, textiles, leather, food, cosmetics and pharmaceutical industries.

1.3.2 Challenges to the environment

The dye usage is highly warranted in many industries, which is already described in detail in previous sections. However, associated with their effective use in industries and market growth, there are several environmental challenges and concerns. These synthetic dyes account for water pollution, which leads to the ecological imbalance. Instead of discussing in more detailed

insights on the environmental challenges, glimpse of environmental challenges is summarized shortly in the following bulletins (Uday et al. 2016).

- The water bodies' interaction with dye effluents produces byproducts.
- Causes several health issues (allergy, carcinogenic etc.) due to toxic nature.
- Effects natural photosynthesis process due to its high resistivity towards light.
- Effects marine life and depletes oxygen in water bodies.
- Hazardous for animals and human being.
- Difficult to remove azo dyes from water bodies due to their high stability, and lead to long time accumulation in water.

1.4 Congo red (CR) dye

Paul Bottinger, who was working as dye chemist in Friedrich Bayer Company in Ebersfeld, Germany discovered Congo red (CR) dye in 1883. It is a first direct dye. The molecular compound was patented in the name of Bottinger and sold to AGFA (Actiengesellschaftfu ¨r Anilinfarbenfabrikation), Berlin a dye manufacture company in 1885. Variety of direct dyes are available in the market, and bright colors are being offered by dye industries, in particular, these dyes are mostly used for cellulosic fibers. Azo, stibene, oxazine, phthalocyanine and few thiazole and copper complex, which belong to chromophoric group of direct dyes whereas, maximum number of direct dyes belong to an azo group. In the similar way, CR dye consists of azo group and it is a diazo dye, which has been explored for various industrial applications.

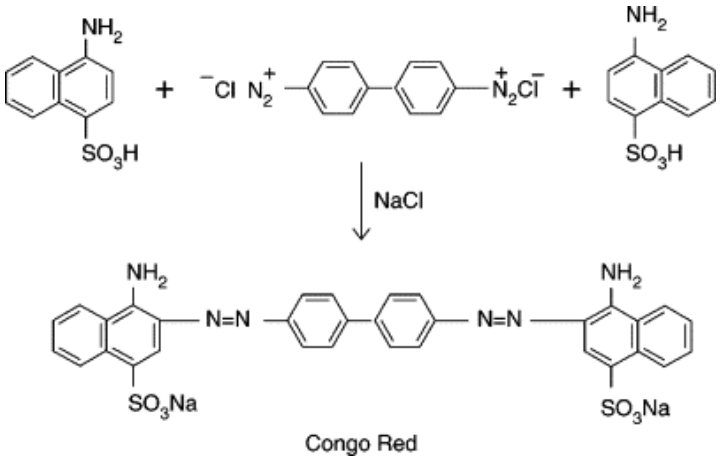


Figure 1: Synthesis reaction and Molecular structure of Congo red (Chattopadhyay 2011).

Synthesis of direct dyes are done by coupling hydroxyl, amino or other groups with an aromatic diazotized base. CR dye is a diazo dye. It is synthesized by combining bis-diazotised benzidine

with two molecules of napthionic acid. Blue dye is obtained. Further, Sodium chloride salt converts this blue dye into red disodium salt formally known as Congo red dye (Chattopadhyay 2011).

1.4.1 Uses of Congo red dye

Congo red dye, is enormously practiced in histological studies in medical field i.e. tissue staining for microscopic investigation. It is also used in chemical laboratories as an acid-base indicator, it converts red in alkaline whereas, blue in acid solutions (Mera and Davies 1984). CR dye is significantly used for cotton dyestuffs and cellulosic fibers.

1.5 Congo red impact on ecology- Insights on human health

Untreated textile effluents contain organic and inorganic pollutants, which contaminate the water bodies due to direct release. CR dye is recalcitrant towards light, and stay for long time in the water bodies. Release of CR dye effluents into rivers, lake and ponds makes them colorful, which resist light penetration into it, and effects the photosynthesis process, causes depletion of Dissolve oxygen (DO) and increases the Chemical oxygen demand (COD) due to thin layer formation of discharged dye over surface. Such synthetic dyes have aromatic structure due to which disappearance of color from dyestuffs do not take place even after coming in contact with soap, sweat, water, light and any oxidizing agent (Hernández-Zamora et al. 2016). Moreover, it is also unaffected with temperature and microbes due to presence of xenobiotic compounds accumulated in the ecosystem. Presence of genotoxic and toxic compounds in dye industrial effluents have made it as a source of significant pollutant to the environment across the world. Therefore, effective pretreatment for dye removal prior to discharge into the environment is quite essential. Discharge of dye effluents into environment by different industries is depicted in Figure 2 (Katheresan et al. 2018).

CR dye is toxic, carcinogenic and mutagenic in nature and is also hazardous to humans, animals and aquatic life. Its inhalation causes hemolytic anemia, hyperbilirubumia, nausea, vomiting, mental disorder, methemoglobinemia and sudden failure of kidney in humans (Chung 2016). It can also cause permanent inaction of eyes of humans and animals as well. Bafana et al. (2009) confirmed the toxicity of CR dye using HL-60 cells (Huma leukemia cell line) wherein intermediate metabolites such as benzidine and 4-aminobiphenyl induce the cell apoptosis (Bafana et al. 2009).

The discharge of textile dye contained water into lakes immediately affects the aquatic biotic, and the impact is extended even on health of animals, birds and humans. The misbalance of the hydrobionts is caused due to the presence of recalcitrant dyes in the water bodies. The colored dyes do not allow the penetration of light, which adversely affects the photosynthesis. This effect of photosynthesis eliminates the green algae, which is subsequently this effect passed onto the symbiotic aquatic life. The available literature on the effect of Congo red dye on aquatic biota is limited. However, the toxic effect of Congo red dye on aquatic biota has been demonstrated by restricting too few species of aquatic system. Zebra-fish embryos when exposed to CR dye elucidate the impact of dye on fish, and the observations made were production of yolk sac edema, stopped the larvae hatching, skeletal deformities and heart beating. This confirmed the toxic effect of CR dye. *P. subcapitata* (algae) was cultured in the presence of CR dye, the decrease in the photosynthetic pigment concentration and algae population observed, demonstrated clearly the toxic effect of dye on algae (Hernández-Zamora and Martínez-Jerónimo 2019).

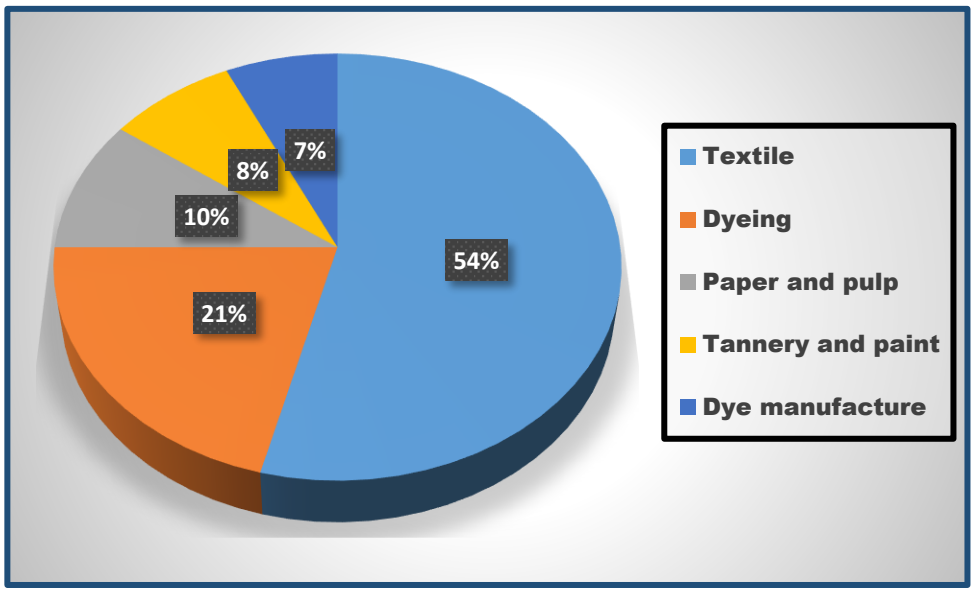


Figure 2: The percentage of dye effluents discharged into the environment by various industries.

1.6 Treatment approaches for the removal of Congo Red Dye

In the past, until nineteenth century, the government guidelines were not formulated well in extent of textile wastewater permissible limits to discharge into the environment from industries. However, it cautioned the industries to take measures for minimizing the discharge and adopt purification methods. To this end, equalization and sedimentation purification

methods have been employed. After establishment of dye effluent standards, improved dye removal methods were discovered such as degrading filter beds and activated sludge treatment process (Mezohegyi et al. 2012; Katheresan et al. 2018). These traditional methods were adopted effectively by various industries but high cost and maintenance is required. At present, dye removal methods can be categorized into three different types namely physical, chemical and biological. A short note on physical, chemical and biological methods are given in the following sections. A brief outline is given on the biological methods and trending technologies for dye degradation studies.

1.6.1 Physical methods

Physical methods are based on mass transfer mechanism. These methods are conventional and frequently adopted due to their simplicity and effectiveness. In comparison to chemical and biological methods small amount of chemicals are required in physical methods for dye removal. Adsorption, coagulation or flocculation, ion exchange, irradiation, membrane filtration, nano filtration or ultra-filtration and reverse osmosis are the examples of physical dye removal methods (Robinson et al. 2000; Katheresan et al. 2018). Few physical methods for Congo red dye removal are discussed in Table 1.

Table 1: Description of various physical methods for CR dye removal.

Physical methods	CR dye removal methods	
Adsorption	**Nanoparticles**	$Fe_xCo_{3-x}O_4$ (Liu et al. 2019), biosynthesized zinc oxide (Debnath and Mondal 2020)
	Nanocomposites	Guar gum/ activated carbon (Gupta et al. 2020), Polypyrrole and polyaniline (Aliabadi and Mahmoodi 2018)
	Agricultural waste	Sugarcane bagasse (Zhang et al. 2011), Rice hull ash pellet (Chou et al. 2001), Cabbage waste powder (Wekoye et al. 2020)
	Hydrogel	Chitosan hydrogel beads (Chatterjee et al. 2009), pineapple peel hydrogel (Dai et al. 2020)

	Activated carbon	Activated carbons (Kannan and Meenakshisundaram 2002), Jujube seed activated carbon (Aminu et al. 2020)
Coagulation or flocculation	**Coagulant**	Aluminum sulfate nano coagulant (Garvasis et al. 2020), Surjana seed powder, maize seed powder and chitosan (natural coagulant) (Patel and Vashi 2012), Chitosan/PVA/zeolite composite coagulant (Habiba et al. 2017)
	Flocculent	Dextran based flocculent DAB (Zhao et al. 2018), microbial flocculent (Wang et al. 2020), cyclodextrin-based acrylamide polymer flocculant (Su et al. 2020)
Nano-filtration or ultra-filtration	Polypyrrole/sintered pozzolan ultrafiltration membrane (Derouich et al. 2020), Polypiperazine amide nanofiltration (PA–NF) membrane (Hairom et al. 2014)	
Osmosis	Forward osmosis hybrid system (Ding et al. 2020), (Li et al. 2020)	
Irradiation	Solar irradiation (Jiang et al. 2020), visible light irradiation (Zamani et al. 2020)	
Ion exchange	Ion exchange resin (Jia et al. 2020)	

1.6.2 Chemical methods

Chemical theories and knowledge acquired in chemistry has led to various chemical treatment methods for dye effluent removal. Advanced oxidation process, electrochemical destruction, Fenton reaction dye removal, oxidation, ozonation, photochemical and ultraviolet irradiation are the examples of chemical dye removal methods (Robinson et al. 2000). Chemical treatment methods are expensive compared over physical and biological methods. Consumption of high electrical energy from equipment and reactors, consumption of huge chemicals and reagents and requirement of specific equipment are limitations of chemical dye removal methods. Discarding secondary metabolites generated after effluent treatment is another additional major

issue of this method (Katheresan et al. 2018). Few chemical methods for Congo red dye removal are discussed in Table 2.

Table 2: CR dye removal through different chemical methods described.

Chemical Methods	CR dye removal methods
Oxidation process	Thermally activated persulfate (PDS) oxidation (Luo et al. 2020), UV/H_2O_2-based advanced oxidation process (Mullapudi et al. 2020)
Fenton method	Photo Fenton (Molla-Babaker and Idreesb 2020), Fenton reagent (Askarniya et al. 2020)
Ozonation	Hybrid system (bioremediation + ozonation) (Goswami et al. 2020), Moving bed biofilm reactor (MBBR) coupled with ozonation (Dias et al. 2020)
Photochemical/ photocatalytic	Tin dioxide for photocatalytic degradation (Ma et al. 2020)

1.6.3 Biological methods

Biological methods are cheapest and easiest methods compared to physical and chemical methods for dye effluent treatment. Microorganisms are the biological agents used for the dye effluent treatment. Based on the importance of oxygen for microbial growth the biological methods are classified into aerobic, anaerobic and synergistic (both aerobic and anaerobic). Combination of aerobic/anaerobic treatment alone known as a conventional biological method has been employed for dye effluent treatment (Bhatia et al. 2017). This conventional method alone is not potential enough to remove the toxic effect completely from dye wastewater. Other biological conventional methods like adsorption on microbial biomass, algae degradation, enzyme degradation and use of fungal cultures, microbial cultures (pure and mixed culture) are used for dye removal. The culture maintenance, handling the cultures for large scale is quite essential with followed guidelines of current good laboratory practices (cGLP) and current good manufacturing practices (cGMP). The favorable conditions and maintaining those conditions are vital factors to be considered in the development of biological methods for any treatment in particular dye effluent treatment. However, the growth rate of microorganism is an important factor for dye removal process which decides the removal rate of dye and reactions involved

(Robinson et al. 2000; Katheresan et al. 2018). Few biological methods for Congo red dye removal are discussed in Table 3.

Table 3: List of various biological methods for CR dye removal.

Biological methods	CR dye removal methods
Pure culture	*Bacillus sp*. (Hanis et al. 2020), *Bacillus sp*. (Mutant) (Gopinath et al. 2009), *Shewanella xiamenensis* BC01 (Ng et al. 2014), *Aspergillus terreus QMS-1* (Laraib et al. 2020)
Mixed culture/ consortium	Microbial consortium (Lade et al. 2015a), Mixed aerobic and anaerobic sludge (Hou et al. 2011)
Immobilized culture assisted degradation	Immobilized Terminalia arjuna seed biochar in Packed bed bioreactor (Goswami et al. 2020), Immobilized Polyurethane foam in upflow Column bioreactor (Lade et al. 2015a)
Microbial fuel cell assisted degradation	Aerobic and anaerobic sludge as inoculum (Cao et al. 2010), *Bacillus subtilis* (Prajapati and Yelamarthi 2020), activated sludge from sewage treatment plant (Senthilkumar et al. 2020)

The development of ecofriendly technology for dye removal from wastewater is a great need of present time (Rodríguez Couto 2009). Biological method is a road way for developing eco-friendly, low cost method for complete mineralization of pollutants (organic/inorganic). Biological dye removal is achieved by various microorganism like fungi, bacteria, yeast and algae under certain condition of environment (Pandey et al. 2007).

1.6.3.1 Microbial degradation of dyes

Biodegradation of dye is defined as microbial mediated breakdown of chemical dyes. It breaks the dye molecules into various byproducts with the help of various enzymes produced and is also an energy consuming process (Kaushik and Malik 2009). Under biodegradation process, these synthetic dyes are decolorized and fragmented into very simplified molecules, thus forming byproducts. Breakdown of chromophoric center of dye is known as decolorization (Kaushik and Malik 2009). Various microbes (bacteria, yeast, fungi and algae) have different potentials towards different types of dyes. The effective microbial decolorization can only be achieved through microbial adaptability into dye environment and its activity towards dye

(Chen et al. 2003b). Moreover, the enhanced potential degradation of dyes can be gained by selective strain under favorable conditions (Novotný et al. 2004).

1.6.3.2 Mixed microbial degradation of dyes

The mixed cultures had shown an effective degradation and almost complete mineralization towards azo dyes due to interactive metabolic activity between microbes. Due to complex structure of dyes, it is difficult to trace out the complete metabolic pathways involved in dye degradation when mixed cultures are used for the treatment. However, microbial consortium (a combination of individual strains) has found to be more effective than single strains. An individual strain of consortium may break the dye molecules from different positions or utilize the breakdown products by another strain. Consortium of mixed cultures are potential candidates for the removal of dye effluents and utilize the organic and inorganic compounds as a source of carbon, energy and nitrogen.

1.6.3.3 Immobilized microbial degradation of dyes

Cell immobilization technology is adopted for scale up of wastewater treatment process. Bio-carriers provide support to microbial growth colonization and facilitate interaction with dye effluents. Entrapment and attachment are the cell immobilization techniques, which are widely explored. Entrapment of cells are performed onto the porous martial or trapped inside fibrous materials, whereas, in attachment of cells, cultures are attached onto the surface of bio-carriers. The immobilized cultures possess more activity and are highly adaptable to the environmental changes, i.e. concentration of dye and pH. Immobilized cultures are more potent than suspended cultures.

1.6.3.4 Microbial fuel cell degradation of dyes

Microbial fuel cell technology is the recently emerged research for wastewater treatment. The concept of using MFC for dye removal from textile industrial effluent was evolved by considering the facts of potential use of microbial cultures of dye effluent removal and electricity generation. The current research trend wishes to bring a technology self-sustainable, cost effective and efficient for the removal of textile dye effluents. In this direction, MFC based approach for textile wastewater treatment is an ideal treatment approach to fulfill the aforesaid requirements. Various dyes that include di-azo dyes, mono azo dyes, anthraquinone dyes have been studied for dye degradation using MFC. Various microbes like bacteria (Franks et al. 2010), Fungi (Sekrecka-Belniak and Toczyłowska-Mamińska 2018), yeast (Schaetzle et al. 2008), algae (Lee et al. 2015), anaerobic-aerobic sludge, mixed consortium, pure culture

(Solanki et al. 2013) have been used in MFC as biocatalyst and are extensively investigated for the performance of microbial cultures. MFC system for dye degradation is a promising technology used for various applications such as wastewater treatment (Gude 2016), recovery of valuable products (Choi and Cui 2012), environmental sensor development (Shantaram et al. 2005), bioremediation (Solanki et al. 2013), hydrogen production (Tartakovsky et al. 2008) and renewable electricity production from biomass (Zhang et al. 2009).

1.6.4 Factors influencing biodegradation of dyes

Changes in the ecosystem depends on dynamic abiotic environmental conditions i.e. temperature, oxygen, pH, salts, metals, etc. Microorganisms are responsible for carbon, nitrogen and sulfur cycles in ecosystem, and significantly affected with change in abiotic conditions. These changes also affect decomposition rate of microbes present in environment. Therefore, these parameters should be optimized for finding potential microbial candidate for degradation of xenobiotic compounds. Hence, these optimized abiotic conditions play vital role in scaling up of bioreactors for real-time application.

1.7 Scale up of biological based treatment methods using Bioreactors

The development of sustainable, economically viable process with minimal time consumption are the major concerns for scaling up of the microbial mediated dye degradation (Kivaisi 2001; Ertuğrul et al. 2009). Ideally, bioreactors are used to well govern the optimal process parameters to enhance the treatment efficiency. Usage of bioreactors for textile wastewater treatment has attracted the attention and is especially aimed to provide optimal process parameters for microbial growth, colonization and improved biological interaction with dyes. Overall, bioreactors have been used for dye degradation studies in two ways, namely free cell systems in which microorganisms are directly inoculated into the bioreactor, and immobilized systems in which microorganisms either encapsulated in bio-carrier support or grown on the surface of bio-carrier (Georgiou et al. 2005; Mohanty et al. 2006). The immobilized system has advantages like more available surface area for microbial growth, enhanced efficiency, and better monitoring of process parameters. The immobilized microbial carriers in the bioreactor are the best choice for dye degradation due to the improved stability of microbes at extreme environments including pH and temperature and the process conditions can be tailored for treatment at higher concentrations (Ramsay et al. 2005). Various bioreactors using immobilized systems have been studied for azo dye decolorization, which include MBBR, PBR, fluidized

bed bioreactor (FBBR) and other reactors. The advantages and disadvantages of these reactors are shown in Table 4.

Table 4: Different types of Bed based bioreactors for dye decolorization.

Bioreactor Type	**Advantages**	**Disadvantages**	**Decol. (%)**	**Ref.**
Moving Bed Biofilm reactor (MBBR)	High nitrification rate Low Residual sludge production Stable and reliable operation	Settling issues Fouling	96.2%	(Zhang et al. 2017)
Packed Bed Bioreactor (PBR)	Translation into practical application is easy	High pressure drop Large dead zones	98.04%	(El-naas et al. 2014)
Fluidized Bed Bioreactor (FBBR)	Improved biological interaction with effluent under fluidized media Enhanced treatment efficiency with reducing hydraulic retention time (HRT) - No bed accumulation, oxygen transfer - Higher surface area available for biological interaction	High energy requirement Higher fluid velocity is not desirable	70-80%	(El-naas et al. 2014)
Up-flow fixed bed reactor	The multiple cycles do not hamper the decolourization efficiency	Continuous mode of operation with higher retention time, which reflects the time needed for diffusion of dye	95%	(Kurade et al. 2019)

		molecule to interact with microbial cells		
Up-flow anaerobic fixed bed bioreactor	Low-cost Environment and user-friendly approach	Higher HRT is required to improve decolourization efficiency of higher concentrated industrial dye effluent This bioreactor performs well at lower concentration of dye loading.	95%	(Khelifi et al. 2009)
Up-flow column bioreactor	Cost effective Eco-friendly	Achieving the optimal flow rate for enhanced decolourization efficiency is challenging.	99%	(Bedekar et al. 2014; Lade et al. 2015a)
Air bubble column bioreactor (Agar-Alginate Beads)	Air bubble improved the decolorization efficiency	The efficiency of the reactor was confirmed with lower concentration of dye	97%	(El-Naggar et al. 2004)
Airlift bioreactor	Immobilized enzyme and bead stability maintained at high flow rate Ensured good circulation for improved colour removal efficiency	The optimization of oxygen transfer rate to achieve maximum decolourization decides the reactor performance	100%	(Teerapatsakul et al. 2017)

		The optimal flow rate varies from pilot scale to industrial scale plant due to varied volume of treatment plants.		

The conventional bioreactor based treatment approaches for dye reduction follows different decolorization mechanisms using reductive agents including enzymes, redox mediators, and biogenic reductants (Pandey et al. 2007). The azoreductase enzyme responsible for azo dye reduction reaction under anaerobic condition, wherein the electrons generated by bacteria accounts for breaking of azo linkage resulted for dye decolorization.

1.8 Road way in developing Multistage Restricted Fluidized Bed Bioreactor: Concept and Hypothesis

FBBR has attracted attention for effective dye degradation, owing to its advantages of uniform mixing, enhanced mass transfer, particle distribution, uniform temperature, and availability of large active surface area. The immobilized cell-mediated approach has been chosen for effective removal of pollutants using FBBR, wherein enlarged surface area and effective mass transfer are essential to be provided to improve the removal efficiency. Herein, the porous structure of PUF foam improves mass transport of substrate and nutrients, which facilitate microbial growth and colonization on the surface. Inexpensiveness, effective cell attachment, and colonization are the advantages of PUF to be used as bio-carrier support (Feng et al. 2012; Lade et al. 2015a; Padmanaban et al. 2016). In addition, PUF has high porosity, good mechanical strength.

The major disadvantage of lower density PUF foam is non-availability of foam matrix at the bottom of the reactor due to the floating nature of the foam. It is essential to provide a homogeneous distribution of foam in the reactor for microbial colonization and biological interaction to enhance the biodegradation of dye. The modulation of existing fluidized bed bioreactor to make it available the PUF foam throughout the reaction volume is warranted. The restriction of PUF foam by changing the design of reactor is one of the possible alternative

strategy. The detailed concept, methodologies, efficacy of multistage fluidized bed bioreactor for dye decolorization was discussed in detail elsewhere in this dissertation work.

1.9 Microbial Fuel Cell for Dye Degradation

In general, MFC can be categorized into Dual chamber MFC and single chamber MFC. Dual chamber MFC is comprised of two chambers made up of acrylic sheet where, one is anaerobic (anodic) chamber and another is aerobic (cathodic) chamber. Both the chambers are separated by proton exchange membrane (PEM). Microorganisms produce electrons and protons in anodic chamber after utilization of substrate. Electrons move to cathode chamber via electric circuit attached to cathode, which acts as electron acceptor. Protons pass through PEM to cathode side here, hydrogen ions get attached to oxygen molecules and form water molecules. Anolyte solution at anode chamber is inoculated with anaerobes with substrate like glucose whereas, catholyte solution at cathode chamber is inoculated with aerobes or electron acceptor medium or oxygen. pH of 7 is maintained at both the chambers of MFC (Li et al. 2010; Solanki et al. 2013).

Single chamber MFC consists of anode chamber, it has simple design and reduces construction cost. Microfiltration membrane is attached in between anode chamber and air cathode. One side of cathode is exposed towards anolyte and another side in the air covered completely with a plexi sheet plate, which consists of holes to contact oxygen on cathode surface. Anode chamber is inoculated with cultures or mixed sludge and substrate. The azo bonds of dyes are broken using electrons and protons during power output, which results in colorless solution (Sun et al. 2009; Hou et al. 2011).

The electrons generated in MFC are carried out to the electrode via possible ways (He et al. 2017): (1) Direct electron transfer (through outer membrane cytochromes) (2) Electron transfer through artificial mediators (3) Electron transfer via nanowires of microorganism (4) Metabolic intermediates.

1.9.1 Azo dye decolorization in MFC

The biological methods can possibly overcome the limitations of existing physical and chemical methods with a higher removal efficiency of dye effluents. In addition to this, bioenergy production is feasible to make a self-sustained wastewater treatment plant to reduce the process cost for industries (Solanki et al. 2013; He et al. 2017). Thus, microbial fuel cell (MFC) systems are forecasted as a viable alternative way for the removal of dye effluents along with power

generation (Chandrasekhar et al. 2017; Pandit et al. 2017). Besides, this system can decrease 50-90% of solids generation and also minimize their disposal (Rabaey and Verstraete 2005). MFC is a novel and emerging technology for the wastewater treatment. This bio-electrochemical system can convert organic pollutants present in wastewater to bioelectricity. The phenomenon followed to generate electricity in the MFC is-the bacterial cellular respiration through its metabolic activity attributed to convert organic substrate into electricity, which aids for wastewater treatment process (Du et al. 2007). MFC is a very promising technology which is being used in powering gadgets (Aelterman et al. 2006), biosensors (He et al. 2006) and especially in wastewater treatment (Rabaey and Verstraete 2005).

Complete mineralization of azo dyes under biological condition at anode chamber under anoxic condition is only possible for few dyes i.e. acid orange 7 (AO7). Whereas, azo dyes generate sulfonated aromatic amines under anaerobic condition. Due to its toxic effect it should be further treated under aerobic condition which is a major concern for environment and problematic to MFC biotreatment also. Hence, sequential anaerobic- aerobic treatment for azo dye is required for decolorization and degradation process. However, until now partial degradation of azo dyes are only reported.

There are various factors, which affect the performance of MFC and dye decolorization efficiency. Dye concentration and type of dye decides the performance of MFC. It is observed that with increase in dye concentration the decolorization efficiency is decreased. Mu et al. (2004) studied the effect of pH on dye, and found that decolorization efficiency started decreasing with increase of pH of cathode (Mu et al. 2004). Performance of MFC is also affected with different dye structures. External load, hydraulic retention time (HRT), co-substrate, wastewater quality are also few parameters responsible to the performance and decolorization.

1.9.2 Detailed Mechanism of dye decolorization in MFC

Dyes can be decolorized in both the chambers (anode or cathode) of MFC. Dye effluents contain organic and inorganic substances, which act as fuel for MFC. It is observed from the literature that substrate i.e. as glucose, acetate, molasses etc. are essential for microbial growth whereas, toxic azo dyes exploit as co-metabolites.

1.9.2.1 Anodic mechanism of azo dye

Dye degradation mostly depends upon the co-metabolism reaction where, electrons are produced under oxidation process anaerobically. In general, few electrons are transferred to electrochemical active bacteria gathered (biofilm) on anode where, electrons travel to cathode and generate current. Whereas, the other part of electrons attacks the azo bond of the dye structure for reductive cleavage of bond. Hence, the competition arises for electrons in between dye molecules and anode in MFC (Sun et al. 2009; Hou et al. 2011). MFC based dye degradation is mainly because of the oxidative metabolism of bacteria other than sorption of dye by live or dead microbes (Sun et al. 2009). Dye degradation in MFC is either specific or non-specific. In case of non-specific reduction reaction, there is no relation between decolorization rate and molecular weight. The electron transport mechanism present in bacterial cell membrane or reduced compounds produced by anaerobic biomass generates the electron, and subsequently the dye accepts the generated electron. The azo dye reductase enzyme produced by bacterial metabolism contributes for the reductive reaction and the cell uptake of dye is essential to make available the dye for reduction process, which is executed by the intercellular azoreductase enzyme. Some bacteria produce extracellular azoreductase for dye decolorization. The improved performance of MFC for dye decolorization depends on the stabilization and complete microbial adoption, colonization on the bioelectrode. Anaerobic based MFC treatment can be used for both extra and intra cellular reduction of dyes at anode (Pandey et al. 2007).

1.9.2.2 Cathodic mechanism of azo dye

Dual chamber MFC has provided the dual treatment approach for azo dyes in single setup. The decolorized metabolites obtained from anodic chamber are further treated into cathode chamber abiotically for complete degradation/ mineralization. Moreover, in cathode chamber azo dyes fulfill the dual role by facilitating the cathode for electron acceptance and simultaneously decolorizing itself. The reduction of azo bond at cathode side takes place by utilizing two or four electrons that reduce to either hydrazo or amines, as discussed in equations (1) and (2).

$-N=N- + 2e^- + 2H^+ = -NH-NH-$ (1)

$-N=N- + 4e^- + 4H^+ = -NH_2-NH_2-$ (2)

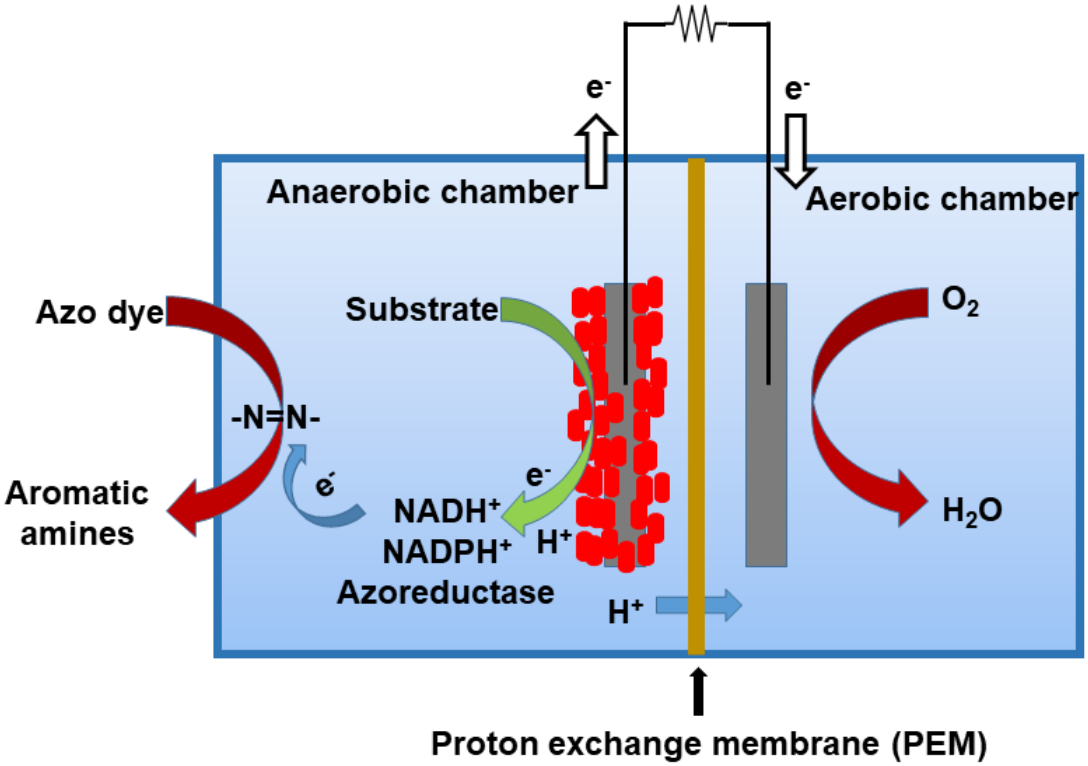


Figure 3: Schematic representation of mechanism of Microbial fuel cell assisted dye decolorization.

1.10 Trends in emerging concepts for complete degradation of Congo red

Several researchers to intensify the azo dye degradation process have demonstrated various combined anaerobic and aerobic biological treatment methods. In majority of cases, anaerobic condition is favorable and effective for dye decolorization and commercially successful but its reductive mechanism processes are very slow moving and generates methane rich gas. Due to high redox potential of aromatic amines generated during anaerobic process, they cannot be fully degraded. These partially degraded aromatic amines are known to be carcinogenic for human health and hazardous to environment. These aromatic compounds can be further mineralized by unspecific enzymes (Easton 1995) through hydroxylation and ring-opening under aerobic condition (Zissi and Lyberatos 1996).

Upflow anaerobic sludge blanket reactor/completely stirred tank reactor (UASB/CSTR) sequential anaerobic-aerobic system (Işk and Sponza 2003), mesophilic upflow anaerobic sludge blanket reactor/ mesophilic aerobic sequencing batch reactor (SBR) (Da Silva et al. 2012), UASB (ADQS free)/ ADQS supplemented reactor (Costa et al. 2010), anaerobic- fixed bed column reactor/aerobic reactor (Rajaguru et al. 2000) are few studies on combined

anaerobic and aerobic based bioreactors used for CR dye treatment. The development of sustainable technologies for wastewater treatment is the need of the hour. The idea of using microbial fuel cell technology has conceived for textile wastewater treatment since the generated electricity can be used for the maintenance of the treatment plant, and it comes under green technology platform owing to the application of biological degradation. Several studies have been conducted using microbial fuel cell technology for dye degradation with aim of developing sustainable technology for large scale textile wastewater treatment application. In particular, the concept of coupled microbial fuel-bioreactor cell system is emerging research work for textile wastewater treatment owing to the feasibility of complete degradation along with generation of electricity.

Li et al. (2010) had demonstrated dual chamber microbial fuel cell as a sequential anaerobic-aerobic system for CR dye removal. The authors reported that azo bond present in CR dye is cleaved under anaerobic condition using anaerobic sludge and then further these aromatic amines are treated in anode chamber with help of aerobic sludge (Li et al. 2010). Several studies were conducted to investigate the effect of glucose concentration, which was used as co-substrate for dye decolorization. Electrochemical systems have attracted the attention for dye decolorization studies, for example, CR dye decolorization was studied using combined bioanode-biocathode electrochemical system (Kong et al. 2014). The significance of glucose co-substrate addition and tailored electrodes for enhanced dye decolorization using single and dual chamber bio-electrochemical system (BES) has been explored (Kong et al. 2014). The development of economically feasible method relied mainly on electrode cost, electron donor (co-substrate), and membrane. Effective decolorization takes place with suitable biocatalyst, electrode arrangement assembly and combined treatment methodology. The combination of these two technologies along with agricultural waste as an electron donor could possibly enhance the dye removal process and make the process economically viable. The research findings on dye decolorization using MFC and bioreactor alone or in combinations significantly have given the insights for carrying out further research towards complete degradation of dyes. The available knowledge in the field is substantially useful for devising the work plan of the present thesis. The combined approach of using MFC-bioreactor integrated system would be the potential approach, which is an emerging research concept in the field of textile wastewater treatment. Recently, studies have been conducted using integrated bioreactor-bioelectrical systems for complete dye degradation.

1.11 Synchronized/Integrated Bioreactor and MFC based approaches for Cong Red degradation

Many azo dyes have been investigated for decolorization and degradation studies to elucidate the cleavage of molecular structure into simple compounds. The major conclusion from the previous studies is that either aerobic or anaerobic treatment approach alone could not degrade the azo dye completely. By considering the toxicity of the compounds formed during the decolorization, the quest for understanding the integrated treatment approach for complete dye degradation has been developed. The reduction of azo dye using anaerobic treatment requires co-substrate, which acts as electron donor. For example, glucose, organic waste materials and acetate have been used as co-substrate in the anaerobic treatment of azo dye (Fernando et al. 2014). These aromatic amines if kept under the anaerobic treatment even after achieving the decolorization, these compounds remain present in the water and may not degrade further. The oxidative environment established under aerobic condition facilitate for further degradation of these aromatic amines. The sequential treatment, which comprises of anaerobic and aerobic treatment may assure the complete biodegradation, but it is quite essential to make these integrated systems more economically viable, providing easy operation, and be able to scale up the technology.

Microbial fuel cell (MFC) technology has been proved as an economically viable system for the treatment of wastewater. The concomitant treatment of wastewater and electricity generation have attracted the attention to develop the MFC technology for dye degradation studies. Several studies have been conducted on the MFC based treatment for dye degradation and yet the concluding remarks of the findings so far confirmed that MFC based treatment alone could not completely degrade the azo dye (Parthasarathy and Narayanan 2014; Huang et al. 2017; Yuan et al. 2017; Thung et al. 2018; Mani et al. 2019). The discharge of the treated water using MFC is environmentally toxic, and hence it is imperative to achieve the complete degradation of aromatic amines formed after the MFC treatment. The intervention of bioreactors for aerobic treatment is a potential solution for achieving the complete degradation of aromatic amines formed during the MFC treatment. Given this, the research has aimed to develop integrating MFC and bioreactors for complete degradation of azo dye, wherein the anaerobic and aerobic treatments have been included in the integrated technology (Cui et al. 2012; Fernando et al. 2014; Sultana et al. 2015; Das and Mishra 2019). However, the coupled anaerobic and aerobic treatment strategy has not been studied for CR dye degradation and few

studies have been explored to demonstrate the use of agricultural residue for azo dye degradation (Saratale et al. 2009; Zeng et al. 2011; Vats and Mishra 2017). It was hypothesized that the integration of in-house MFC and MRFBBR can be used to execute the anaerobic and aerobic treatment, which could be the potential approach for complete degradation of CR dye. In the present study, MFC and MRFBBR coupled system has been developed and studied for CR dye degradation. Here, the authors report for the first time that the integrated model consists of anaerobic and aerobic treatment for complete CR dye degradation which is cost-effective, user-friendly and sustainable. The pretreated corncob was used as a co-substrate instead of using glucose in the MFC and MRFBBR.

2. Literature Review

Biological wastewater treatment has several advantages over physicochemical treatment such as: eco-friendly and cost-effective method, results in reduced sludge production and complete mineralization and less consumption of water for dilution of effluents (Hayat et al. 2015). Hence, biological treatment is considered as a potential approach for CR dye degradation. The development of sustainable, cost-effective, eco-friendly approach for dye degradation is possible using biological treatment (Saratale et al. 2011a). However, a thorough understanding on the selection of microorganism, choice of co-substrate, improved biological response using immobilization, governing the optimal conditions for improved biological treatment, and finding effective strategy for complete degradation of CR dye is essential for the development of a novel approach. The search for biological waste usage for dye degradation either in bioreactor or in microbial fuel cell has shifted the research with an aim of developing cost-effective, and sustainable method. The choice of biowaste used for any treatment process depends on the abundant availability, easy processing for their use in the treatment, possibility of scaling up the technology, and that, importantly the treatment efficiency is not compromised. The basic outline of the present study was framed only by considering the postulated concepts in the literature review that created the foundation for the present study. Thus, the complete literature survey pertained to developing an innovative approach for complete degradation of CR dye using coupled anaerobic-aerobic system discussed in detail in subsequent sections.

2.1 Biological methods for Congo Red Dye Degradation

Biological method for dye removal denotes the use of living microorganisms or enzymes for the treatment of wastewater contained with dye effluents. These methods are considered as eco-friendly, and the advances in biochemical engineering has directed towards the development of biological methods for dye degradation. In this direction, various microorganisms have been explored for Congo red dye treatment like; **Bacteria:** *Bacillus sp.* (Hanis et al. 2020), *pseudomonas sp. Mutant* (Gopinath et al. 2011), *Acinetobacter baumannii YNWH 226* (Ning et al. 2014; Li et al. 2015), *Bacillus thuringiensis RUN1* (Olukanni et al. 2013), *Shewanella xiamenensis* BC01(Ng et al. 2014), **Fungi:** *Aspergillus terreus QMS-1* (Laraib et al. 2020), *Aspergillus Flavus* (Singh and Singh 2010), *Phanerochaete chrysosporium* (Bosco et al. 2017), *Aspergillus niger* (Asses et al. 2018; Hamad and Soliman 2020), **Algae:** *Arthospira maxima, Haematococcus sp., Chlorella sp., Chlorella vulgaris, Scenedesmus obliquuss, S. officinalis,*

*and S. quadricaud*a (Mahalakshmi et al. 2015), *Chlorella vulgaris* (Hernández-Zamora et al. 2015), **Yeast:** *Pichia sp.* (Victor et al. 2020). Other than microbial cultures **enzymes** such as, Laccase (Lopez-Barbosa et al. 2020), ligninolytic enzymes (Sosa-Martínez et al. 2020), are demonstrated for CR dye removal studies. The understanding on the microbial and enzymatic dye effluent treatments and the advances in the use of genetic engineering tools for enhanced process efficiency has emerged the research in line of recombinant microbial cultures for the dye degradation. Removal of Congo red dye has been also studied through genetic mutation of microorganisms (Gopinath et al. 2009; Ng et al. 2014). The extensive studies on the dye effluent treatment using microbial cultures assessed the significant factors influencing the treatment efficiency, importantly optimal conditions for microbial growth and the metabolic pathway triggers the dye utilization through breakdown of the complex dyes (Gao et al. 2018). Azoreductase, laccase, peroxidase and exo-enzymes have been identified as responsible enzymes in cleavage of complex dye into simpler molecules, and further these simpler compounds can be utilized by microbes (Shabbir et al. 2017).

Azoreductase and laccase are found to be potential enzymes for dye decolorization and degradation (Singh et al. 2015). Sosa-Martinez et al. (2020) extracted ligninolytic enzymes from *Phanerochaete chrysosporium CDBB 686* for Congo red dye decolorization via solid state fermentation. Corncob was utilized as carbon source as well as support material for enzymatic production in packed bed bioreactor and decolorization was achieved at optimal conditions (Sosa-Martínez et al. 2020). CR dye degradation can be achieved under aerobic, anaerobic or anoxic condition. Generally, azoreductase enzymes are responsible for azo dye degradation. NAD (P) H: flavin oxidoreductase are confined inside bacterial cell membrane to intracellular or extracellular sites. NADH, NADPH and $FADH_2$ are the reducing agents which catalyze the azoreaction in their presence and also act as electron donor (Singh et al. 2015). These azoreductase enzymes transform colored dye solution into carcinogenic colorless aromatic amines (Ajaz et al. 2020). In aerobic condition NADH molecules are inhibited due to presence of oxygen and barricade the transfer of electrons from NADH to azo bond (Singh et al. 2015). The experimental conclusions using microbial and enzymatic dye treatment methods have emerged the notion of developing advanced technologies such as; Bioreactors, Bio-electrochemical systems, Hybrid systems, and anaerobic-aerobic systems for CR dye degradation.

2.1.1 Suspension cultures for Congo Red Degradation

Suspension cultures are the free cells used for degradation of dyes. CR dye is water soluble anionic azo dye, which is highly hazardous to human and animals (Mittal et al. 2014). Free cell CR degradation studies are employed for different ways of treatment methods i.e. static, flask, bioreactors, MFC, and synchronized approaches. The research outcome of CR degradation using various microorganisms is compiled in Table 5.

Table 5: Bacterial free cells employed for CR dye removal.

Microbes	Conditions	Results/Comments	Ref.
Pseudomonads	ambient temperature; anaerobic-aerobic	134.9 mg/l per day maximum degradation rate.	(Rajaguru et al. 2000)
D. alaskensis	37°C, static; Strict anaerobic condition	Lactase, sulfate and iron at optimized condition enhanced the dye decolorization.	(Diniz et al. 2002)
pseudomonas sp. mutant ACT 1	pH, 7; 30°C, anoxic condition	Ion concentration: Cr (VI), Zn (II) and Cu (II) were 0.8958, 0.3028 and 0.204 g/l respectively were obtained at optimized condition.	(Gopinath et al. 2011)
Bacillus thuringiensis RUN1	pH,7.2; static anoxic condition	72.84 ± 3.25% decolorization was achieved within 12 h for 100 mg/L CR dye.	(Olukanni et al. 2013)
Shewanella xiamenensis BC01	37°C, 180 rpm, pH, 4.2, anoxic condition	96% decolorization achieved at 200 mg/L CR dye concentration. Nanowire structure of bacterium accelerated the electron transfer for CR reduction.	(Ng et al. 2014)

Acinetobacter baumannii YNWH 226	29°C; DO: 2.6 mg/L, aerobic condition	96.3% CR removal and 52% COD removal was obtained for 400 mg/L CR dye.	(Li et al. 2015)
Bacillus subtilis	pH: 8; 37°C; aerobic condition	92% decolorization and 86.6 % COD removal achieved in 24 hours for 100 mg/L CR dye.	(Shalini and Y. 2019)
Bacillus sp.	pH: 7.55; 30°C; anoxic condition	83.12% decolorization achieved within 5 days at 25 ppm.	(Hanis et al. 2020)

Bacillus species are found to be highly potential candidatures for azo dye degradation due to their ability to produce azoreductases and NADPH reductases (Chengalroyen and Dabbs 2013). In another study, *Bacillus sp.* from tannery industry has shown higher degradation and was reported that the role of mutagenesis approach in *Bacillus* was found to be a potential alternative for CR degradation (Gopinath et al. 2009). Apart from these approaches, several studies on azo dye degradation using immobilized cells were found to have improved dye degradation (Lade et al. 2015a; Hameed and Ismail 2018). In the previous reports, it is seen that immobilization of microbial cells/consortium showed higher CR removal compared to free cells. Free cells have disadvantages like; higher dye loading rate and biotic condition. Therefore, free cells are not potential enough to overcome with high rate of concentration for dye degradation and hence immobilized techniques are adopted to strengthen the cells.

2.1.2 Immobilized cultures for Congo Red Degradation

Immobilization of cells takes place on bio-carriers, which are the supporting materials used for holding up the cells and accumulation of microbes takes place for biofilm formation to activate the treatment process efficiently. It provides high cell mass density, which enhances the decolorization rate in bioreactors. Immobilized cells have several advantages over free cells like; improved activity, viability and productivity, long term cell maintenance and operation, automatic generation of cells, stability, stable operation, protection from shear forces and abiotic environmental changes (Zhu 2007). Talha et al. (2018) had compared free cell and immobilized cell for CR dye degradation in packed bed bioreactor and coconut shell biochar was used as immobilized carrier material. The results concluded that the free cells could be able

to attain 65.95% removal at 100mg/L of CR concentration whereas, bio-char immobilized cells achieved 63.25% of CR dye removal at 300mg/L CR concentration. Hence, it was concluded that immobilized cell methods are effective for dye degradation up to high concentration. The large surface area of bio-char improved the contact between microbes and dye, which significantly improved the CR dye removal at higher rate. In addition, the active sites of bio-char significantly helped to capture the dye pollutant in the scaled solution. Further, dye degradation takes place on active pore sites and also subsequently renews the pores for absorption (Abu Talha et al. 2018). In another study, natural luffa sponge was used as natural bio-carrier material for CR dye degradation using *Aspergillus terreus* QMS-1. It is a dried form of vegetable fibre of Luffa cylindrica or Luffa aegyptiaca. Luffa fibers have parallel and antiparallel arrangements. The spatial structure provides large opening and free spaces to facilitate the nutrients inside microbes and oxygen diffusion up to inside sites for biofilm formation. However, 100 percent CR decolorization was achieved at 100 mg/l at 48h HRT (Laraib et al. 2020). Different bio-carriers employed for CR degradation studies are depicted in Table 6.

Table 6: Advantages and disadvantages of bio-carrier over Congo red dye removal.

Microbes	Bio-carriers	Advantages	Disadvantages	Ref.
Microbial consortium	Polyurethane foam (PUF)	High porosity, good mechanical strength.	Low density	(Lade et al. 2015a)
Brevibacillus parabrevis	Coconut shell bio-char	Large surface area, more active surface area for immobilization, low cost.	Effected by the dye concentration and abiotic conditions.	(Abu Talha et al. 2018)
Providencia stuartii MG1	Terminalia arjuna seed biochar	Large number of free pores, large surface area, improved bioremediation and enhanced dye removal efficiency.	Carbon, hydrogen and nitrogen were increased after CR degradation, biofilm formation on the pores depends upon	(Goswami et al. 2020)

			substrate concentration, pH and temperature.	
Aspergillus terreus QMS-1	Luffa cylindrical (natural luffa sponge)	Parallel and antiparallel arrangement of fibers provides spatial arrangement, high porosity.	Low density and mechanical strength.	(Laraib et al. 2020)

2.1.3 Polyurethane Foam as Immobilized matrix for Congo red Degradation

Polyurethane foam has all qualities required of an ideal support matrix for immobilization (Patil et al. 2006). Application of carriers are widened because of high surface area for complete cell immobilization, avoid washing of cells, stability, withstand and protect the biomass in wider range of temperature, pH and dye concentration (Ramsay et al. 2005). However, in bioreactors it is necessary to select an appropriate bio-carrier due to large reaction volume (Lade et al. 2015a).

The immobilized cell-mediated approach has attracted the attention due to effective removal of pollutants, wherein enlarged surface area and effective mass transfer are essential to be provided to improve the removal efficiency. Herein, the porous structure of PUF foam improves mass transport of substrate and nutrients, which facilitate microbial growth and colonization on the surface. Low cost, effective cell attachment, and colonization are the advantages of PUF to be used as bio-carrier support (Feng et al. 2012; Lade et al. 2015a; Padmanaban et al. 2016). Also, PUF has high porosity, good mechanical strength and also maintains proper diffusivity of dissolved oxygen inside PUF (Feng et al. 2012).

Lade et al. (2015) investigated the immobilized mixed bacterial consortium in upflow column bioreactor for mineralization of CR dye and real textile wastewater (RTW). Complete CR decolorization was achieved from microbial immobilized PUF-consortium. Here, PUF supported microbes under microaerophilic condition and at different dye concentration, pH and additional carbon and nitrogen sources showed ability to enhance the decolorization. Hence, Biofilm based reactor performance and efficiency relied on attachment of cells and its favorable growth conditions (Lade et al. 2015a).

Ratio between PUF carrier packaging rate and bioreactor capacity also decides the rate of CR decolorization. The removal efficiency of CR dye was increased with filling ratio of PUF. The result showed that 69% dye removal was obtained at 10% filling ratio and it increased to 89% with 35% filling ratio at 55mg/L of dye in Moving bed biofilm reactor (MBBR). However, increase in dye concentration diminished the rate of dye removal efficiency. Good removal efficiency rate was obtained with increase in PUF filling ratio because of enhanced surface area with increased rate of PUF, which resulted in higher and stable biofilm formation (Feng et al. 2012). The pore size of PUF and its dimensional shape and size are also determining factors for the wastewater treatment (Nguyen et al. 2010). Therefore, the packaging rate is likely to be lower in case of highly porous PUF carrier (Feng et al. 2012).

Table 7: Hypothesis behind the immobilized PUF matrix system for CR dye removal.

Microorganism	**Hypothesis**	**Conclusion**	**Ref.**
Microbial consortium	Development of low-cost economical system using PUF and wheat bran (WB) growth medium for CR dye mineralization and detoxification.	The PUF-immobilized consortium system showed effective decolorization of 100 mg/L CR dye in WB medium (agricultural waste) which can be used for further treatment of azo dye-based system at lower cost.	(Lade et al. 2015a)
Bacillus sp. MH587030.1	Development of PUF-PP optimized system for enhanced CR decolorization using Central composite design (CCD) and Response surface methodology (RSM).	CR Removal efficiency was found to be excellent at optimum conditions. Filling ratio of PUF has shown an adverse effect on colonization of microbe and was found to decrease with increase in dye concentration.	(Goswami et al. 2020)

2.2 Conventional bioreactors for Congo red degradation

Bioreactors are the devices or vessels where biochemical reaction takes place in order to convert raw materials or substrate into different byproducts. Biocatalysts i.e. enzymes, microorganisms, animals and plant cells are responsible for the conversion. Bioreactors are being widely used for scaling up the process and optimistically the process parameters were well governed to facilitate the microbial growth, colonization and improved biochemical response. The development of dynamic systems and setting up the parameters as per the desired process for enhanced treatment efficiency can be achieved using bioreactors. Thus, bioreactors have enormously been studied for dye degradation studies, especially immobilized biocarrier based approach has proven as an effective strategy owing to their ability to avoid washout of microbes with great durability and stability towards broad range of pH and dye concentration (Feng et al. 2012; Lade et al. 2015a). These conventional bioreactors are explored into different industries like food industries, industrial and domestic wastewater treatment, production of antibodies, vaccine and chemicals. Textile effluents are highly toxic and cause environmental issues. Hence, Various types of bioreactors are used for dye effluent treatment either with free cell or as immobilized bed bioreactors (Chisti and Moo-Young 2003).

Free cells in the bioreactors cannot stand with sudden strokes of higher dye concentration which leads to substrate inhibition and cells get damaged due to toxic effect which resulted in low dye removal efficiency. These free cells can be supported with large surface area of bio-carriers to enhance the performance of bioreactors. Biochar (Terminalia arjuna seed) has surface area of 170.0 m^2/g and pore size of 2.873 nm. It plays a dual role in CR dye degradation process due to presence of adsorption sites or free pores onto the AS-biochar surface (Goswami et al. 2020). These pores help in dense biofilm formation as well as to absorb the CR dye molecules on their active sites, which facilitate the microbes to improve the bioremediation process and enhance the bioreactor efficiency. Immobilized AS-biochar for CR dye decolorization was studied in a packed bed bioreactor (PBBR). PBBR is a reliable and practical bioreactor for real time wastewater treatment for scale up and closed system process. Here, immobilized cell gave better result than free cells in PBBR for CR removal of 65.95% and 85.39% respectively. It is due to the effective interaction between large active surface areas of AS-biochar and CR molecules (Goswami et al. 2020).

In another study, CR dye was effectively treated in upflow anaerobic sludge blanket reactor (UASB) under anaerobic condition. UASB reactors are highly suitable for dye wastewater treatment under anaerobic condition, and have low concentration of suspended solids. Here, effect of glucose-COD and concentration of $NaHCO_3$ alkalinity was investigated on CR dye removal in UASB reactor. Approximate 100% dye removal was obtained at 100-500 mg/L of glucose concentration whereas, efficiency of COD removal was decreased from 78 to 68% with decrease in glucose concentration from 500 to 100 mg/L. Also, effect of bicarbonate alkalinity on CR dye removal efficiency and COD removal efficiency was studied. More than 99% CR removal efficiency was obtained at different sodium bicarbonate concentrations (250-3000 mg/L) but there was no significant difference in the COD removal efficiency (84-89%) reported while alkalinity was found to be decreased from 3000 to 500 mg/L. Thus, UASB reactor could be able to decolorize 100% CR dye at 100 mg/L dye concentration (Işik and Sponza 2005). The combination of activated sludge process and biofilm reactor provides an efficient growth to biomass with provision of no further recycling, and can be obtained together only in moving bed bioreactor (MBBR). MBBR system has several advantages; it provides large surface area for colonization and high biomass activity, less head loss, no filter channeling requirement and no fixed essential period requirement for back washing. It has been very successfully utilized in treatment of domestic and industrial wastewater and also useful in upgrading the small-scale level industrial plants (Feng et al. 2012). Also, MBBR support together, free and immobilized cell for biodegradation of pollutants. MBBR has salient features in terms of excellent mass transfer, highly concentrated loading rate, and small space size bioreactor requirements (Goswami et al. 2020).

Sonwani et al. (2020) had studied the significant effect of various process parameters on CR dye degradation in MBBR. PUF-PP filling ratio, concentration of CR dye, and pH were optimized using RSM-CCD designed method. The interactive effect of pH and initial CR concentration over dye removal efficiency (RE) revealed that either acidic or alkaline pH is not favorable for the cells. RE was found to be decreased at both conditions. Neutral pH 7 was found to be optimum at 10 mg/L CR dye and 35% filling ratio but increase in dye concentration diminished the RE. Then, the interactive effect of pH and PUF-PP filling ratio found that the pH 7 is favorable for the MBBR system at 35% PUF filling whereas, the PUF filling above 35% decreased the RE. This could be due to dense packaging of PUF-PP inside the MBBR because, it occupied void space inside the bioreactor mostly and limited the movable free space and oxygen diffusion inside PUF. Further, interactive effect between CR concentration and

PUF filling ratio demonstrated that the increase in PUF filling ratio increased the RE at 55 mg/L because the surface area was increased with increase in PUF filling, which causes active, stable, and more biofilm formation on the surface of PUF. However, with increase in dye concentration RE was found to be decreased due to CR dye toxicity and also inhibition of substrate and enzymatic activity. Finally, at optimized condition MBBR performance was evaluated under continuous condition by changing the flow rates. The optimized conditions of MBBR reactor were 7.1 pH, 50 mg/L CR dye concentration, and 45% PUF-PP filling ratio. In order to achieve high performance of MBBR towards CR dye different flow rates (25-100 mL/h) were studied. Flow rate of 25, 50, 75,100 mL/h showed 95.7%, 91.8%, 88.9% and 72.2% of RE respectively. This decrement is found due to short Hydraulic retention time (HRT) inside MBBR. High flow rate in bioreactor reduces the interaction between carrier and dye molecules, decreases the growth of microbes above the carrier surface. UV-spectrometry and FTIR analysis were used for CR dye analysis and Modified Stover–Kincannon model was used for understanding the CR degradation kinetics (Sonwani et al. 2020). Different kinds of bioreactors studied for CR degradation are elaborated in Table 8.

Table 8: Various types of bioreactors for CR dye degradation.

Bioreactor type	Bio-carrier	Optimized biotic conditions	Result	Ref.
Upflow anaerobic sludge blanket reactor (UASB)/ completely stirred tank reactor (CSTR); anaerobic-aerobic	Sludge	0.486 days HRT; 6.656 kg COD m^{-3} day^{-1}; ambient temperature; pH neutral.	95% color removal and 77% of COD at 100 mg/L.	(Işk and Sponza 2003)
Upflow anaerobic sludge blanket reactor (UASB); anaerobic treatment	sludge	37 °C temperature; 6-6.6 pH	58% COD, 39% TAA removal efficiencies and 380 ml/day methane production rates and	(Işik and Sponza 2005)

			100% dye decolorization at 100 mg/L.	
Upflow column reactor; Anaerobic treatment	Polyurethane foam	7.0 pH; 30 ± 2 °C temperature.	99% dye decolorization; TOC 83%; COD 89% at 100 mg/L.	(Lade et al. 2015a)
Moving bed bioreactor; aerobic treatment	Polyurethane foam-polypropylene (PUF-PP)	7.1 pH; 50 mg/L CR dye concentration; 45% PUF -PP filling ratio; 25 mL/h flow rate at continuous mode.	95.7% decolorization achieved at 50 mg/L.	(Sonwani et al. 2020)
Stirred tank reactor; aerobic treatment	Luffa cylindrica	5 pH; ambient temperature.	97% dye decolorization at 100 mg/L.	(Laraib et al. 2020)
Packed bed reactor; Hybrid (biological and ozonation) treatment	Terminalia arjuna seed biochar	30 °C temperature; 248 h process time, and 3 × 105 CFU/mL inoculum size.	92.0 ± 5.0% decolorization in hybrid system; 63.3% at 300 mg/L in batch mode of PBBR; 88.9% at 500 mg/L in continuous mode of PBBR.	(Goswami et al. 2020)

2.2.1 Metabolites obtained into bioreactor after CR dye degradation

The initial step for degradation of CR dye through microbes is to cleave the azo bond, which leads to CR decolorization. Aromatic amines are generated after decolorization as end product; which are potentially hazardous and colorless. Second stage involves degradation of aromatic amines through oxidative enzymes under aerobic condition. The initial possible conversion of

CR dye after azoreductase catalysis of azo bond forms biphenyl-1, 4′-diamine [A] and unknown intermediates [I] (Chung et al. 1992). GC-MS analysis of CR dye resulted in three different intermediate metabolites after degradation includes, biphenyl-1,4′-diamine, biphenyl and naphthalene (Lade et al. 2015a). In another study, LC-MS analysis of CR decolorized samples gave four possible different intermediate byproducts after treatment i.e. 2-((4'-aminobiphenyl-4-yl) diazenyl) naphthalen-1-amine; 4, 6-diaminonaphthalene-1-sulfonate; 1-(biphenyl-4-yl)-2-(naphthalene-2-yl) diazene and benzidine.

2.3 Effect of parameters responsible for Congo red decolorization in bioreactors

2.3.1 Effect of Inoculum size

Talha et al. (2018) studied the effect of inoculum size, which was varied from 1.0×10^5 to 5.0×10^5 CFU/mL for CR dye removal. The degradation efficiency significantly increased up to 3.0×10^5 CFU/mL, and there was no significant improvement in the degradation rate with respect to increase in the inoculum size above 3.0×10^5 CFU/mL (Abu Talha et al. 2018).

2.3.2 Effect of CR dye concentration

Dye concentration is one of the vital factors to determine the dye degradation efficiency in the bioreactor due to its effect on the microbial growth and metabolism. Goswami, et al. (2020) investigated the effect of CR dye concentration in packed bed bioreactor (PBBR) with varied dye concentrations from 100 to 500 mg/L. The authors tried to elucidate the importance of dye concentration on CR dye removal and hypothesized that determination of the optimal concentration is essential for enhanced dye removal efficiency. It was reported that 82.38% and less than 48% removal was obtained at 100 mg/L and 500 mg/L respectively, and concluded that the treatment efficiency is poor due to the microbial growth inhibition at above optimal dye concentrations (Goswami et al. 2020). The higher loading rate of the dye restricts the enzymatic activity of microbes and effects the bioremediation process in the system (Sonwani et al. 2020).

2.3.3 Effect of pH on performance

The microbial growth and metabolism depend on the optimal pH because the enzymatic production is responsible for dye effluent removal significantly which is interlinked with its favorable environment. CR dye removal at pH 7 and 8 showed only 1% (97% and 98% respectively) difference in the decolorization (%) whereas, at extreme acidic (4-6) and alkaline

(9-10) pH retarded performance of bioreactors was noticed, due to reduction in metabolic activity of the bacteria (Lade et al. 2015a).

2.3.4 Effect of temperature

As like with pH, temperature also is a vital factor for microbial growth and metabolism. Generally, for fungi 25-30°C and bacteria 35-37°C are the most favorable temperatures for the dye removal. High or low temperature of the system reduces the cell viability, metabolic pathways get inactivated for producing responsible enzymes to improvise the dye degradation or altered enzymatic activity of the cells (Laraib et al. 2020). In one of the studies of CR decolorization optimum incubation temperature was found to be 30 ± 2°C, and efficiency at 25, 37 and 40 ± 0.2 °C resulted in 96%, 97% and 86% removal. Bioreactors are designed to well govern the process parameters at optimum level, the studies concluded that maintaining optimal temperature in the bioreactor system is highly essential for enhanced efficiency of biodegradation of dye (Lade et al. 2015a).

2.3.5 Effect of media

In general, synthetic media is used for dye removal studies. Media is a source of salts and nutrients for microbial growth. However, this is a cost determining factor for scale up studies. Lade et al. (2015) have replaced the salt media with wheat bran media (WB media) for development of an upflow column bioreactor system for CR dye decolorization studies. Here, 99% removal was obtained at 100 mg/L in wheat bran media successfully. Therefore, agricultural waste also can be utilized for the microbial growth media in dye degradation studies (Lade et al. 2015a).

2.3.6 Effect of carbon source

Glucose is a carbon source (co-substrate) utilized in the bioreactor to facilitate in generation of reducing enzymes (NADH, NADPH, FAD and FMN) and electrons to reduce the azo bond cleavage of CR dye after getting oxidized (Shalini and Y. 2019).

2.3.7 Effect of ratio of biocarrier to bioreactors

Ratio of biocarrier to bioreactors is also one of the major parameters for CR dye decolorization. However, this ratio is dependent on type of carrier and bioreactor. The effect of biocarrier ratio to moving bed bioreactor on CR dye removal was investigated and reported that optimization of biocarrier filling in the reactor is a significant factor which influences the dye removal. The dense filling in the bioreactor decreases the free space present inside the reactor, which restricts

the oxygen transfer into the biocarrier. This study found that above 35% filling of PUF in MBBR stabilized the removal efficiency of CR dye (Sonwani et al. 2020).

Other various parameters like hydraulic retention time (HRT), effect of time over decolorization, effect of additional nitrogen sources are also responsible for performance of bioreactors studied in different reports for various azo dyes.

2.4 Electrochemical approach for CR dye degradation

Microbial fuel cell is an electrochemical device efficiently used by researchers for CR dye degradation studies. CR dye is treated into either dual chamber fuel cell or single chamber fuel cells or bio electrochemical devices. Li et al. (2010) demonstrated MFC as anaerobic-aerobic combined system for CR dye degradation. Here, CR dye decolorized in anode chamber and produced colorless aromatic amines which were further, treated in cathode chamber (Li et al. 2010). Carbon sources are oxidized through microbes and generate electrons. Therefore, production of electrons depends on type of co-substrate feed to biocatalyst. Glucose, sodium acetate and ethanol were compared as co-substrate on CR dye for their effect on decolorization process in air cathode single chamber MFC. Glucose was found to be suitable co-substrate among all, followed by ethanol and sodium acetate. 98% CR removal was achieved in 36 h with 103 mW/m^2 power density at 300 mg/L. The result suggested that suitable co-substrate for MFC application at optimized condition can enhance the MFC performance and decolorization (Cao et al. 2010).

Microbial enrichment procedure (EP) is also one factor reported for CR dye decolorization and MFC performance. Two different procedures were adopted for the inoculation of single chamber MFC, EP1: 500 mg/L glucose containing salt media initially then replaced with 500 mg/L glucose and CR dye (100-300 mg/L) containing salt media and EP2: salt media containing 300 mg/L CR dye and 500 mg/L along with suspended sludge until end of anode enrichment. Both EP1 & EP2 took 170 h for 90% decolorization at 300 mg/L of CR dye concentration. EP2 achieved 75% higher Power density than EP1, 192 mW/m^2 and 110 mW/m^2 respectively. The scanning electron microscope (SEM) morphology of EP1 & EP2 directly correlated the condition of microbes, EP2 has chain like colonies with smooth surface which might have helped in transferring electron to anode whereas, EP1 has depressed bacterial surface. However, it resulted that enrichment procedure on decolorization had negligible effect but significantly affected the power output (Hou et al. 2011).

Anode surface area also decides the MFC performance and dye decolorization. Effect of three different anode surface areas (18 cm^2, 36 cm^2 and 72 cm^2) were examined for CR dye decolorization. CR dye decolorization was accelerated with increase in anode surface area. Anode surface area of 18 cm^2, 36 cm^2 and 72 cm^2 took 168 h, 72 h and 26 h respectively for CR dye decolorization. Here, doubling of anode from 18 to 36 cm^2 enhanced the rate of CR decolorization from 1.6 to 4.2 mg/L/h (increase of 160%) and four-fold of anode surface area to 72 cm^2 enhanced the rate to 11.5 mg/L/h (increase of 600%). Increment of surface area provides large surface area for colonization of cells, which increased the decolorization rate and also lowered the anode impedance. However, this could not affect power output performance of MFC significantly, which could be due to the cathode limitation or ineffective utilization of anode. Therefore, optimal MFC performance can be achieved through higher macro surface area or optimized cathode (Sun et al. 2012). Further, effect of aerobic biocathode and air cathode MFCs were compared. In terms of decolorization, biocathode system achieved 96.4% CR removal within 29 h where, the same removal was achieved within 107 h by air cathode MFC. This could be due to the presence of facultative aerobes on the surface of air cathode MFC and absence in biocathode MFC. It indicates that oxygen diffusion from cathode to anode chamber was more serious in air cathode rather than biocathode MFC whereas, the performance of MFC was found to be opposite. Here, air cathode MFC (324 mW/m^2) exhibited 166% better performance than biocathode MFC (122 mW/m^2) (Hou et al. 2012).

Investigation of appropriate microorganism is also one of the major challenges for dye decolorization with simultaneous MFC performance. Moreover, the interaction of pure culture or mixed culture or co-cultures with MFC and dye demonstrate the microbial interaction and its mechanism between electron and mass transfer (Xu et al. 2013). Single chamber bio-electrochemical system (BES) is a combined bioanode and biocathode system, and can also accelerate CR dye decolorization. Modified arrangement of electrodes into the single chamber BES is potentially effective in azo dye treatment. Modified electrode systems can possibly improve the surface area of anode and cathode and also facilitate electron transfer (Kong et al. 2014). Various hypotheses behind electrochemical approach for CR decolorization is summarized in Table 9.

Table 9: The outcome of CR decolorization in MFC behind the hypotheses.

MFC type	Hypothesis	Outcome	Ref.
Dual chamber MFC	Investigation of sequential anaerobic- aerobic treatment in MFC system can degrade the aromatic amines obtained at anaerobic stage.	✓ Azo bond cleaved in anode chamber. ✓ Aromatic amines degraded in cathode chamber using electrons and protons.	(Li et al. 2010)
Air cathode single chamber MFC	Search for appropriate co-substrate for recalcitrant dye and its effect on MFC performance.	✓ The rate of decolorization was higher with glucose followed by ethanol then sodium acetate. ✓ Glucose served the higher power density and ethanol least.	(Cao et al. 2010)
Air cathode single chamber MFC	To find out effect of microbial enrichment procedure on CR decolorization and MFC performance.	✓ The result indicated that activity of bacterial enrichment procedure could not affect seriously on decolorization. ✓ However, it affected the power performance.	(Hou et al. 2011)
Air cathode single chamber MFC	To find out the effect of anode surface area on CR decolorization and MFC performance.	✓ Increase in anode surface highly improved the rate of decolorization due to more active sites availability and MFC performance. ✓ But power output was limited, may be due to the cathode limitation and incomplete surface utilization of anode.	(Sun et al. 2012)

Air cathode single chamber and dual chamber MFC	Comparative study of platinum coated cathode and biocathode, and its effect on CR decolorization and MFC performance.	✓ Power density of Pt cathode was higher than biocathode MFC. ✓ The decolorization achieved by biocathode was in less time interval (29 h) compared to Pt cathode (107 h).	(Hou et al. 2012)
Air cathode single chamber and dual chamber MFC	Investigation of function and its interaction behind the predefined pure and mixed co-culture microorganisms inside MFC and its effect on CR decolorization and MFC performance.	✓ Pure culture rate of decolorization was slower than mixed co-cultures but opposite in case of MFC performance.	(Xu et al. 2013)
Air cathode single chamber MFC	Effect of long time period operation on MFC performance in presence of CR dye and glucose (co-substrate).	✓ The cathode potential decreased while anode was not measurably affected. ✓ MFC performance decreased over time.	(Sun et al. 2013)
Air cathode single chamber MFC and combined bioanode and biocathode BES	Development of combined bioanode and biocathode electrochemical system (single BES) and effect of electrodes arrangement on CR decolorization and its comparison with dual chamber MFC.	✓ The result concluded that single BES system with suitable electrode arrangement could potentially enhance the azo dye decolorization. ✓ The decolorization in BES system was 98.3 ± 1.3%, which is higher compared to dual chamber MFC (67.2 ± 3.5 %) within 23 h.	(Kong et al. 2014)

Dual chamber microbial fuel cell	To investigate effect of carbon rods obtained from zinc-carbon (1050-D) batteries as electrode and effect of CR dye concentration and catholyte solution on MFC performance.	✓ Zinc-carbon rods have shown good electrochemical activity and also have good biocompatibility observed from SEM images. ✓ At optimized condition maximum power density and current density of 0.90Wm^{-2} and 1.65 Am^{-2} were achieved at 100 mg/L$KMnO_4$ of catholyte solution.	(Senthilkumar et al. 2020)

2.5 The need of Biowaste Derived Electrode and its role in improving MFC performance for Dye Degradation

Microbial fuel cell (MFC) is a promising technology, which is being explored in the wastewater treatment area owing to its potentiality for the removal of toxic compounds from wastewater and electricity generation which addresses the environmental and energy challenges. Electrodes play a vital role in the performance of MFC. Various electrodes include platinum (Hou et al. 2012), metal alloys, carbon and other transition materials for effluent treatment studies. The synthetic electro active material is toxic and cost intensive. The development of cost-effective electrodes and their use in dye removal is the need of the hour. Carbon based materials show the ideal properties like high microbial adhesion, light weight, high porosity, thermal stability, biocompatibility and low cost (Li et al. 2017). Biowaste derived material, known, as bio-char are rich in carbon. Bio-char is a material of low-cost material, with high carbon content and large surface area (consists of macro, meso and nano-pores). Generally, bio-chars can be obtained from raw materials like agricultural waste, forest raw waste, milling waste etc. (Huggins et al. 2014). The performance of MFC depends on particle size of bio-char because power density of MFC is inversely proportional to the particle size. Overall surface of bio-char are negatively charged, which initially inhibit the microbial attachment on the surface. Bio-char

can be activated by thermal or chemical method. Hence, activation of bio-char enhances surface area by 137% compared to non-activated bio-char (Chakraborty et al. 2020).

The primary limitation of MFC is the cost-effective electrode and another limitation is scaling up of MFC. Natural based materials can be used as anode and can potentially substitute to mitigate the limitations of existing synthetic material to improve the MFC performance. Biowaste derived electrodes such as; natural luffa sponge (Yuan et al. 2013), Tubular bamboo charcoal (Zhang et al. 2014), Pomelo peel (Chem et al. 2012) and carbon material derived from king mushroom; wild mushroom; corn stem (Karthikeyan et al. 2015) which are few carbon-based materials procured from nature, have been widely demonstrated for the electricity production. The effect of coconut shell biochar derived from coconut shell was studied on methylene blue (MB) dye degradation in microbial fuel cell and was reported. MFC performance increased with increase in biochar dose in MFC. The highest voltage of 722 mV and 1.07 mA current was obtained at 1g of biochar dose. At 25 mg/L MB concentration, power density of 61.469 mW/m^2 (1.38-fold), 57.3 mW/m^2 (1.29-fold) and 44.3 mW/m^2 was obtained for 1g, 0.5 g and 0 g biochar respectively. Maximum decolorization of 93% achieved at 0.5 g biochar was found to be having optimum active sites available for decolorization and degradation. Adsorption, break-down of dye molecules and reformation of active sites are the three steps involved for MB degradation in MFC. Biochar molecular structure facilitate for the treatment of organic contaminants through chemical reactions, which are duly triggered due to the presence of zig-zag carbon atoms. The functional surface properties enhanced with chemical activation, and the oxygenated functional groups upon activation improved the adsorption ability. The surface activity enhances the organic content adsorption and further initiate the chemical activation process for the degradation of organic effluents and all the intermediate compounds (Sophia Ayyappan et al. 2018).

Similar finding was obtained from corncob-based biochar used for real textile wastewater treatment. Here, corncob biochar improved the electrode surface area and facilitated dual role in the MFC with simultaneous oxidation and adsorption of organic matters. In addition, MFC internal resistance was reduced due to the improved surface area. In anaerobic chamber dyes trap the oxidized electrons and are reduced into intermediate aromatic compounds. Free radicals of biochar combine with intermediates and get mineralized (Sonu et al. 2020). Further, biomass derived from Water Hyacinth was utilized as cathode catalyst in MFC. It has a porous structure, high surface area, and distinct chemical composition. Electrochemical activity of biochar

towards oxygen reduction reaction (ORR) activity was promising in neutral phosphate buffer solution. The produced power density was half of Pt/C catalyst i.e. 12.3 mW/m^2 and 24.7 mW/m^2 respectively (Allam et al. 2020). The advantages of natural based electrodes are ease in availability, abundant in nature, natural precursor, lower cost and conversion of undesirable waste into value added products. Generally, fibrous waste biomass materials transform into highly porous carbon due to richness of fibers.

Ficus religiosa (FR) tree is also called as sacred fig, bodhi tree, peepul tree, and pippala tree in Indian subcontinent. FR is a semi-evergreen tree that is native to tropical Asia having an average life time of 900–1,500 years. Ficus Ficus religiosa leaves (FRL) is the renewable biowaste available abundant in nature and low-cost precursor for production of porous carbon. Biowaste used as a functional material in energy applications would be the ideal way of handling solid waste management. Herein, FRL porous carbon was derived from dead leaves intended to be used as anode material for dye decolorization application in MFC.

2.6 Effect of parameters responsible for azo dye decolorization in MFC

2.6.1 Effect of dye concentration

MFC performance is relied on concentration of dye and its type. Sun et al. (2009) investigated effect of ABRX3 (Active Brilliant Red X3) dye concentration on MFC. The results of this study represented that the increase in dye concentration affects the microbes after certain point of concentration. Within 48 h, ABRX3 dye concentration from 300 mg/L to 900 mg/L slightly declined from 90% to 86% whereas, 100 mg/L gave 90% result in 12 h only. At, 1500 mg/L decolorization rate decreased to 77% and took more than 48 h time. So, it is noteworthy that higher concentration of dye strongly induces the microbial growth (Sun et al. 2009).

2.6.2 Effect of pH on dye

Catholyte pH in MFC decides the power output. Liu et al. (2009) studied the effect of catholyte pH (3-9) on power density for methyl orange (MO) dye, orange I and orange II. Complete reduction of MO, orange I and orange II dye found at pH 3, slightly less than pH 3 and pH 5 respectively since, MO dye is highly subjected towards electrochemical reduction among all three. Hence, power output of MFC evidently correlated to the azo dye reaction rate. Maximum power density at pH 3 reached 34.77 mW/m^2 and 1.51 mW/m^2 at pH 9. Cyclic voltammetry (CV) analysis of MO indicates that reduction reaction is higher at lower pH compared to higher

pH. Results concluded that increase in catholyte pH declined the power density. Therefore, proper buffer of catholyte should be maintained to avoid increase in the pH (Liu et al. 2009).

2.6.3 Effect of co-substrate

Glucose, sucrose and confectionery wastewater (CW) were examined individually in air cathode single chamber MFC. Among the three, glucose gave higher decolorization rate followed by sucrose than CW (Sun et al. 2009). Similarly, Cao et al. (2010) also found glucose as suitable co-substrate than sodium acetate and ethanol (Cao et al. 2010). Therefore, literature suggested that carbohydrates are the suitable co-substrates for dye decolorization and are bacterial specific. Carbohydrates and organic matters are good electron donors whereas; acetate and other fatty acids are very poor electron donors.

2.6.4 Effect of external resistance

Sun et al. (2009) studied the effect of external resistance on ABRX3, the decolorization rate was faster at lower resistance. External resistance of 50, 500 and 5000 ohm decolorized more than 90%, 90% and 85% took 24, 36 and 48 h respectively. At lower resistance, more coulombs are recovered from the substrate compared to higher resistance due to higher substrate conversion rate (Sun et al. 2009).

2.6.5 Effect of hydraulic retention time (HRT)

HRT is an important factor in dye wastewater treatment because, it helps in estimation of substrate concentration and dissolved oxygen (DO) level present in wastewater. In other study, the effect of six different HRT (44.4, 22.2, 14.8, 11.2, 8.8 and 7.4 h) on power density, COD and CR dye decolorization was reported. It was reported that power density and COD varied with change in HRT, and maximum power density of 552.2 mW/m^2 was achieved at 14.8 h HRT. Open circuit potential of anode chamber increased from -431 to -283.8 mV when, HRT increased from 14.8 to 44.4 h with decrease in substrate concentration. Reduction of 50.4% was observed in maximum power density when HRT decreased from 14.8 h to 7.4 h and apparently, the reduction in COD and decolorization was reported. COD and CR removal was relatively higher at HRT 11.2 h, further increase in HRT to 44.4 h only improved the CR removal. Finally, 14.8 h HRT was obtained as an optimal value based on all parameters (Li et al. 2010).

2.6.6 Effect of electrode

Electrode size plays vital role, and enhancement of anode surface area could accelerate the CR dye removal but did not affect the power output. It could be due to the limitation of cathode

surface area (Sun et al. 2012). Electrode potential was used to measure at different current densities to investigate its performance at different dye concentrations. Both anode and electrode potentials with respect to varying dye concentration was investigated and reported that anode potential increased quickly with increase in dye concentration from 600 mg/L to 1500 mg/L at higher current densities but cathode potential was not affected much. Anode potential was increased due to the constant electron supply to anode affected by increase in dye concentration, which also affects the MFC performance in terms of electricity generation. Here, increase in dye concentration consumes more electrons for azo bond breakage which creates over potential on anode due to less electrons transferred to the anode (Sun et al. 2009).

2.7 Microbial fuel cell-Bioreactor coupled systems for Dye Degradation

Complete dye degradation has not been achieved using either aerobic or anaerobic treatment alone. The integrated system coupled with anaerobic and aerobic treatment has attracted the attention for complete dye degradation. Guang et al. (2017) developed an integrated system; microbial fuel cell coupled with a catalytic oxidation reactor (COR). System was filled with activated granule of FePc (Iron phthalocyanine)-catalyst to degrade CR dye and glucose was used as an electron donor. Integrated MFC-COR reactor achieved CR dye removal of 91.3 ± 4.3 % within 72 h. The MFC-COR system found that H_2O_2 was generated at cathode side and oxygen residuals together utilized as oxidant for CR dye degradation. H_2O_2 and O_2 pass to the COR reactor while circulating CR dye to COR reactor where they react with FePc catalyst and produce high-valent iron-oxo species. Congo red dye decomposes into smaller molecules with the help of iron-oxo species and mineralize organic pollutants also (Yuan et al. 2017).

In another study, molasses used as electron donor for acid orange 7-dye degradation in MFC, aerobic two-stage bioreactor system at ambient temperature. Molasses is cheap and sustainable electron donor. The system, in continuous mode at different loading rates of acid AO-7 was successfully operated up to 150 days. Loading rate of AO-7 was varied between the 70 g m^3 day^{-1} to 210 g m^3 day^{-1}. The results showed that integrated system worked efficiently without any deterioration on dye removal and COD. Above 90% decolorization was obtained even at 210 g m^3 day^{-1} AO-7 loading. MFC resulted in recalcitrant compounds which further mineralized into two-stage aerobic system (Fernando et al. 2014). Summarization of integrated systems studied earlier for different dyes is presented in Table 10.

Table 10: Summarization of integrated systems studied for dye degradation.

Integrated system	Dye and co-substrate	Hypothesis	Outcome	Ref.
Microbial fuel cell and aerobic two-stage bioreactor system	Acid orange-7 and Molasses	Development of low-cost co-substrate based integrated system to achieve AO-7 degradation at ambient temperature.	Molasses is low cost sustainable electron donor for dye removal. Integrated system is highly significant for higher loading rate of dye.	(Fernando et al. 2014)
Microbial fuel cell and catalytic oxidation reactor	Congo red and Glucose	Development of integrated system using FePc catalyst to enhance the CR degradation.	FePc catalyst reacted with H_2O_2 and O_2 and produced Fe-oxo species mineralized CR dye resulted in 91.3 % removal.	(Yuan et al. 2017)
Dual chamber microbial fuel Cell and aerobic system	Remazol navy blue and salt media	Degradation of RNB into less toxic compounds in integrated system.	Complete mineralization of RNB achieved 97.89% in 14 h.	(Das and Mishra 2019)

The current research aims to provide cost effective, user friendly, and sustainable integrated system for complete dye degradation. Biological reduction of aromatic compounds (remain present after MFC treatment) requires the electron donor, which is provided using co-substrate in the system (Chengalroyen and Dabbs 2013). Glucose, acetate and other sustainable waste materials have been used as co-substrate and limited research was conducted on the use of agriculture residue as co-substrate for azo dye degradation (Cao et al. 2010; Lade et al. 2015a). Molasses (Fernando et al. 2014) and pure form of glucose (Yuan et al. 2017) are being used for dye degradation studies. The abundant availability of agriculture residues at low cost has triggered the research towards their effective utilization in dye degradation. The glucose addition as co-substrate in pure culture-based treatment approach hampered scaling up of the

process. The established protocols for the pretreatment of agriculture biomass for the enhanced production of reducing sugars (RS) are available and detailed studies have been conducted on their effective utilization in bioenergy and biofuel production (Liu et al. 2010; Potumarthi et al. 2014). The use of agriculture residue for the replacement in growth media in mixed culture consortia or as co-substrate in pure culture-based treatment approach is the emerging research for developing the sustainable biological treatment for dye degradation.

2.8 Logistical plan derived from the lacunae noticed in existing literature to develop a potential approach for Congo Red Degradation

- ✓ Immobilization of *Bacillus subtilis* using biocarrier for CR dye decolorization was not studied. PUF has significant advantages like higher cell attachment and cell-colonization, enhanced bioavailability for the dye removal. PUF immobilization-based approach is not yet elucidated for the CR dye degradation.
- ✓ The major disadvantage of lower density PUF foam is non-availability of foam matrix at the bottom of the reactor due to the floating nature of the foam. It is essential to provide a homogeneous distribution of foam in the reactor for microbial colonization and biological interaction to enhance the biodegradation of dye. Considering the advantages of FBBR and PUF immobilization, and at the same time, due to disadvantages associated with the use of lower density, development of a novel bioreactor configuration is needed. Earlier investigators in this direction have given no attention.
- ✓ Biocompatibility and cost are the major concerns for the selection of electrode material to develop MFC based treatment approach for dye effluent removal. Using biowaste derived material as an electrode in MFC based treatment is a novel approach and this needs to be explored for concomitant removal of dye and electricity production.
- ✓ The electron donor such as glucose has been well explored for enhanced dye removal and reported that co-substrate is essential for dye degradation. Limited research was conducted on the use of agriculture residue as co-substrate in azo dye degradation for reducing cost-economics of treatment set up. Hence, a detailed study using more agriculture residue as co-substrate is needed.
- ✓ An integrated MFC and bioreactor MFC approach is needed for complete degradation of CR dye which involve anaerobic and aerobic treatments. A combination of MFC and MRFBBR approach has not been studied earlier.

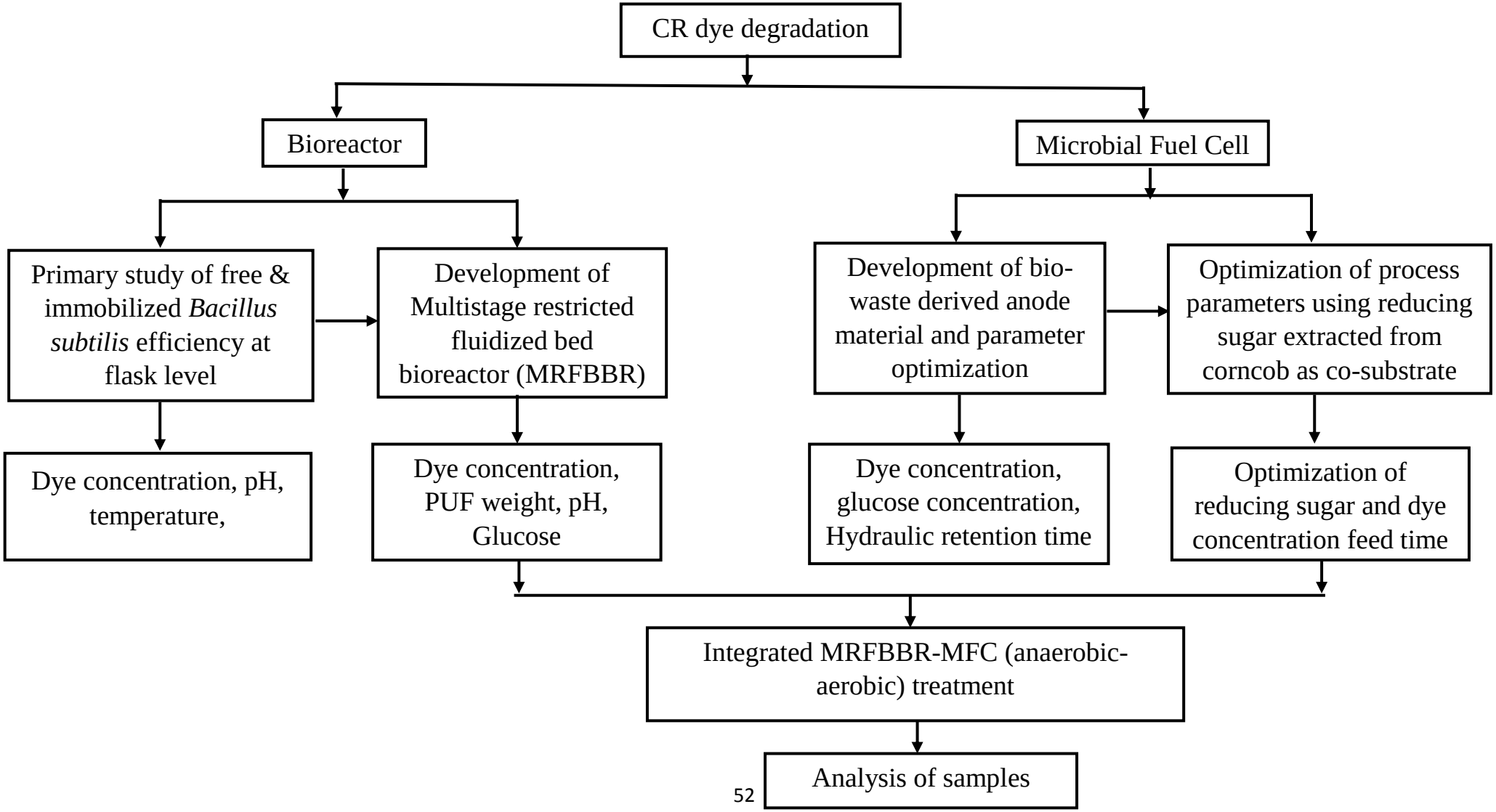
2.9 Objectives of the present study

The overall objective of the present study is to develop an integrated MFC-Bioreactor system for Congo red degradation using biomass derived electrode and corncob residue as co-substrate for sustainable technology. The achievement of the present goal is represented into four prime objectives.

Objectives:

1. Development of immobilized bacterial method for improved Congo red dye decolorization.
2. Design and development of multistage restricted fluidized bed bioreactor (MRFBBR), optimization of process parameters for enhanced Congo red dye decolorization.
3. Development of cost effective and efficient anaerobic treatment for Congo red dye decolorization using microbial fuel cell based on biowaste derived electrode.
4. To study integrated MFC-MRFBBR system for CR dye degradation using corncob agro residue as co-substrate to make the process sustainable.

2.10 Work plan of the thesis



3. Materials and Methods

3.1 Chemicals and reagents

Congo red (CR) (C.I. No. 22120, Mol. Formula C32H22Na2N6O6S2, Mol. Wt. 696.66), Polyurethane foam (PUF) purchased from the local market (Bhatia stores, Warangal), Phosphate buffer saline (PBS), Carbon cloth (1071 HCB), Nafion membrane (N117) as a PEM, and 5% Nafion solution for preparation of catalytic ink. Copper oxide (CuO, mol. wt. 79.55, particle size 30–50 nm, 99% purity) nanoparticles were purchased from Alfa Aeasar, Thermo Fisher Scientific. All chemicals and reagents used while conducting experiments were of analytical grade.

3.2 *Bacillus subtilis* culture maintenance

Bacillus subtilis (NCIM: 5433) was procured from National Collection of Industrial Microorganism (NCIM), Pune. Slant culture obtained from NCIM was regularly sub-cultured on Nutrient agar plates at an interval of every two months and stored in the refrigerator at 4°C for further use.

3.3 Inoculum Preparation

Bacillus subtilis was cultured in 100 mL nutrient broth in a 250 mL conical flask and kept at 37 °C in a rotary shaker at a speed of 130 rpm for 24 h for the optimal growth and 10% v/v was used as an inoculum for initial startup of all experiments.

Table 11: Composition of Nutrient Broth.

Composition of Nutrient Broth (g/L)	
Beef extract power	10
Sodium chloride (NaCl)	5
Peptone	10

3.4 Synthetic textile wastewater (STW)

Synthetic textile wastewater (STW) was prepared to evaluate the decolorization efficiency of the process. Congo red dye was added to the minimal salt media (MSM) to prepare the STW.

Table 12: Composition of synthetic textile wastewater.

Composition of Minimal salt media (MSM) (g/L)	
K_2HPO_4	6.3
KH_2PO_4	1.8
$NaNO_3$	1.0
$MgSO_4.7H_2O$	0.006
Yeast extract powder	5.0
Dye	Varied concentrations

3.5 Decolorization analysis

Samples were collected at equal time intervals and centrifuged at 8000 rpm in centrifuge. The supernatant was scanned at absorbance value of 490 nm using UV-Spectrophometer. Decolorization percentage analysis was estimated by using the formula,

$$Decolorization\ (\%) = \frac{Initial\ absorbance - Final\ absorbance}{Initial\ absorbance} \times 100$$

3.6 Chemical oxygen demand (COD) analysis

COD analysis was conducted using closed reflux method. The reagents used for COD analysis i.e. Standard potassium dichromate reagent as digestion solution, Sulphuric acid reagent as catalyst solution and standard ferrous ammonium sulphate solution for titration were prepared as per standard protocols (Baird et al. 2017). Water sample (Blank) or diluted sample of 2.5mL, 0.1 N potassium dichromate ($K_2Cr_2O_7$) of 1.5mL and 3.5mL of H_2SO_4 reagents were added into a digestion bottle and then left for cooling at room temperature. Then the digestion solution was kept in the COD digester in a block heater for 2 hours at 150°C (Thermoreactor CR 2200 WTW, Germany). After completion of digestion it was left for normalization of temperature. Digestion samples were transferred into the beaker, and then two drops of farrion indicator were added followed by titration against ferrous ammonium sulphate. End point appears when solution turns color from bluish green to reddish brown and reading of ferrous ammonium sulphate consumed was noted down. The following formula is used for estimation of concentration of COD (mg/L),

$$Concentration\ of\ COD = \frac{(A - B) \times N \times 8000}{V} (mg/L)$$

Whereas,

A= Volume of ferrous ammonium sulphate consumed for blank (ml)

B= Volume of ferrous ammonium sulphate consumed for sample (ml)

N= Concentration of ferrous ammonium sulphate in Normality,

V = Sample volume in (ml)

3.7 FTIR analysis

Fourier transform infrared (FT-IR) spectrophotometer (Shimadzu, Perkin Elmer 1000) was used for the analysis of the samples. The parameters used in the FT-IR instrument were 2 cm^{-1} resolution, and 400-4000 cm^{-1} frequency range. Method of KBr pellets was used to make the pellets.

3.8 SEM analysis

After immobilization, PUF cubes were submerged in 3% of glutaraldehyde for cell fixation and incubated for 2-3 h. Subsequently, PUF cubes were washed twice with PBS. Sequential dehydration was carried out with acetone 30, 50, 70, 80, 90, and 100 vol% for 15 minutes in each step. PUF cubes were immersed in hexamethyldisilazane and kept for overnight in a fume hood. Samples were then coated with gold in mounted carbon tube and imaged using SEM (FEI/Philips XL-30 ESEM) at 20 kV.

3.9 Congo Red Decolorization studies using free cell and Immobilized Cell

3.9.1 Free cell mediated decolorization

10% (v/v) of inoculum was added to MSM media containing dye. The different dye concentrations such as 25 mg/L, 50 mg/L, 75 mg/L, and 100 mg/L were considered to study the effect on decolorization efficiency of the CR dye. The effect of pH (6, 7, 8 and 9) and temperature (25, 30, 35, 37, 40, and 45 °C) on CR decolorization was studied. In all the cases, 10% (v/v) pre-grown culture inoculum in NB broth added to MS medium for the experimental study of free and immobilized cells. All the samples were collected at regular intervals of 3 hours and the clear supernatant liquid absorbance values were recorded using UV-spectrophotometry. The concentration of cell mass was analyzed using spectrophotometry, and absorbance was recorded at OD 600 nm (OD_{600}).

3.9.2 Immobilization of *Bacillus subtilis* on PUF for decolorization

The PUF (size 1 cm^3) cubes were soaked in distilled water for 2-3 h. These foams were washed with distilled water before sterilization at 121°C for 15 min (Silveira et al. 2011; Lade et al. 2015a). The cells were grown in nutrient broth (NB) for 24 h. 1 g of sterilized PUF cubes and 10% inoculum were added to 100 mL of NB media for immobilization purpose and incubated for 24 h in a static condition at 37 °C. The immobilized PUF cubes were washed twice with PBS for the removal of non-adhered microbial cells. These cubes were then transferred into media containing dye and the optimal conditions from free cell assisted degradation experiment were used to assess the efficiency of immobilized cell mediated decolorization. The samples were collected at regular time intervals of 2 h to record the absorbance values at 490 nm using UV spectrophotometer (Lovibond XD7500). After completion of the experiment, the PUF cubes were washed with PBS in a laminar airflow chamber and then stored at 4 °C to reuse them for subsequent decolorization experiments. FTIR spectra (400-4000 cm^{-1}) was recorded to check the change in functional groups before and after decolorization.

3.9.3 Statistical analysis

Graph pad prism 7.0 was used to perform the statistical analysis. All the experiments were conducted in triplicates (n), and Mean ± SD values were considered to plot the graphs. Free cell and immobilized cell assisted decolorization with the optimal conditions were evaluated for statistical significance using the two-tailed t-test. The probability value $p<0.05$ indicates the statistical significance.

3.10 CR decolorization in Multistage Restricted Fluidized Bed Bioreactor (MRFBBR)

3.10.1 Dye decolorization studies in bioreactor

Bacillus subtilis immobilization on PUF matrix was done as described in the section 3.9.2. Immobilized PUF cubes were transferred into a reactor. The total reaction volume of the reactor was 1500 ml, which is composed of dye and MS media maintained at 37 °C with an air flow rate of 0.2 LPM. Effect of dye concentration (50, 100, 150, 200 mg/L), weight of PUF (3, 5, 7 g), pH (6, 7, 8, and 9), and glucose concentration (0, 2, 4, 6 g/L) on dye decolorization was studied. The samples were collected, and absorbance at 490 nm using UV Spectrophotometer was recorded.

3.10.2 Real textile wastewater (RTW)

Real textile wastewater was obtained from local textile industry from Prime Textile, Warangal, filtered from whatman filter (grade 1) paper to remove large suspended particles and then stored in refrigerator at 4 °C until further use. The characteristics of the RTW is presented in Table 13.

Table 13: Characteristics of real textile wastewater.

Parameters	pH	TDS	TSS	BOD	COD	TOC
RTW	7-8	1210 ppm	830 ppm	700 ppm	3250 mg/L	1751 ppm

3.10.3 Bioreactor set-up for dye decolorization

Schematic representation of Fluidized-bed bioreactor (FBBR) is presented in Figure 4. The bioreactor was made up of glass. The dimensions of the re

actor are 0.5 m in height, internal diameter (ID) of 93 mm and an outer diameter of (OD) of 100 mm. Air circulation in the reactor was provided by air sparger (Built-in glass sparger) at 0.2 LPM through air pump (MODEL HSV (2), 230/50 Hz 1PH, AMP MAX 0.8; Air filter: Built-in micro air filter). The process parameters like temperature, pH, and dissolved oxygen (DO) concentration were governed by using temperature controller, pH meter and DO meter respectively. The cooling jacket is provided to circulate water to maintain the set temperature in the reactor. Two clamps were fixed, one at sample collection tube and the other at air supply tube. Clamp A would be removed once the air pump was turned on, and clamp B would be fixed after collecting the sample. Silicon tubing was used for connection in the reactor.

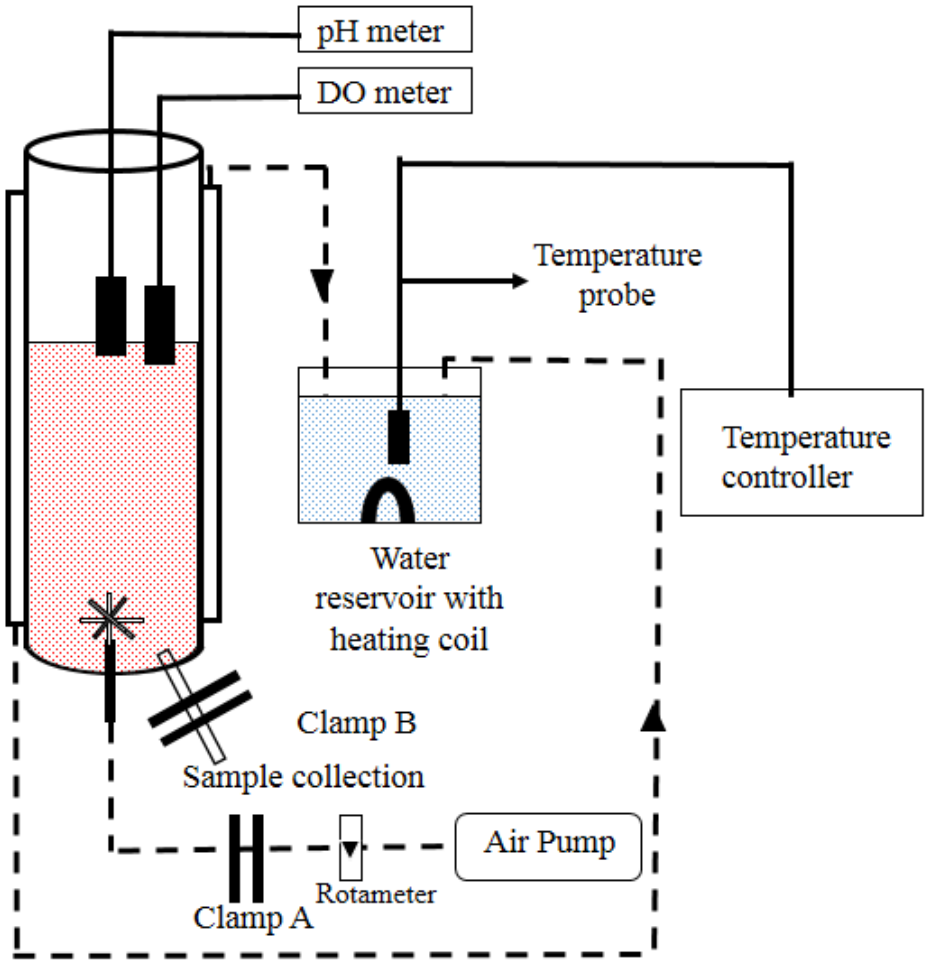


Figure 4: Schematic representation of Bioreactors set up for dye decolorization studies.

3.10.4 Design and development of restricted immobilized porous bed

The existing bioreactor was tailored for improved biological interaction, especially to enable the availability of low-density bio-carrier throughout the reaction mixture. A porous stainless steel box with a dimension of height 25 cm and diameter 8.5 cm was fabricated and inserted in the reactor named as " Restricted immobilized porous bed" (Fig. 5). The custom-made box holds equal partitions to localize the PUF in the respective partitions. It is made up of stainless steel (SS 316), which is a widely used material for bioreactor fabrication. The hook is placed on the top surface plate of the box to adjust the reaction chamber height by tying with stainless steel wire, and to avoid covering the air sparger by the custom-made box. This box is autoclavable, cost-effective, and customizable for different reaction volumes.

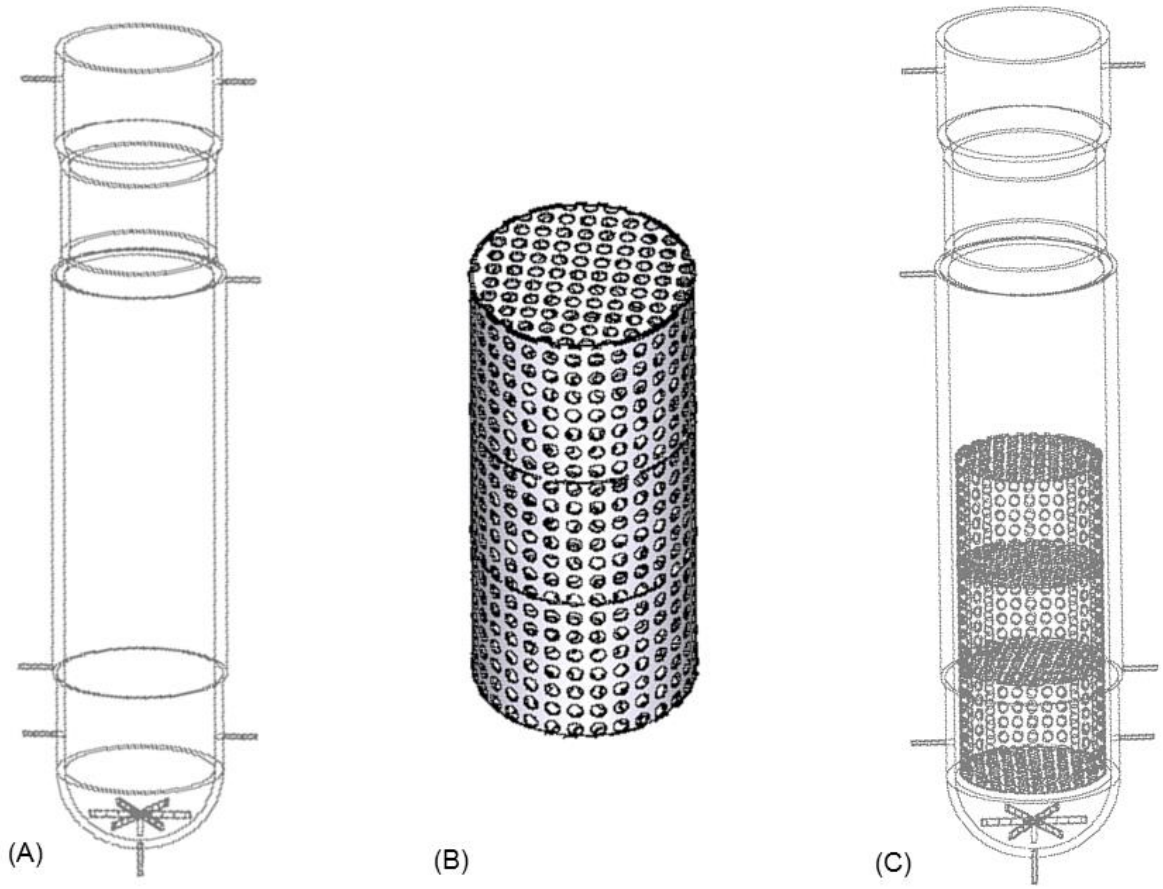


Figure 5: Schematic representation of (A) Bioreactors, (B) Customized box for localization of immobilized polyurethane foam (PUF) and (C) Box inserted into the reactor.

3.10.5 Active surface area modulation by varying foam arrangement

The active surface area for biological reaction is reduced in two ways: the non-availability of PUF for biological interaction in closely packed system, and non-availability of immobilized PUF at the bottom of the reactor due to its floating behavior owing to its low density (Fig. 6). Though it is difficult to provide the realistic information of available active surface area for biological interaction, the reduction of the active surface area due to the accumulation/gathering of foams on top of the reactor was noticed. Thus, a restricted immobilized porous bed-in-a bioreactor was designed for maximizing the accessible active surface area for microbial degradation. However, the active surface area also depends on the matrix size, shape, and packing method. PUF of 1 cm^3 cube was chosen for immobilization, which has 6 faces. In the free form, 6 faces of the cube are available for the biological interaction. In a closely packed system, all faces were not available for biological interaction. Thus, the volume of foams decides the active surface area.

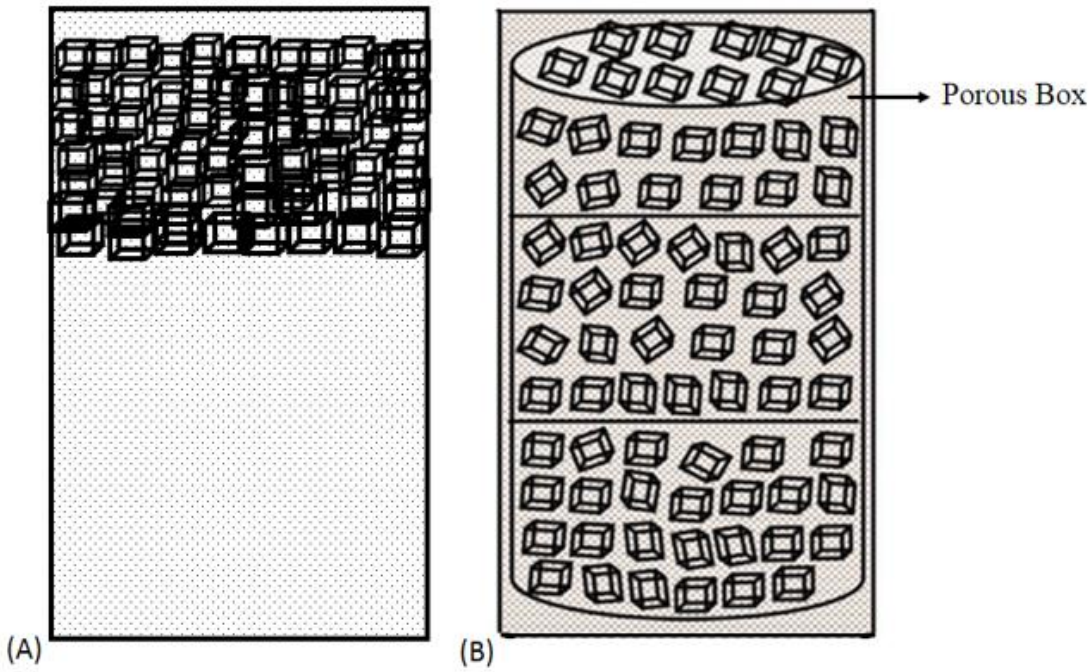


Figure 6: The immobilized PUF in the reactor with and without porous box. A) Gathering of low density PUF cubes on the top surface and left over space at the bottom of the reactor. B) The immobilized PUF localized in custom made porous box.

3.10.6 Mineralization analysis

TOC analysis for synthetic wastewater and real textile wastewater was observed using TOC ICPN analyzer, Shimadzu, Japan and BOD was obtained by 5-day incubation method (Baird et al. 2017).

3.11 CR decolorization in Microbial Fuel Cell (MFC)

3.11.1 Dual chamber MFC fabrication and operation

A dual chamber microbial fuel cell (DC-MFC) was fabricated in laboratory with two identical rectangular chambers made up of plexi sheet (Fig. 7). The working volume of each chamber is 126 mL with dimension of 4.1 cm × 4.1 cm × 7.5 cm and both the chambers were separated with proton exchange membrane (PEM). PEM was pretreated sequentially for 30 min in 30% of H_2O_2, and 1% of H_2SO_4 to improve the porosity. Carbon cloth was pretreated in 30% of HCl for 3 h. After distilled water wash, again it was soaked in pure acetone for 3 h. The pretreated carbon cloth (2 cm× 2.5 cm) was used as anode (FRL coated carbon cloth) and cathode (CuO coated carbon cloth). The anolyte solution consists of MSM media with dye and distilled water

as a catholyte solution. Anode and cathode were connected to digital multimeter by using stainless steel wire (SS316) for continuous observation of voltage. Anolyte solution was continuously stirred at 70 rpm to facilitate interaction between cells and dye solution.

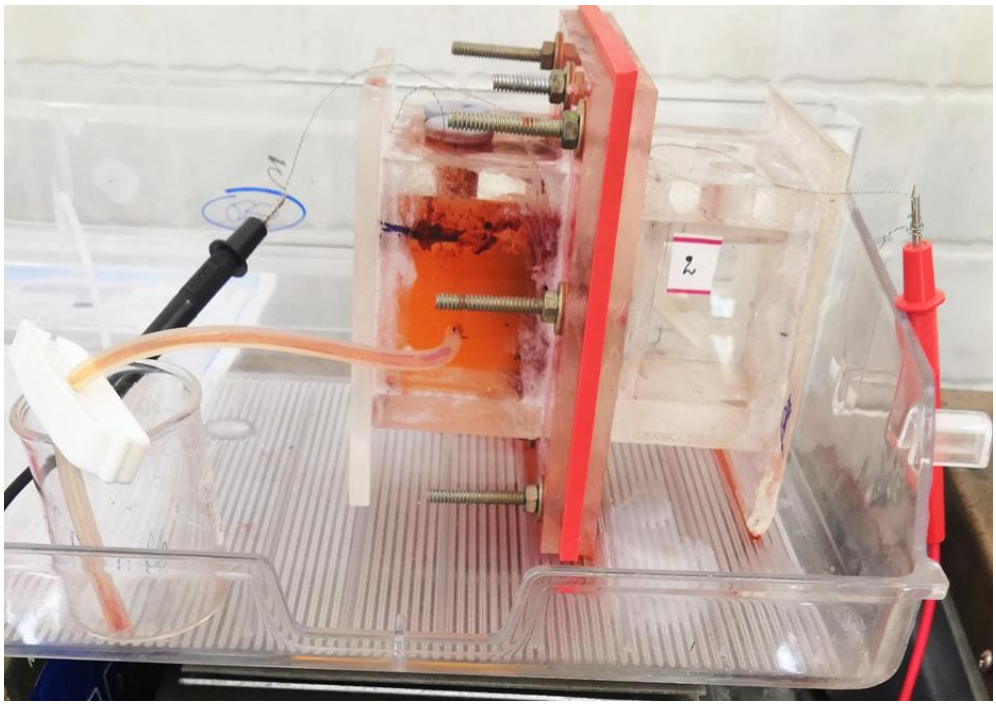


Figure 7: Microbial fuel cell setup used for dye decolorization studies.

MFC was operated in abiotic and biotic conditions (with and without glucose) to study the dye decolorization of CR dye at 100 mg/L concentration. The dye adsorption due to the FRL anode and the significance of glucose addition for effective decolorization was studied at room temperature. The effect of dye concentration, which was varied from 300 mg/L to 900 mg/L on decolorization in MFC was also studied, wherein glucose concentration of 2 g/L and HRT of 18 h was maintained. Further, dye concentration of 700 mg/L was used to study the effect of glucose concentration (0-3 g/L) on decolorization efficiency via MFC treatment. The decolorization efficiency was studied at different HRT time points 12, 15, 18, 24, 36 h for 700 mg/L dye concentration at 2.5 g/L of glucose concentration. Then, experiments were conducted at optimal HRT, and concentration of glucose and dye in triplicate.

3.11.2 Decolorization studies in MFC

Synthetic wastewater composed of CR and Minimal salt media (MSM) composed of K_2HPO_4 6.3 g/L, KH_2PO_4 1.8 g/L, NH_4NO_3 1.0 g/L, $MgSO_4.7H_2O$ 0.006 g/L and Glucose 2.0 g/L were used for decolorization experiments. All the experiments were conducted in triplicate in fed-

batch mode wherein, 100 mL of synthetic wastewater was kept in DC-MFC at room temperature. The decolorized samples were collected at various time intervals and results were recorded.

3.11.3 Preparation of Ficus religiosa porous carbon

The dried fallen Ficus religosa leaves were collected locally. The collected leaves were washed with mineral water 3-4 times followed by hot air oven drier at 60 °C for overnight and then crushed in mixer grinder. Dried powder was carbonized under Ar atmosphere at 750 °C for 2 hours. The obtained carbon was washed thoroughly with distilled water and 1M HCl for removing impurities and dried in vacuum oven at 80 °C for overnight (Senthilkumar and Selvan 2015).

3.11.4 Preparation of electrode

In order to prepare FRL coated carbon cloth electrode (anode); FRL carbon and VulcanXC-72 carbon were mixed thoroughly in the ratio of 70:30 using mortar and pestle. To prepare the catalytic ink, initially 500 µL isopropanol and 500 µL of acetone were added to the required amount of mixed carbon, followed by sonication of 1 h. Further, 300 µL of 5% Nafion Solution was added and kept for 1 h sonication. The obtained catalytic ink was loaded at the rate of 5 mg/cm^2 on the pretreated carbon cloth and dried at 50 °C for overnight to prepare the anode. In case of cathode, copper oxide and VulcanXC-72 carbon were mixed in the ratio of 70:30 using mortar and pestle. The rest of the protocol for preparation of cathode followed the protocol for anode preparation.

3.11.5 Characterization studies

The morphology of FRL carbon coated carbon cloth and anodic biofilm was examined by Scanning electron microscope (SEM) (VEGA 3 LMU, TESCAN). FT-IR instrument (Shimadzu, Perkin Elmer 1000) was operated at 2 cm^{-1} resolution and 400-4000 cm^{-1} range. X-ray diffraction (XRD) analysis of FRL carbon was studied using XRD-7000 (Shimadzu).

3.12 CR Degradation in MFC-MRFBBR

3.12.1 Raw material

Corncobs were purchased from local fields (Warangal, India) having 10-20% moisture content. Raw corncobs were washed with fresh water in order to remove toxic and interferences compounds and sundried before use.

3.12.2 Pretreatment of Corncob

Pretreatment of corncob for extraction of reducing sugar was performed as per the standard NREL protocol (NREL/TP-510-42619) (Sluiter et al. 2008). Extracted reducing sugar was determined by using the 3,5-dinitrosalicylic acid (DNS) method. 3.76±0.5 g of reducing sugar was obtained after pretreatment of 10 g corncob, and glucose standard was used as a reference for measuring total amount of reducing sugar obtained (Miller 1959).

3.12.3 Procedure for extraction of reducing sugar

Sundried corncobs were ground into small granules, and were then autoclaved for 60 minutes in 5M NaOH solution (1g: 7ml) for alkali treatment in order to remove lignin. Biomass separated after alkali treatment was washed thoroughly from water. Further, for acid treatment biomass was submerged into 72% H_2SO_4 and was incubated for 1 hour at 60°C. It was then diluted to 4% H_2SO_4 by adding distilled water and autoclaved for 60 minutes. The pH of obtained solution was maintained to 7 and filtered reducing sugar solution was used for dye degradation studies. Extraction procedure of reducing sugar (NREL/TP-510-42619) (Sluiter et al. 2008) is represented in Figure 8.

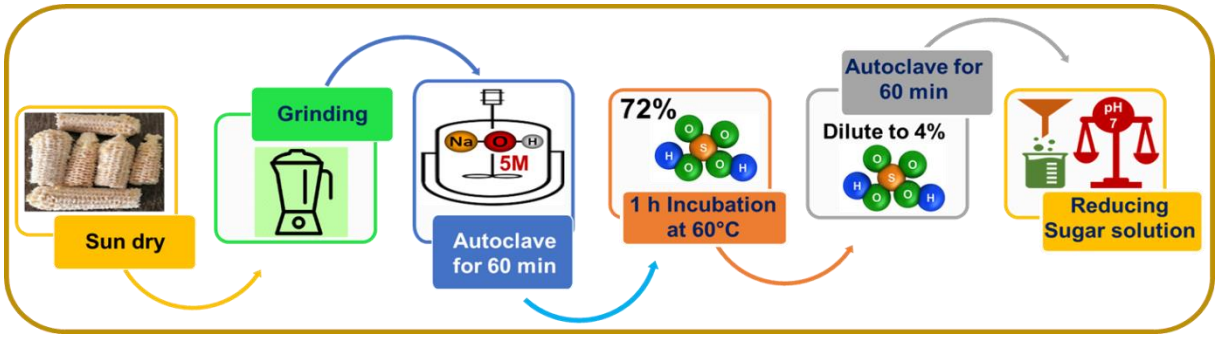


Figure 8: Representation of extraction method for reducing Sugar from corncob.

3.12.4 Electrode preparation

FRL carbon supported with VulcanXC-72 carbon was mixed in the ratio of 70:30. Then, 500 µl isopropanol and acetone were added for the required amount (loading rate: 5 mg/cm^2) of mixed carbon. Afterwards, 300 µl of 5% Nafion Solution was added, followed by 1h sonication. Catalytic ink was loaded on pretreated carbon cloth and dried in vacuum oven at 50 °C overnight (Nandy et al. 2019).

Cathode was prepared as per the similar protocol maintained above whereas, copper oxide (CuO) was used for cathode.

3.12.5 Integrated MFC-MRFBBR system design and operation

Three identical MFCs (600 mL volume) were developed in-house for CR dye decolorization. For MFC fabrication, the two identical chambers were made with acrylic sheet and proton exchange membrane (PEM) was fixed in between these two chambers. Carbon cloth with dimensions of 5×5 cm^2 was used as an electrode in MFCs. The anode and cathode were coated with biowaste derived carbon from *ficus religosa* (FRL) and copper oxide respectively. The electrodes were connected to multimeter using stainless steel wire and open circuit voltage (OCV) was recorded. The design and fabrication of this dual chamber MFC was done at in-house laboratory. In the previous study of the authors, In-house fabrication of MRFBBR and its potential application for CR dye decolorization was already reported (Shalini and Y. 2019). MFC and MRFBBR were connected using silicone tubing and the peristaltic pump was used to feed the treated water in MFC into the MRFBBR. The schematic representation of integrated MFC-MRFBBR system is depicted in Figure 9.

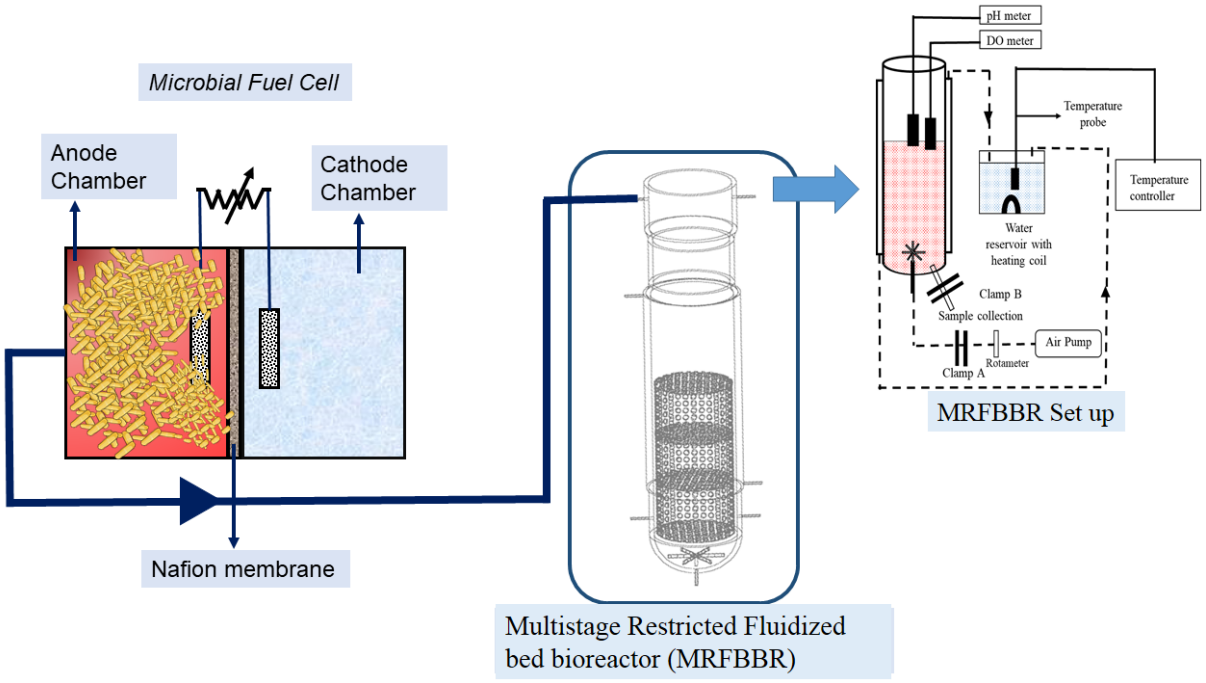


Figure 9: Schematic representation of coupled MFC-MRFBBR setup used for CR dye degradation studies.

Initially, MFC was operated in batch mode with 5 g/L of reducing sugar until the stable voltage was achieved at room temperature. Thereafter, all the experiments were conducted in fed-batch mode for 200 mg/L concentration of CR dye. The effluents treated from MFC stage at optimized

condition were transferred to the MRFBBR for second stage treatment. MRFBBR was operated at 0.2 LPM air flow rate and 37 °C temperature.

3.12.6 Synthetic wastewater and co-substrate

Minimal salt media (MSM) (composed of K_2HPO_4- 6.3 g/L, KH_2PO_4- 1.8 g/L, $NaNO_3$- 1.0 g/L, $MgSO_4.7H_2O$- 0.006 g/L) was used for the preparation of synthetic wastewater. Herein, CR dye concentration of 200 mg/L was chosen for studying the degradation using sequential treatment in MFC and MRFBBR. The reducing sugar was used as a co-substrate and fed into the anodic chamber of MFC. The cathode chamber was filled with water in MFC. The reducing sugar concentration was varied (20-5 g/L) which, acts as an electron donor. pH 7.3±2 was maintained in the anode chamber of MFC.

3.12.7 Decolorization and Degradation studies

The first stage of anaerobic experiment was conducted in dual chamber MFCs in Fed-batch mode and open circuit voltage (OCV) was recorded. CR dye of 200 mg/L of concentration was fixed for all the sets of experiments whereas reducing sugar concentration (20 g/L, 15 g/L, 10 g/L and 5 g/L) and dye feed time (24 h, 18 h, 15 h and12 h) were optimized. The Fed-batch strategy studied is represented in the Table 14.

Table 14: Fed-batch strategy for the CR dye feed in MFC.

S. No.	Total Feed time (h)	Total concentration of CR dye (200 mg/L)			
		50 mg/L	50 mg/L	50 mg/L	50 mg/L
(a)	24	0 h	8 h	16 h	24 h
(b)	18	0 h	6 h	12 h	18 h
(c)	15	0 h	5 h	10 h	15 h
(d)	12	0 h	4 h	8 h	12 h

3.12.8 Polarization study

The open circuit potential between the two electrodes was noted using a digital multimeter. The polarization (Fig. 10) of the MFC was conducted at RS concentration of 10 g/L and 18 h of feeding time. When all three MFCs reached to its maximum OCV at optimized condition then the circuit was connected to various external resistances ranging from 10000Ω to 100Ω for polarization study. Current (I) was calculated using Ohm's law: I=V/R; voltage (V) at resistance

(R). Current and power density were calculated by normalizing current and power values to the projected surface area of anode (25 cm^2).

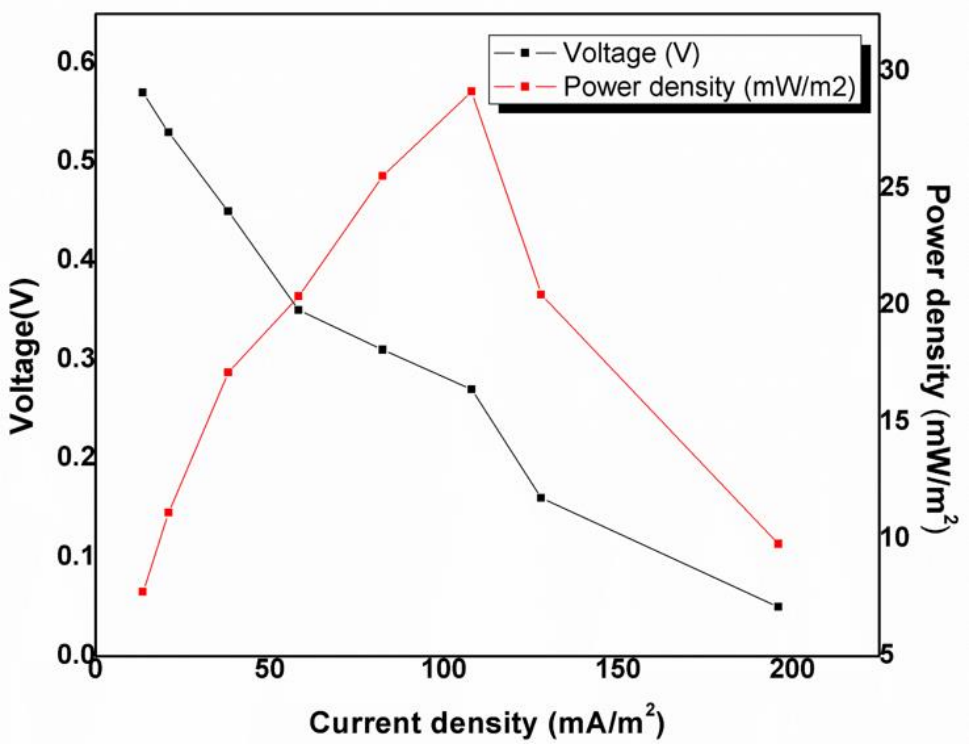


Figure 10: Polarization curve for microbial fuel cell at optimized condition.

3.12.9 Study of CR decolorization and degradation of metabolites in sequential anaerobic-aerobic process

UV-Vis spectrophotometry, Fourier transform infrared spectroscopy (FT-IR) and gas chromatography-mass spectrometry (GC-MS) analyses were used to study the degraded metabolites of CR dye. Samples collected at each stage were centrifuged at 8,000 rpm for 10 minutes and filtered through 0.22-µm syringe filter to separate the suspended biomass. Supernatant collected after centrifuging were directly scanned in UV- Vis spectrophotometry and absorbance at 490 nm were recorded.

3.12.10 Extraction of CR degradation products

10 mL of filtered samples were extracted using ethyl acetate in 1:1 fraction and dried in rotatory evaporator. It was then dissolved in 0.5 mL of HPLC-grade methanol and was used for FT-IR and GC-MS analysis.

3.12.10.1 Fourier transform infrared (FT-IR) analysis

FTIR spectrophotometer was used to understand the chemical nature of dye and the bio-transformed products obtained after treatment were analyzed at each stage.

3.12.10.2 GC-MS analysis

The bio-transformed metabolites of CR degradation at every stage of integrated process were identified by GC-MS analysis. GC-MS analysis was carried out using GCMS-QP2010, SHIMADZU, in temperature programming mode at 70ev ionization voltage. 1.0 mL/min flow rate of helium was maintained. The temperature of oven was maintained from 80 °C to 280 °C at 10 °C/min and held for 10 min. The highest resolution chromatographic peaks were identified by NIST library based on their mass spectrum.

3.12.11 Phytotoxicity effect of CR degradation product from each stage of integrated system

The toxicity effect of dye contained water on seed germination was evaluated using phytotoxicity studies. *Vigna radiate* (Mung bean) was chosen to study the toxicity effect of CR dye contained water before and after treatment. Ten good seeds of *V.radiate* were sprinkled in the sand pot and covered completely. The equal amount of sand was distributed in all plastic pots for experiments. 200 mg/L concentration of dye prepared with water and the same concentration was treated in MFC and integrated MFC-MRFBBR system. The metabolites extracted after treatment in MFC and integrated MFC-MRFBBR were dissolved in 20 mL of distilled water. Phototoxicity experiments were conducted at room temperature. The treated and untreated water was poured regularly into the plants, whereas the domestic water was used as control for evaluation of toxicity. All experiments were conducted in triplicate mode. To check the effect of CR dye and its degraded metabolites formed during the treatment process in MFC and integrated system, root length, shoot length and percentage germination were recorded after 10 days of incubation period.

$$\%\ \text{Germination} = \frac{\text{Number of seeds germinated}}{\text{Number of seeds Sowed}} \times 100$$

4. Results and Discussion

This chapter consists of four sections as follows

1. Immobilization of *Bacillus subtilis* for improved decolorization of Congo Red (CR) compared to free cells.
2. Multistage Restricted Fluidized Bed Bioreactor for Dye Decolorization using Immobilized Polyurethane foam.
3. Microbial Fuel Cell assisted Congo Red Dye Decolorization using Biowaste Derived Anode Material and *Bacillus subtilis*.
4. Microbial Fuel Cell and Multistage Restricted Fluidized Bed Bioreactor Integrated Approach for Congo Red Dye Degradation using Corn Cob Agro Residue as co-substrate.

4.1 Immobilization of *Bacillus subtilis* for improved decolorization of Congo Red (CR) compared to free cells

4.1.1 Effect of parameters on free cells mediated decolorization

The effect of process parameters like dye concentration, pH, and temperature on decolorization of CR was investigated. An increasing trend of dye decolorization was observed over time irrespective of dye concentration (Fig 11). It is confirmed that the dye concentration mostly, is the determining factor in decolorization.

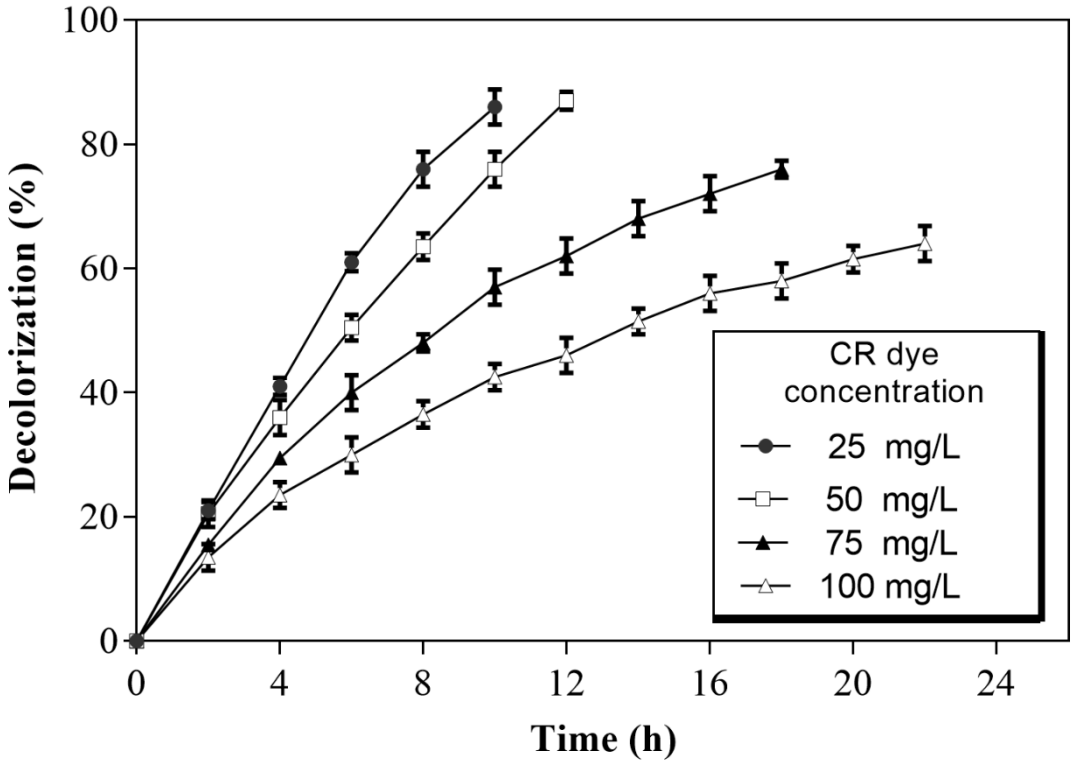


Figure 11: Effect of CR dye concentration on decolorization by *Bacillus subtilis* (n=3).

In the present study, decolorization of approximately 85% was attained within 10 h in case of dye concentration of 25 mg/L. The decolorization efficiency by *Bacillus subtilis* is significantly lowered at the dye concentration of 100 mg/L. Higher concentration resulted in the reduction of decolorization of dye by *Bacillus subtilis*. This could be due to the fact that higher concentration of dye is not favorable for cell growth (Lade et al. 2015a). The current study has considered cell mass profile at each time point to elucidate the effect of cell mass on dye decolorization. Interestingly, it was noticed that dye decolorization is proportionate to cell mass. The cell mass is a vital factor for effective dye decolorization, and the effect of dye

concentration on cell density was recorded at OD_{600} (Fig 12). Dye concentration of 25 mg/L resulted in higher cell mass and decolorization within 10 h, as shown in Figure 12. Dye concentration of 50 mg/L showed higher decolorization compared to 75 mg/L and 100 mg/L. Overall, the results of this study confirmed that 50 mg/L dye concentration is tolerable by microbial cells, and improved cell growth and cellular metabolism favors optimal decolorization. Thus, 50 mg/L was considered for further studies to optimize the pH and temperature. In another study (Tan et al. 2014), it was reported that the time required for a lower concentration of azo dye removal is very less compared to a higher concentration (Tan et al. 2014). In this study, it was observed that dye concentration affects the decolorization behavior and decolorization time also varies.

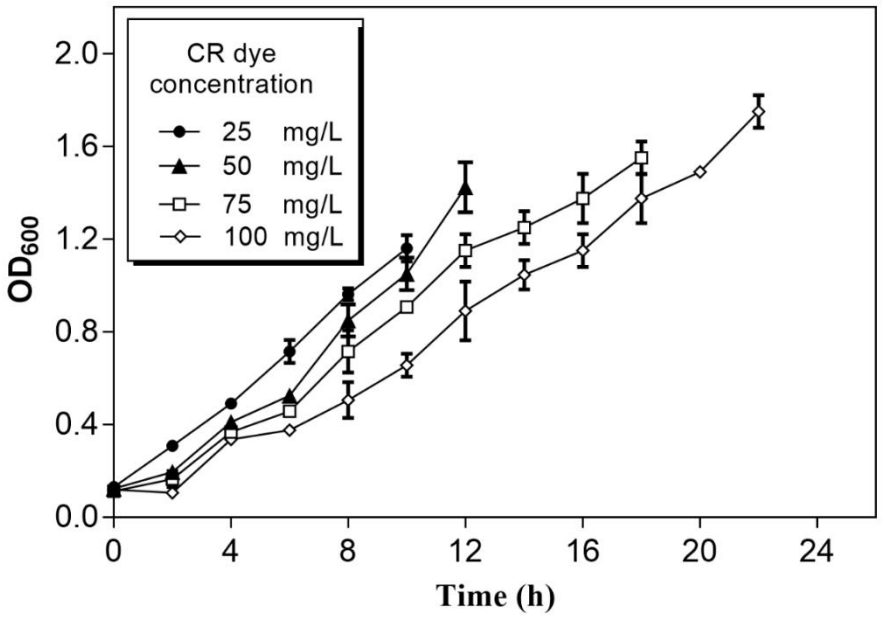


Figure 12: Influence of CR dye concentration on cell mass (OD at 600 nm) (n=3).

pH of the media is the limiting factor for dye decolorization because it helps in dye transportation inside the microbial cell membranes (Lade et al. 2015b). The effect of pH on CR dye (50 mg/L) decolorization is shown in Figure 13. The maximum decolorization of CR, i.e., 92%, was obtained at pH 9. It was found that the acidic pH 6 resulted in 52.32% decolorization, which is approximately one-fold decrement compared to pH 9. The enhanced decolorization was observed at pH 7. Generally, in textile effluents pH is alkaline (Kuhad et al. 2004). Various studies have been conducted to find out the suitable pH for higher decolorization of textile dyes

using fungi, bacteria, and yeast, and was found that pH of 7 or basic is more effective for bacterial decolorization (Agrawal et al. 2017). Also, strongly acidic/alkaline pH does not favor the decolorization (Lade et al. 2015a). The results of the current study affirmed that acidic pH is unfavorable for the decolorization.

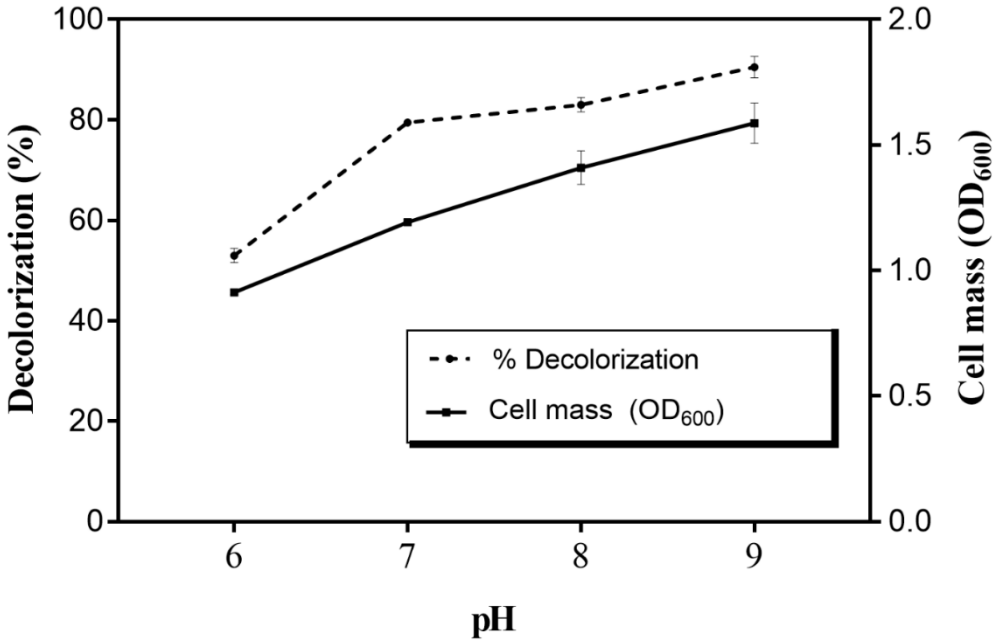


Figure 13: The effect of pH on decolorization of 50 mg/L CR dye and cell mass within 12 h (n=3).

Bacterial dye degradation potential is dependent on bacterial cell growth and its enzymatic activities at its respective temperature (Lade et al. 2015b). Change in decolorization of CR using *Bacillus subtilis* over the temperature range of 20-45±0.2 °C was observed (Fig 14). However, maximum decolorization percentage of 94% CR dye (50 mg/L) was found at 37 °C within 12 hours, while 40%, 45%, 56%,92%, 63% and 54% of decolorization was achieved at temperatures of 20, 25, 30, 35, 40 and 45 °C ± 0.2 °C, respectively. The increase of decolorization was recorded with an increase of temperature from 20 to 37 °C. The decolorization efficiency was found to be lower at both lower and higher temperatures and was observed that corresponding cell mass also followed a similar trend. The microbial cell proliferation and growth is being reduced, and enzyme activity was found to be reduced at a temperature beyond optimum (Agrawal et al. 2017; Meerbergen et al. 2018). Here, the current study indicates that dye decolorization varied with temperature, and cell mass is also affected by temperature. It was confirmed that optimum temperature is required for higher cell mass and

decolorization of CR. i.e., .37 °C. The optimal values of process parameters were found to be dye concentration 50 mg/L, temperature 37 °C, and pH 9 for higher decolorization of CR.

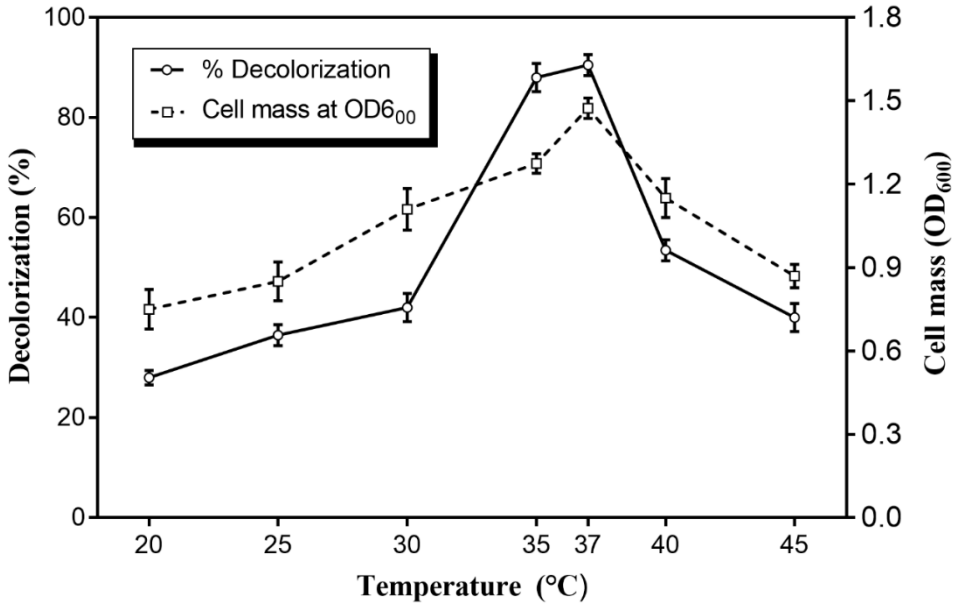


Figure 14: Effect of temperature on decolorization of 50 mg/L CR dye and cell mass within 12 h (n=3).

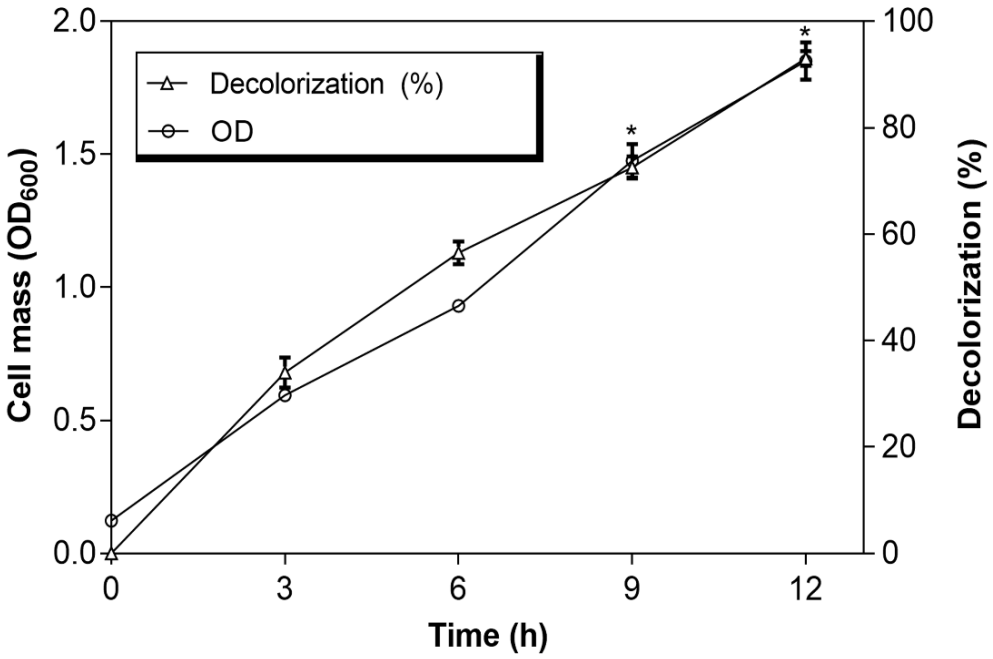


Figure 15: Free cell mediated CR dye decolorization using optimized conditions (dye concentration 50 mg/L, pH 9, and temperature 37 °C). (n=3, * $p<0.05$).

As shown in Figure 15, the decolorization of CR dye was found to be higher at 12 h. The exponential increment was observed in decolorization and cell mass. The optimized conditions favored the microbial growth, which is confirmed by higher cell mass. This led to the improved decolorization by suspended cells, approximately 92% decolorization was achieved, whereas about 82% of decolorization of 50 mg/L CR dye was achieved at pH 7 and temperature 37 °C. The results of this study concluded that the optimum process parameters highly enabled the microbial cells for enhanced decolorization.

4.1.2 Immobilized cells mediated CR dye decolorization

The decolorization of CR dye using immobilized *Bacillus subtilis* was shown in Figure 16. The PUF immobilized cells yielded higher decolorization of 92% within 6 h. A significant improvement in the dye decolorization can be seen from 2 to 6 h. The statistical analysis was performed to assess the decolorization efficiency with respect to time and confirmed that the decolorization at 4 h and 6 h is significantly higher than that of decolorization at 2 h ($p<0.05$). The time required for dye decolorization by immobilized cells is less compared to free cells mediated decolorization. This is nearly equal to one-fold decrement of time compared to free cell decolorization. The absorbance of dye by PUF foam also needs to be considered to evaluate the efficiency of immobilized cell assisted dye decolorization. It was noticed experimentally that approximately 5% of CR dye absorption by PUF was found at 6 h, which indicates that absorption by PUF is negligible. This is also in agreement with the results reported in another study (Lade et al. 2015a), that absorption of dye by PUF cubes without cells is negligible for the decolorization of dye using textile effluent in a reactor. The present study also inferred that immobilized microbial cells removed the dye, whereas the dye absorption by PUF absorption is negligible.

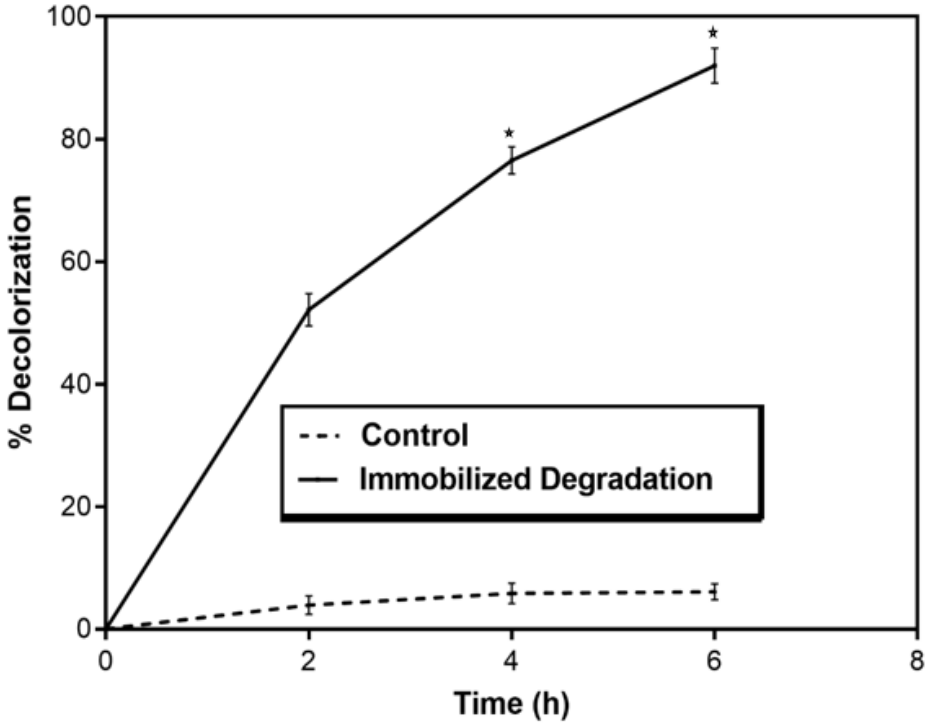


Figure 16: Decolorization of CR dye using immobilized *Bacillus subtilis* within 6 hours. Control represents dye absorbance by PUF without cell. The data represented in Mean ±SD (n=3, * $p<0.05$).

4.1.3 SEM and FTIR analysis

SEM images were taken to confirm the immobilization. A layer formation by *Bacillus subtilis* was found in immobilized PUF (Fig. 17). *Bacillus subtilis* formed chain-like structures and found that each cell was in rod shape (Fig. 17 (b-d)). The layer formation could be seen in Figure 17 (a), and images were recorded on the same foam at different locations using different magnification, which confirmed the bacterial colonization. The microbial cells were attached to PUF and immobilized well, which resulted in enhanced decolorization in less time. Due to this immobilization on PUF, cells were allowed to react with CR dye adequately, and adherence of cells to PUF could support the higher cell growth. The cell-substrate reaction could be higher in the case of immobilized cells, which enhanced dye degradation. It was reported that immobilization of *Brevibacillus parabrevis* on coconut shell biochar provided large surface area for interaction of dye and microbial cells, which resulted in active degradation at higher concentration (Abu Talha et al. 2018). The porous material is a potential candidate for higher cell mass colonization, and hence a porous material PUF has been chosen for immobilization. Present study also showed improved decolorization by immobilized cells compared to free

cells, which attributed to higher surface contact between CR dye and *Bacillus subtilis* facilitated by PUF, and higher cell mass got colonized due to its porous nature.

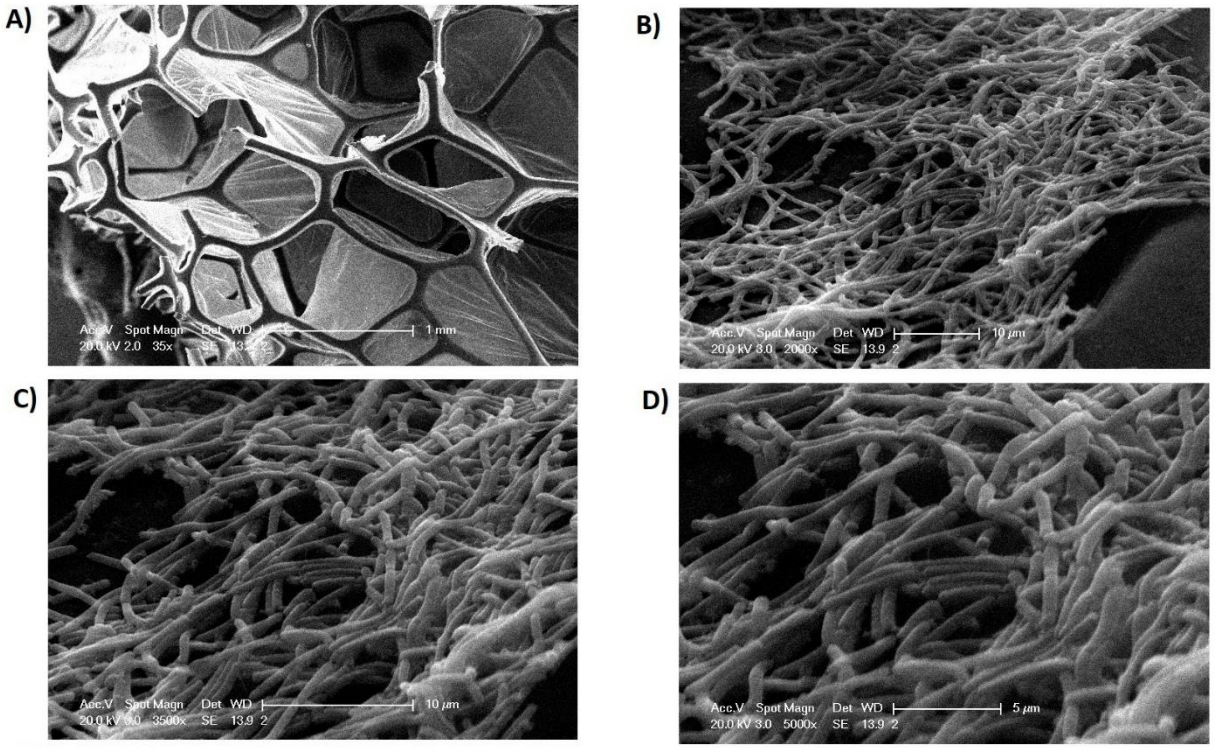


Figure 17: SEM images of a) biofilm (35x), (b, c and d) bacterial colonization on PUF matrix at different magnifications (2000X, 3500X, and 5000X).

FTIR analysis before and after decolorization is depicted in Figure 18. The peaks appeared were predominantly in the range of 630-1593 cm^{-1}. The major shift in the peaks and the disappearance of peaks was observed after decolorization. The presence of a peak at 1638 and 1593 cm^{-1} in dye control sample attributed to azo –N=N– double bond, which disappeared after decolorization (Li et al. 2015; Bartošová et al. 2017). It was reported that the benzene ring structure of Congo red dye is represented in between 880.64 to 698.41 cm^{-1}, and it was noticed that peaks from 840 to 630 cm^{-1} have disappeared after decolorization (Lade et al. 2015a). The stretching peak at 3457 cm^{-1} corresponds to –OH group. There is vast variation seen in control versus decolorized sample confirming that the dye decolorization is achieved and also the reported FTIR results are consistent with the earlier reports on CR dye decolorization (Lade et al. 2015a; Li et al. 2015; Meerbergen et al. 2018).

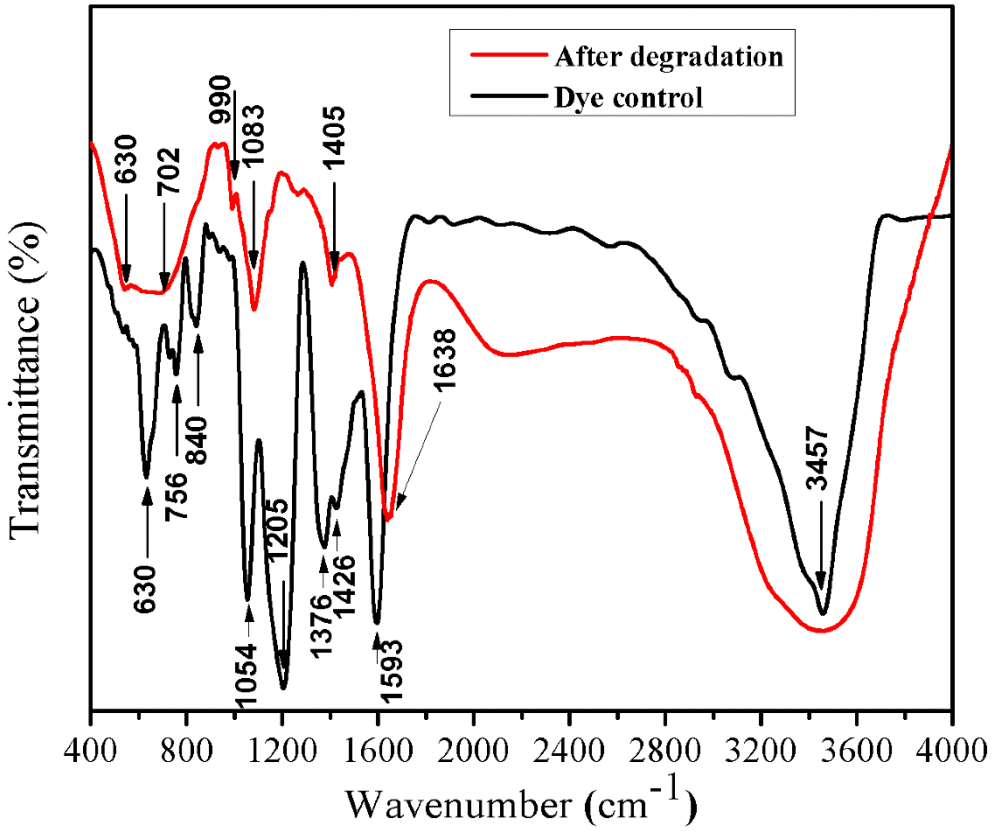


Figure 18: FTIR analysis of samples before and after dye decolorization.

4.2 Multistage Restricted Fluidized Bed Bioreactor for Dye Decolorization using Immobilized Polyurethane foam

4.2.1 Dye decolorization with and without porous box in a bioreactor

Dye decolorization (50 mg/L) using immobilized *B. subtilis* in a bioreactor with and without PSSB over a time period of 16 h is depicted in Figure 19. The results showed that higher dye decolorization was found in PSSB contained reactor at all-time points, and confirmed that decolorization rate is statistically significant ($p<0.05$, two-way ANNOVA). It was observed that PUF cubes were floated and moved to the top surface, which led to improper microbial cell interaction with the dye in a reactor without PSSB. This could be the possible reason for lower decolorization in the absence of PSSB in a reactor. It indicates that PSSB improved the distribution and localization of PUF cubes in a bioreactor throughout the reaction volume. Thus, further dye decolorization studies were conducted using PSSB in a bioreactor.

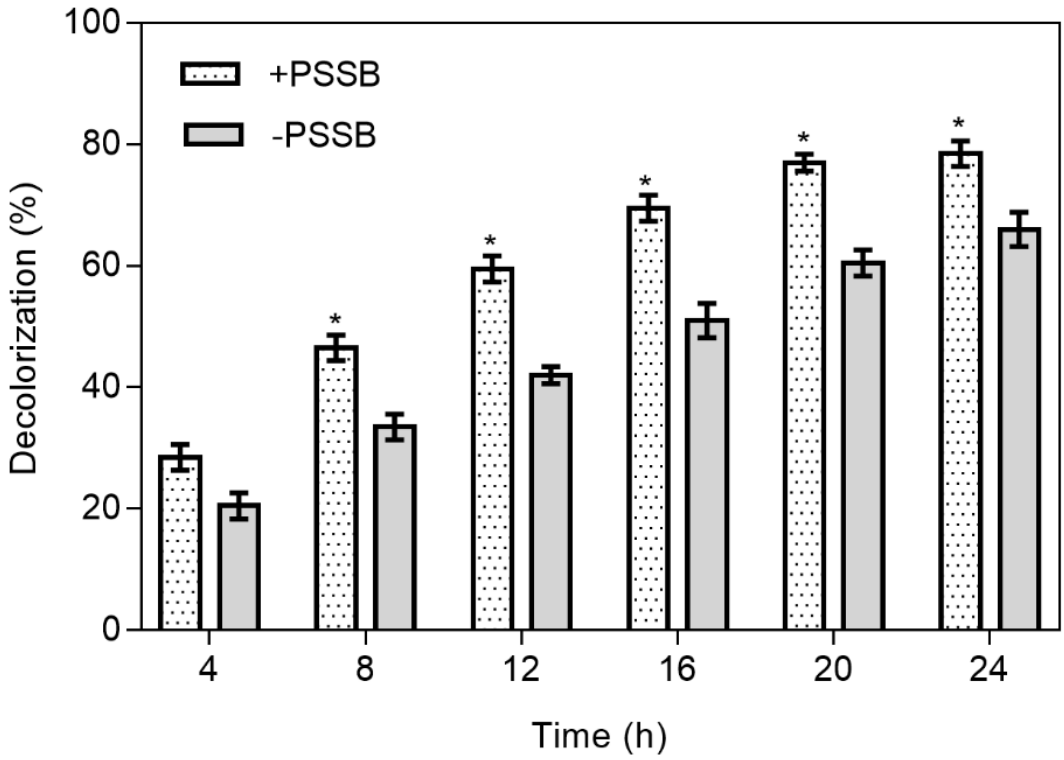


Figure 19: Dye decolorization in the presence and absence of porous stainless-steel box (PSSB) in a bioreactor over a time period of 16 h (n=3, * $p < 0.05$).

4.2.2 Effect of dye concentration, PUF weight, pH and glucose concentration on dye Decolorization

The effect of CR dye concentration (50–200 mg/L) on decolorization was studied in PSSB contained reactor, and results are shown in Figure 20. It can be seen that the percentage of dye decolorization declined with an increase in dye concentration. Bacteria used in the present study can decolorize up to 200 mg/L dye concentration, and the highest decolorization was found at 100 mg/L. Abu Talha et al. (2018) studied the effect of CR dye concentration on decolorization and reported that dye concentration limits bacterial growth. Also, they suggested that it is essential to know the threshold limit of dye concentration for effective decolorization by promoting bacterial growth and colonization on the immobilized surface. In another study (Padmanaban et al. 2016), it was reported that dye concentration resulted in declined dye decolorization beyond 200 mg/L, which confirmed the inhibition of bacteria at a higher concentration of dye. In the present study, a decrease in dye decolorization with increasing dye concentration was observed, and a significant fall in decolorization occurred beyond 100 mg/L.

Thus, 100 mg/L was chosen to optimize other parameters such as PUF weight, pH, and glucose concentration.

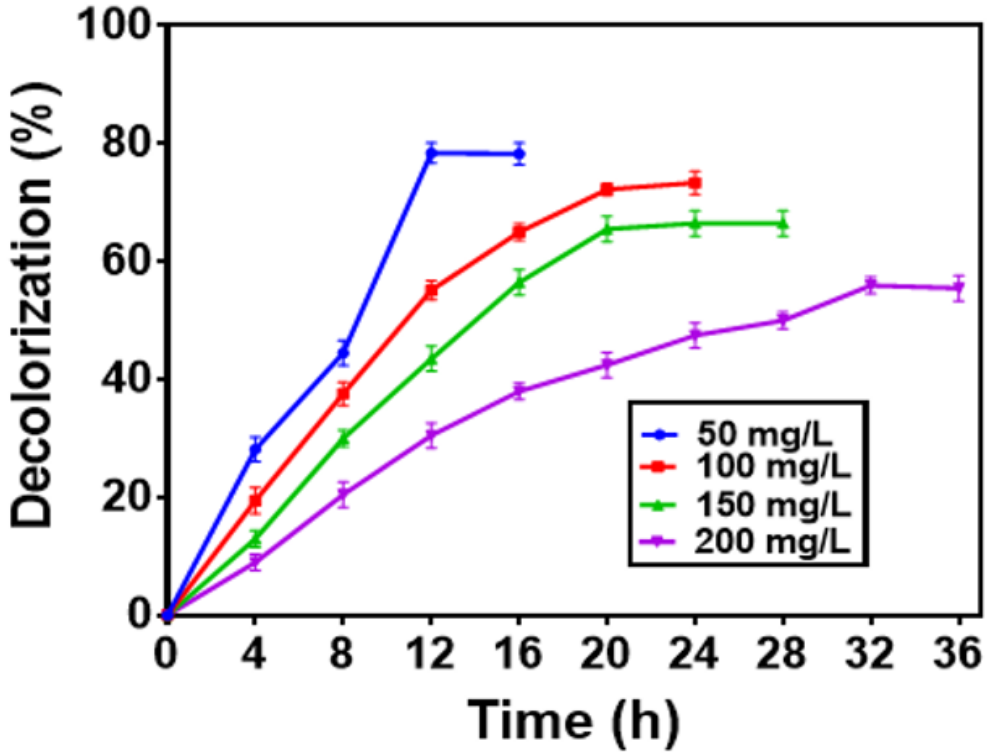


Figure 20: Effect of CR dye concentration on dye decolorization in the porous box contained bioreactor (n=3).

Effect of PUF weight on dye decolorization is presented in (Fig. 21). Dye decolorization was found to be maximum for 5 g of PUF. Though 7 g of PUF is readily available for entire reaction volume, foams got packed fully in PSSB, which reduced the available active surface area for microbial colonization and interaction with dye. 3 g of PUF also resulted in lower degradation, and the available surface area is not enough for effective colonization of bacteria and interaction with dye. Feng et al. (2012) reported that the packing rate is a vital factor for effective dye decolorization and treatment efficiency can be affected by active surface area available for bacterial colonization. In the present study, PUF packing, which depends on the weight of PUF played a vital role in dye decolorization. The available foam throughout the reaction volume was noticed in case of 5 g of PUF, and all faces of each cube were available for microbial interaction, which enhanced bacterial colonization and growth. Also, diffusion and permeability would be improved at an optimal packing rate i.e. 5 g of PUF. Thus, higher dye decolorization was recorded using 5 g of PUF in PSSB. The key observations and understanding of the

phenomenon behind the effect of packing rate on bacterial colonization were in agreement with the published report (Feng et al. 2012).The porosity, size, and type of PUF were pivotal factors in microbial assisted wastewater treatment, and overall biomass growth was found to be higher in aerobic condition. PUF size of 2×2 (in cm) provided the maximum available surface area for biofilm formation and aerobic condition facilitated a good supply of nutrients internally, which led to higher internal biomass in PUF (Nguyen et al. 2010). Though the study on wastewater treatment using PUF is different from the present study, the weight and volume of foam determine the available surface area to improve the efficiency of dye degradation which is an important consideration and is following reports of Nguyen et al. (Nguyen et al. 2010). PUF is a preferable bio-carrier material as it has advantages of high surface area, strength, durability, and good porosity.

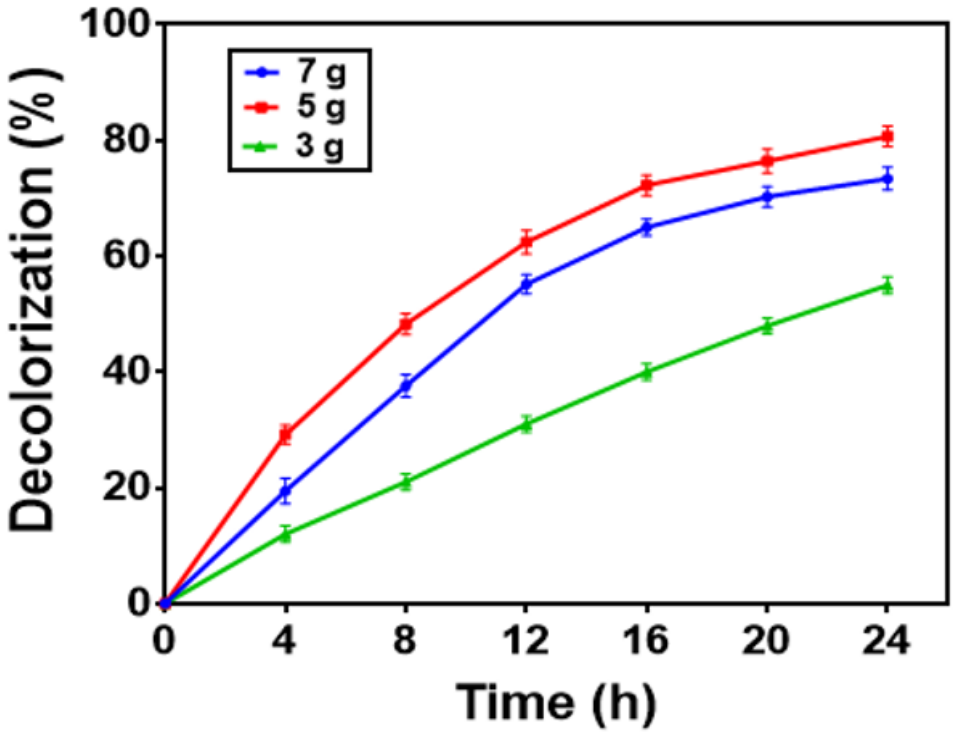


Figure 21: Effect of the weight of PUF on dye decolorization in the porous box contained bioreactor (n=3).

The effect of pH on dye decolorization was also studied. Higher dye decolorization was observed at pH 8 (Fig. 22). The decolorization of 62.01%, 80.73%, 85.10% and 69.96% was achieved at pH of 6, 7, 8 & 9 respectively. Acidic and alkaline pH (6 & 9) was not favorable for the microbial culture, which impedes the decolorization process by 23% and 16%

respectively. Metabolic activity of bacteria could slow down at extreme acidic or alkaline pH. There was an increment of 5% in decolorization when pH raises from 7 to 8. pH resistivity study towards microbial culture is essential since it provides the sustainable environment for growth and treatment for industrial effluents (Aksu and Dönmez 2003). pH 7 to 8, and the alkaline environment is highly favorable for azo dye degradation (Chen et al. 2003a; Kuhad et al. 2004; Lade et al. 2015a).

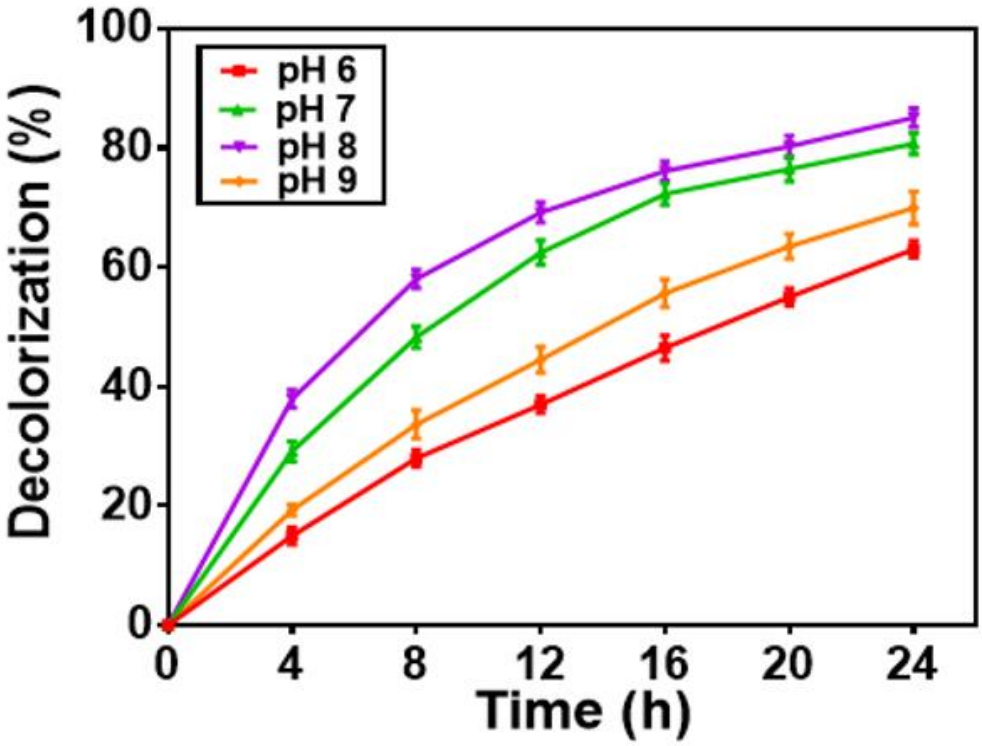


Figure 22: Effect of pH on dye decolorization in the porous box contained bioreactor (n=3).

Dye decolorization was studied by varying concentrations of glucose from 0 to 6 g/L, and higher decolorization was found at 4 g/L (Fig. 23). Dye decolorization was found to be maximum at 4 g/L glucose concentration compared to 0 g/L by a difference of 40%. Dye decolorization has proportionally increased with an increase in glucose concentration from 0 to 4 g/L. The decline in dye decolorization has been found at 6 g/L glucose concentration. Higher glucose content could have inhibited bacterial growth. Decolorization was studied with respect to change in glucose concentration, and the lowest decolorization was recorded at 6 g/L glucose concentration and is in agreement with Tan et al. (Tan et al. 2014). They reported that all microorganisms are not able to use the azo dye, but decolorization of azo dye is possibly achieved by the co-metabolism process using external carbon source. 50.87%, 59.58%, 87.26%

& 64.22% of decolorization were found at 0, 2, 4 & 6 g/L of glucose respectively. *B. Subtilis*, an aerobic bacterium has a tendency to reduce the azo bond cleavage by a reductive mechanism. Glucose, used as co-substrate aided for the formation of reducing factors (FAD, FMN, and NADH) through oxidation of compounds by producing electrons, which in turn enhanced efficiency of wastewater treatment (Carliell et al. 1995). The present study was also conducted at a flow rate of 0.2 LPM to provide the aerobic condition, and co-substrate glucose was used to improve dye decolorization. Several studies were reported stating that the provision of co-substrate is obligatory to donate electrons for azo dye decolorization (Senthilkumar et al. 2011; Ong et al. 2012). The mixed ratio of dye concentration and co-substrate is crucial for microbial metabolism. Though dye concentration is high, more availability of co-substrate does not allow bacteria to utilize dye as a substrate for its growth, which led to decreased dye decolorization at higher glucose concentration (Shi et al. 2012).

The findings of this study suggesting that optimal glucose addition is essential to maximize decolorization. In contrary, the addition of commercial glucose has to be replaced with glucose derived from agriculture residues to provide economically feasible and practically applicable process to translate the technology for industrial scale plants. Mostly, agricultural residues have been widely investigated for lignocellulolytic enzymes and ethanol production, wherein the pretreated biomass can produce higher amount of sugars after hydrolysis. However, there are some studies available on the usage of agricultural residues for textile wastewater treatment, wherein the efforts have also been made to produce biofuel simultaneously. Saratale et al., reported on solid state fermentation approach which enhanced the lignocellulosic enzyme production using rice waste bagasse, which accounted for maximizing ethanol production and was subsequently used for textile wastewater treatment. Herein, the left-over slurry after ethanol production has been investigated for dye decolorization studies. The integration of biotechnological approaches leads to the development of cost effective, sustainable methods with effective utilization of agricultural residues (Saratale et al. 2017).

In another study, sugarcane bagasse was used for reactive blue 172 dye and cellulolytic enzyme hydrolysis, which integrated the bioremediation-biofuel coupled process to adopt the technology for solid waste management, dye degradation and biofuel production (Waghmare et al. 2014). The additional supplement of lignocellulosic biomass contributes for effective dye degradation and also enhances the lingocellulytic enzyme production (Jadhav et al. 2008; Saratale et al. 2011a). The significance of additional carbon source other than pure glucose in

decolorization studies suggests methods to explore further investigations on agro residue mediated dye decolorization using immobilized microbial cells. Given the details of advantages of the proposed decolorization approach in the MRFBBR in the presence of co-substrate glucose and the available literature, it is intended to continue further studies using biomass.

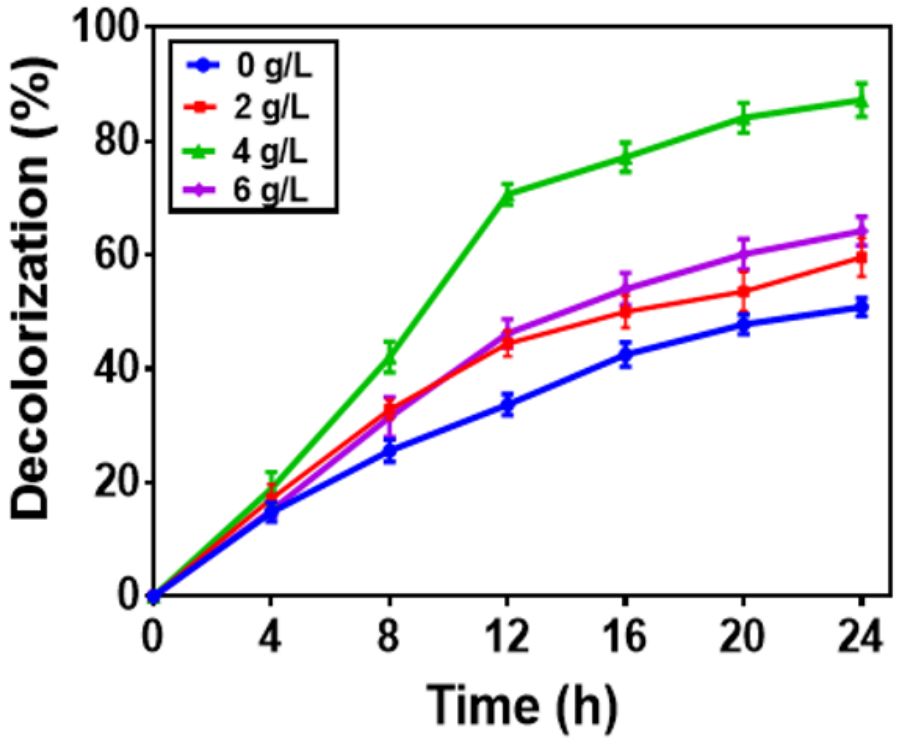


Figure 23: Effect of glucose concentration on dye decolorization in the porous box contained bioreactor (n=3).

Dye decolorization study was performed using optimal conditions (dye concentration 100 mg/L, PUF weight 5 g, pH 8, and glucose concentration 4 g/L) as shown in Fig 24. The maximum dye decolorization of 92% was achieved using PSSB contained bioreactor. Dye decolorization was improved in a bioreactor at optimal conditions, which confirmed that controlled environment and favorable conditions for bacterial colonization and metabolic activity accounted for higher decolorization.

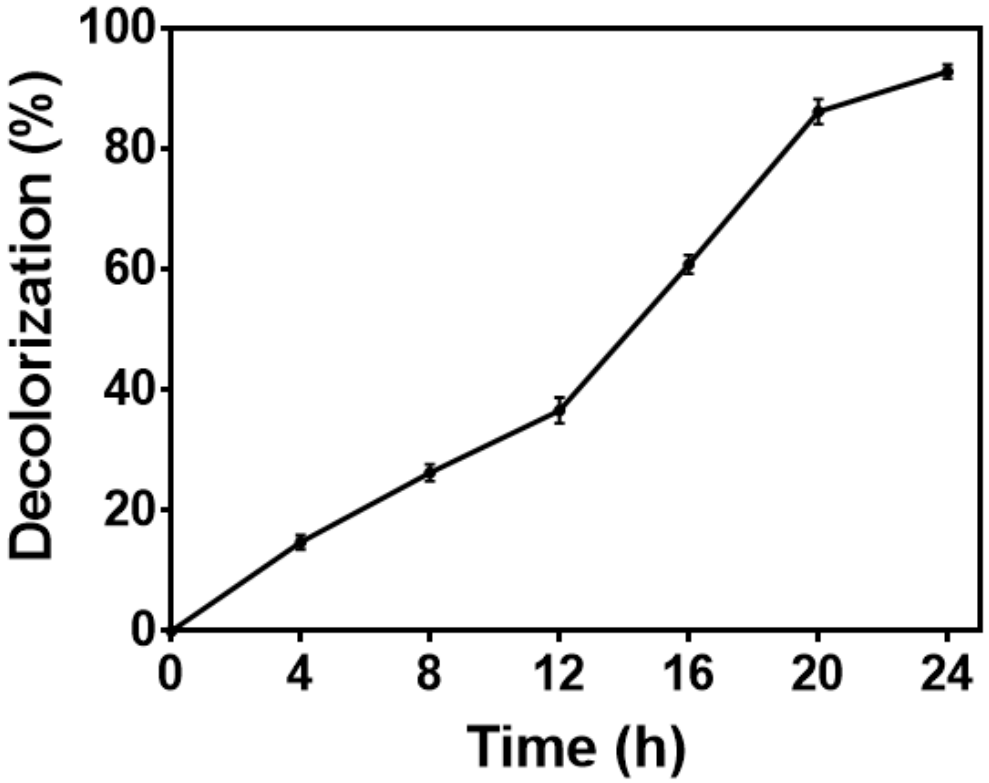


Figure 24: Dye decolorization at optimal conditions (dye concentration 100 mg/L, PUF weight 5 g, pH 8, and 4 g/L of glucose concentration) in a bioreactor using the porous box.

4.2.3. Mineralization analysis

In order to confirm the mineralization of synthetic wastewater and RTW before and after treatment, COD and TOC were analyzed. The initial COD and TOC of synthetic water and RTW are 5760, 3583 and 3250, 1751 (mg/L) respectively. The COD reduction (86.6%, 90.15%) and TOC reduction (57.54%, 69.13%) were obtained for synthetic water and RTW respectively (Fig. 25). The reduction percentage of COD and TOC of RTW are apparently similar to synthetic water as shown in Figure 25. This indicates that *B. subtilis* immobilized on PUF in a MRFBBR improved the decolorization along with significant reduction of COD and TOC. The decolorization and mineralization of CR dye suggest that *B. subtilis* metabolizes the dye, the intermediate compounds involved in mineralization, and MRFBBR facilitated for higher decolorization via immobilized cells. The decolorization approach would be considered for real time industrial application in view of environmental and economic aspects, only when immobilized cells on bio-carrier can utilize the dye and intermediate compounds which contribute for mineralization of the dye (Saratale et al. 2011b). According to this, *B. subtilis*

immobilized on PUF in a MRFBBR approach for dye decolorization would be worthy to be used in practical applications.

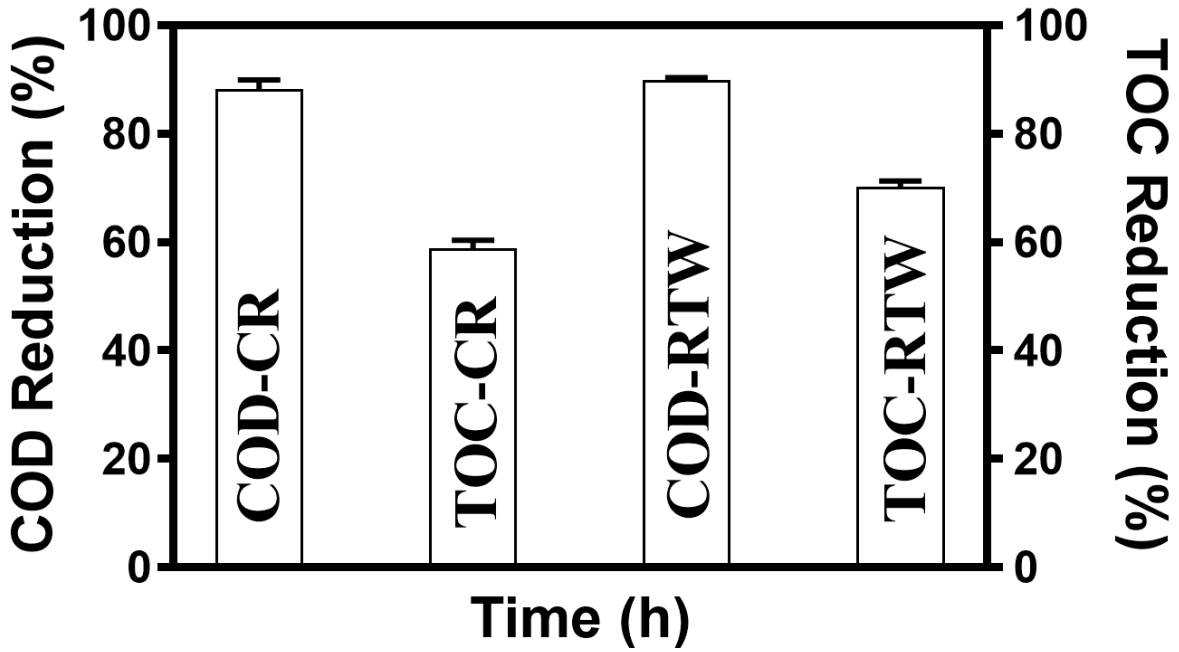


Figure 25: COD and TOC reduction percentage for CR dye and real textile wastewater (RTW) at optimum condition after treatment at 24 h.

4.2.4. FTIR analysis

FTIR was employed to confirm the dye decolorization, for which samples were analyzed before and after decolorization. The stretching peaks were mostly observed in the control sample in the range of 1630-1649 cm^{-1} (Fig. 26). These peaks almost disappeared after decolorization, especially 1638 cm^{-1} peak, which corresponds to azo –N=N– double bond was not found after decolorization (Lade et al. 2015a). The major shift in the peaks and the disappearance of peaks was noticed after decolorization. The peaks in the range of 834 to 640 cm^{-1} denote benzene ring, of Congo Red dye, which did not appear in the FTIR sample after decolorization (Bartošová et al. 2017). The stretching peak at 3457 cm^{-1} corresponds to −OH group. The FTIR results clearly showed a huge variation between samples of 0 h and 24 h. The previous studies on CR dye decolorization using other approaches were also checked for FTIR, which are in line with our findings (Lade et al. 2015a).

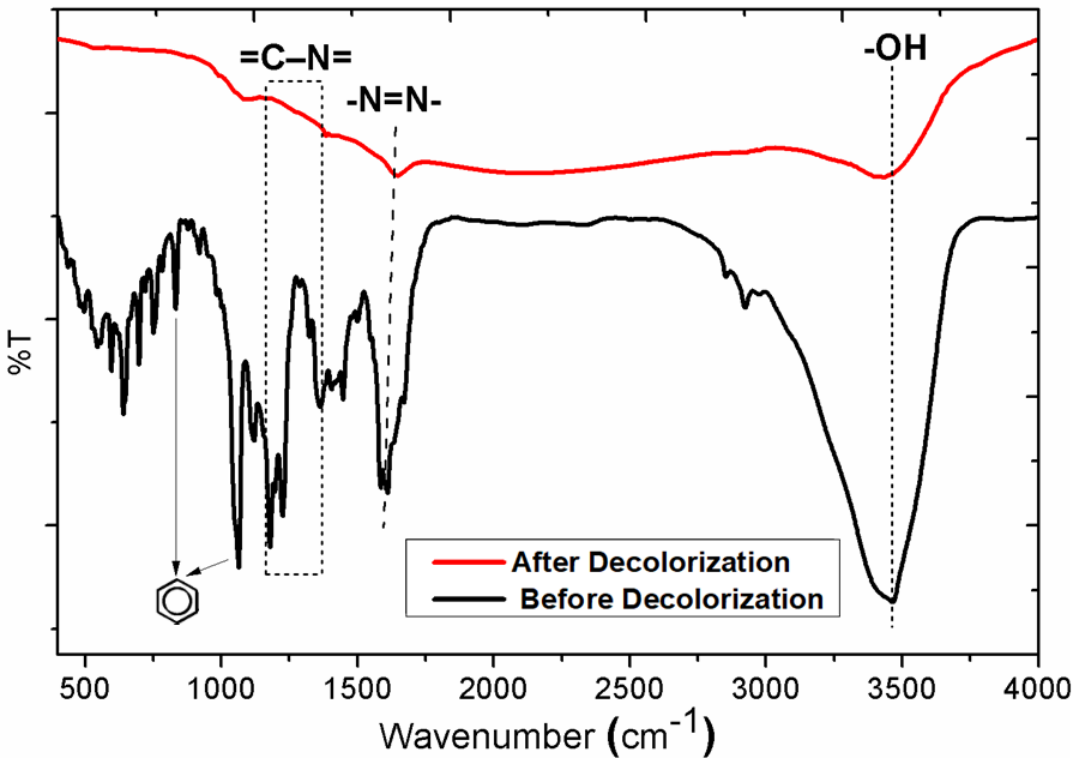


Figure 26: FTIR analysis for CR decolorization at the optimized condition.

4.3 Microbial Fuel Cell assisted Congo Red Dye Decolorization using Biowaste Derived Anode Material and *Bacillus subtilis*

4.3.1 SEM and EDX analysis of FRL carbon

SEM analysis of FRL carbon coated on electrode revealed the distribution on carbon cloth, as shown in Figure 27A. Carbon cloth contains uniformly stacked lamella structures to provide higher surface area. It was observed from Fig 27(A1) that FRL carbon has shown uneven, complex, granular, and porous surface structure. FRL carbon powder was coated on the carbon cloth, wherein the carbon powder got distributed and formed aggregated complex units on carbon cloth (Fig. 27 (A1)). The fibrous structure and carbon powder distribution as an aggregated complex can be seen in Fig 27(A2). The surface area of carbon cloth has got modified using the FRL carbon powder and improved the surface roughness (Fig 27(A3)). The surface roughness aids for higher cell adhesion and colonization, which boosts the electricity generation and dye decolorization (Guo et al. 2015; Sindhuja et al. 2018).The distribution of FRL carbon particles was shown at higher magnification in Figure 27(A3).

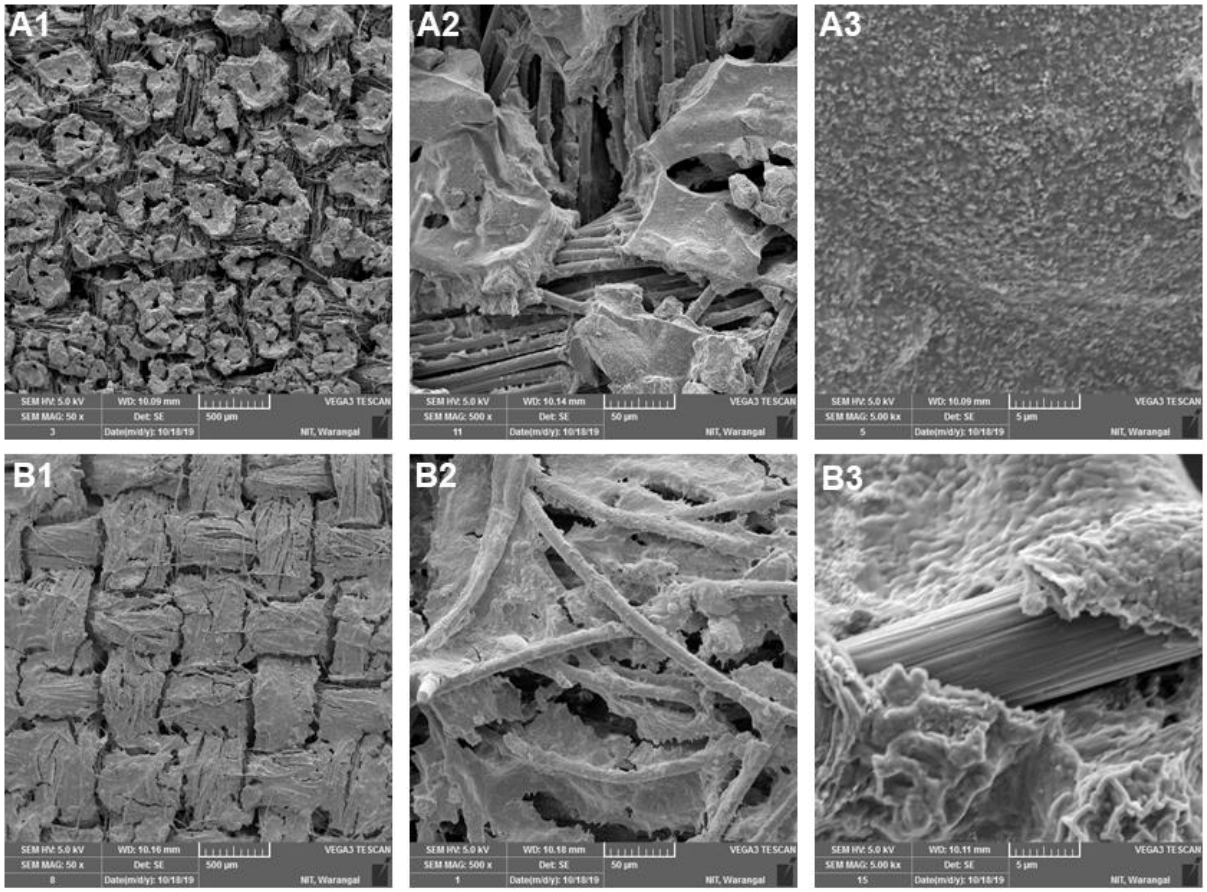


Figure 27: Scanning electron microscopy images at different magnifications. (A) FRL carbon coated electrode (B) Biofilm formed on coated electrode in MFC.

The surface morphology of anode material plays vital role for biofilm formation and current production (Santoro et al. 2017). Biofilm formation on coated carbon cloth is shown in Figure 27(B1). The microbial cells formed as sheets, chain like structures on electrode surface. The fiber of carbon cloth was used effectively by *B. subtilis* to colonize, and evenly formed chain like structures with neighboring fiber (Fig. 27 (B2)). The microbial colonization has occurred throughout the carbon cloth, and thick biofilm was formed on the electrode surface, which resulted in the formation of thick biofilm on the surface of the electrode. The rod-shaped structures, which surrounded the fiber, formed chain like structures, and covered completely the adjacent fiber portion indicating *B. subtilis* colonization (Figure 27 (B3)). Biofilm formation on anode material depends on the porous structure, and surface area, which significantly contribute for bacterial adhesion and colonization (Li et al. 2018). Biowaste based carbon

coating on anode improves hydrophilicity, which subsequently enriches the bacterial attachment and electricity output. In dye decolorization using MFC, the colonized bacteria on anode material supports bacterial interaction with dye and electron transfer, which enhances decolorization and electricity generation (Ilamathi and Jayapriya 2018; Li et al. 2018). Thus, FRL carbon was used for coating the anode material, and the morphology of coated electrode has confirmed the distribution of FRL carbon and microbial colonization on coated electrode (Fig. 27 (A & B)). The elemental analysis of FRL is represented in Table 15 showing the maximum carbon content (67.6 %) compared to other elements.

Table 15: Energy dispersive X-ray (EDX) analysis of FRL porous carbon.

Element analysis of FRL porous carbon							
Elements	C	O	S	Al	Si	Cl	Na
Avg. %	67.6	28.8	1.4	0.63	1.1	0.2	0.43

4.3.2 Characterization of Ficus relogiosa carbon

The chemical properties of carbon powder derived from Ficus relogiosa biowaste was studied using FTIR spectra and XRD analysis. Figure 28 represents the FTIR spectra of carbon powder. The observed peaks in the range of 3000 to 3800 cm^{-1} are the representative peaks of OH bond stretching, and at 2349 and 2890 cm^{-1} are the corresponding peaks of aromatic compound i.e., stretching vibration of C-H bond. The carboxylic and carbonyl groups were noticed at 1504 and 1660 cm^{-1}. The sharp peak at 1103 cm^{-1} was attributed to the C-O bond stretching. XRD analysis of Ficus relogiosa carbon powder was depicted in Figure 29. The broad peak in the range of 15 to 30° represents the disordered aromatic carbon.

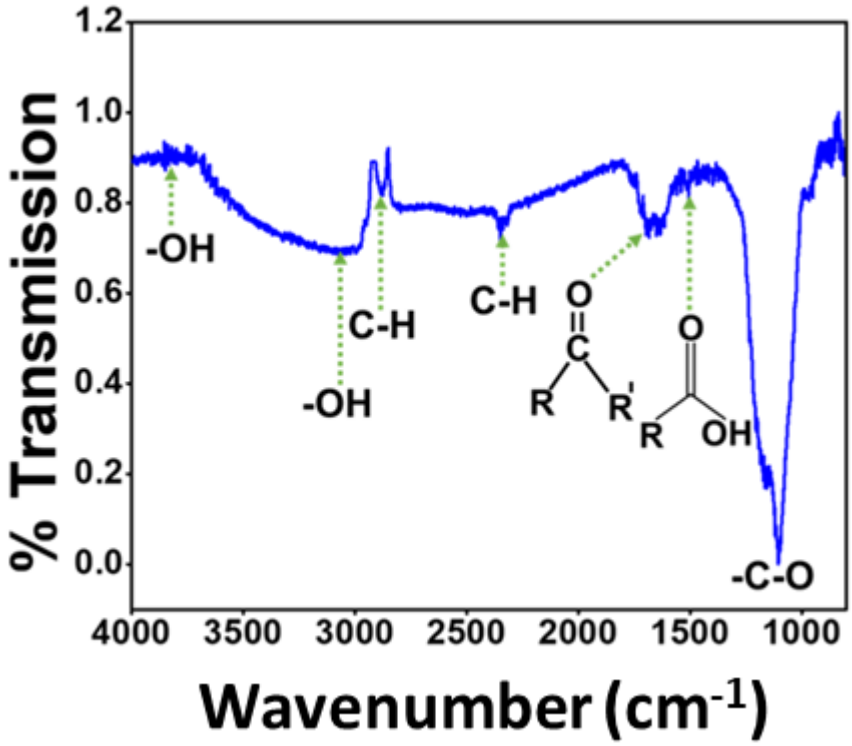


Figure 28: FTIR analysis of carbon powder derived from dead leaves of Ficus relogiosa.

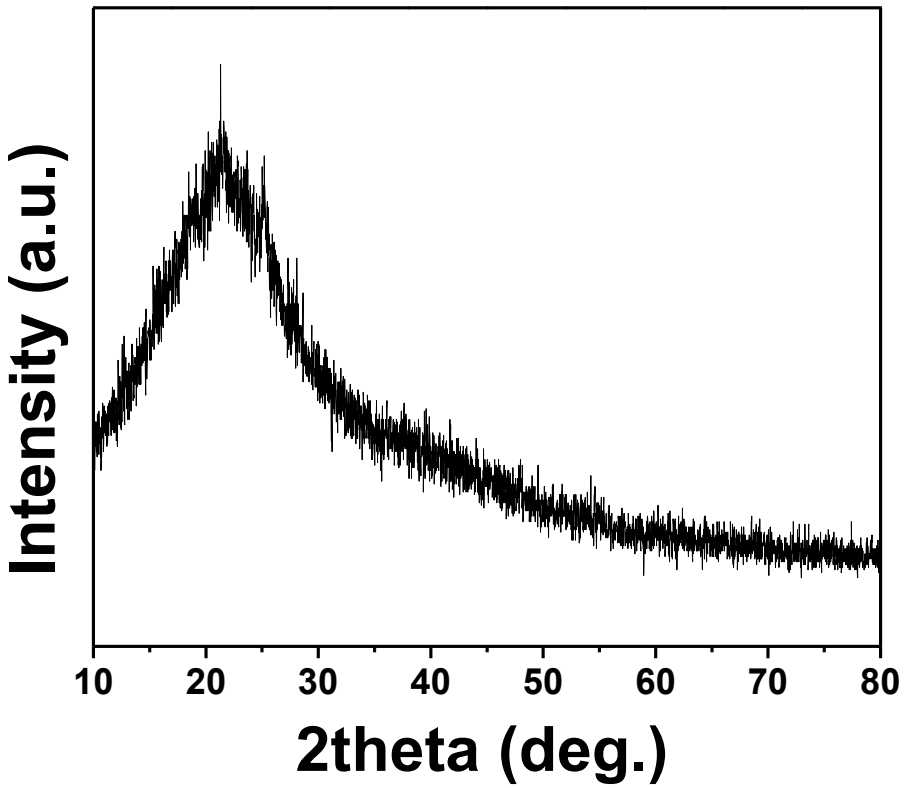


Figure 29: XRD analysis of carbon powder derived from dead leaves of Ficus relogiosa.

4.3.3 Polarization curve

A polarization curve was used to check the MFC performance. The polarization curve gives the outline on change in voltage corresponding to varied current densities, wherein the voltage was recorded at various load conditions. The external resistance was applied when MFC reached the maximum open circuit voltage (OCV) of 0.53 V. In the present study of MFC assisted dye decolorization using *B. subtilis*, the relationship between power density and cell voltage as a function of varied current densities was given in Figure 30. The maximum power density of 70.50 mW/m^2 and current density of 251.79 mA/ m^2 at 2224 Ω were achieved.

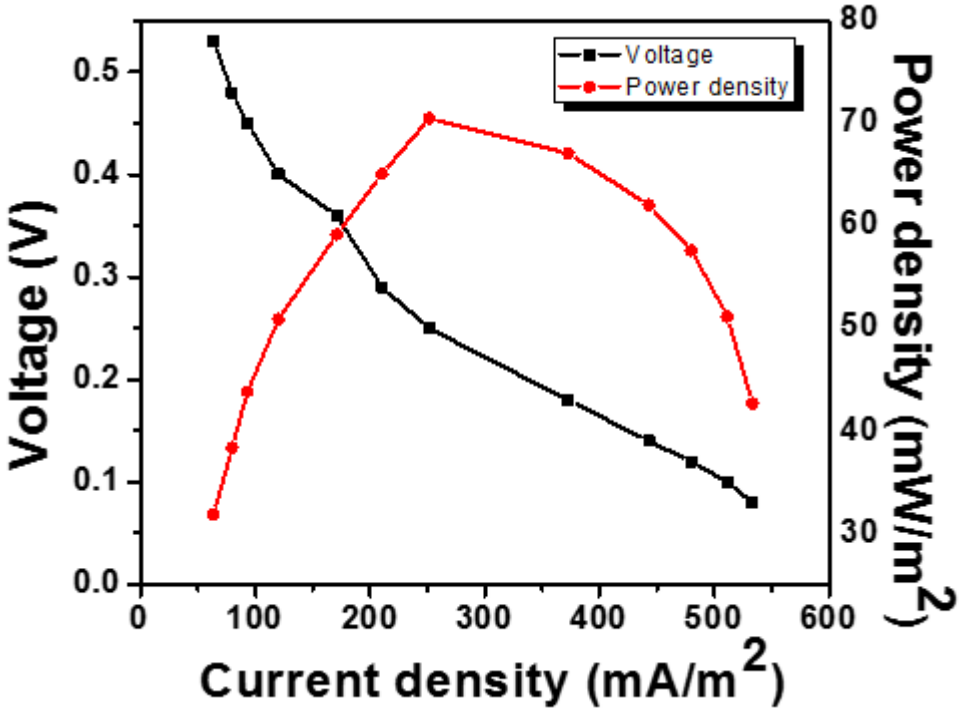


Figure 30: Polarization curve for microbial fuel cell.

4.3.4 Effect of dye concentration, glucose and HRT on dye decolorization

The decolorization due to dye adsorption on FRL electrode and presence and absence of co-substrate (glucose) under biotic condition represented as abiotic control, bioanode with glucose, bioanode without glucose respectively, were shown in Figure 31. The dye concentration of 100 mg/L was used to evaluate the decolorization efficiency. The maximum dye adsorption of 24.38% at 36 h was observed, and there is no significant improvement in the adsorption of dye due to the FRL electrode at 24 h and 32 h indicating that adsorption reached to saturation state. The time required for saturated dye adsorption on electrode has driven us to conduct the

decolorization studies under biotic conditions after saturation time point which gives the accurate decolorization efficiency, wherein the false information of decolorization due to adsorption can be avoided (Kalathil et al. 2011). The effect of co-substrate i.e., glucose on decolorization in MFC under biotic conditions showed that color removal efficiency (95.5%) was higher in the presence of glucose (Fig. 31).

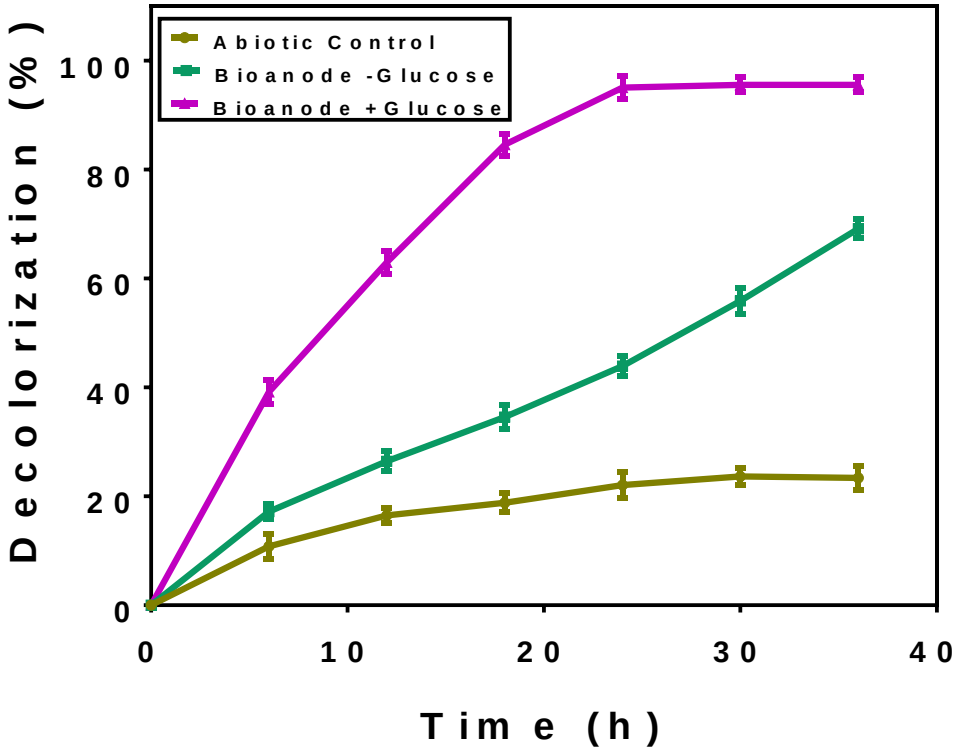


Figure 31: Effect of Congo red decolorization in MFC; abiotic control (100 mg/L), bioanode with and without glucose (2g/L and 0g/L).

It is noticeable that color removal efficiency under biotic condition with glucose is higher at any given time within 36 h. The presence of glucose enhanced decolorization efficiency, which clearly revealed that additional co-substrate contributes for higher electron transfer under biotic conditions. The decolorization rate depends on the added co-substrate, and the mixed or pure culture mediated azo dye treatment requires co-substrate (Hong et al. 2007; Pandey et al. 2007). Several studies have been reported that glucose shows the higher color removal efficiency over other co-substrates (Chung and Rittmann 2007; Cao et al. 2010; Silveira et al. 2011). Generally, the co-substrate addition influences the microbial activity, which proportionally affects the decolorization efficiency. Importantly, the glucose is a potential co-substrate to provide the electrons for the reduction of azo dye (Dos Santos et al. 2005). In the present study, glucose

addition in MFC showed higher color removal efficiency compared over the absence of glucose. This clearly confirmed that glucose co-substrate addition is essential to accelerate the color removal efficiency. Thus, optimization of glucose concentration with respect to dye concentration and hydraulic retention time (HRT) is augmented to improve the decolorization at higher concentration of dye in MFC. Figure 32 shows the effect of dye concentration (300-900 mg/L) on dye decolorization under biotic conditions via MFC treatment. The increase in dye concentration has resulted in lower decolorization rate and increased time for decolorization. The maximum decolorization of 93.7%, 85.8%, 68.3%, and 48.3% was noticed for different dye concentration of 300, 500, 700, and 900 mg/L respectively (Fig. 32). The color removal was almost achieved in 42 h for 300 mg/L dye concentration. At 100 mg/L dye concentration, glucose addition enhanced the decolorization rate. It is essential to optimize the glucose concentration for improved decolorization via MFC treatment to make the setup potential for removal of dye at higher concentration. Thus, 700 mg/L dye concentration has been chosen to study the effect of glucose concentration (0-3 g/L) on rate of decolorization in MFC.

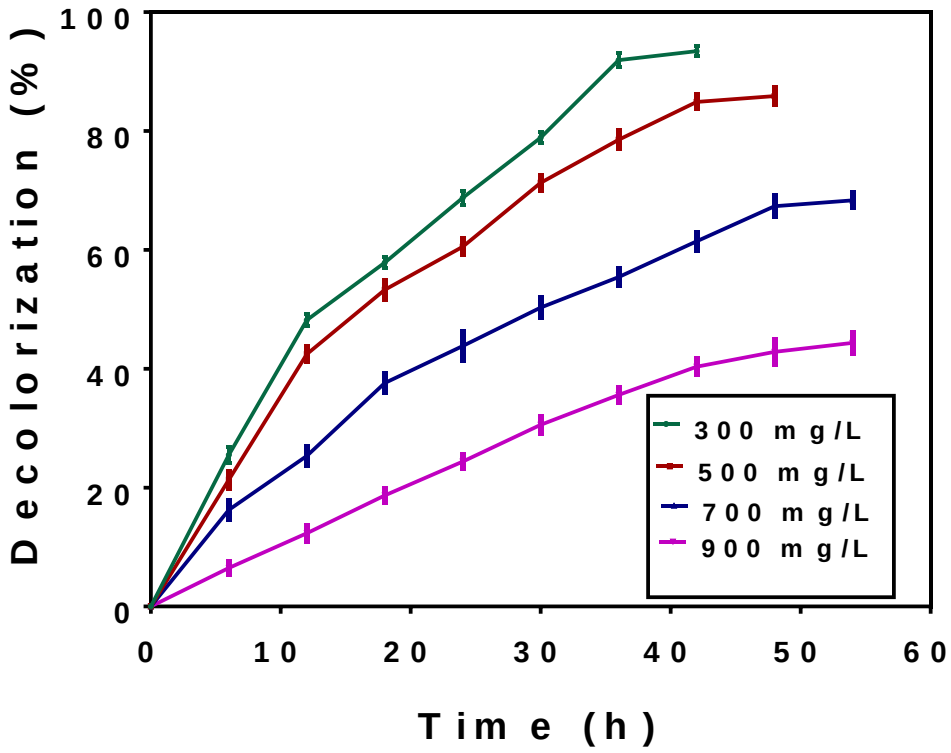


Figure 32: Effect of dye concentration on Congo red decolorization in MFC.

Figure 33 explains the significance of glucose addition for decolorization, the increase of glucose concentration resulted in enhanced decolorization rate. At 2.5 and 3 g/L glucose concentration, color removal percentage of 79.4 % and 84.5% respectively were observed. It was reported that increased glucose concentration improves the decolorization rate and diminish the power output (Li et al. 2010). The recalcitrant CR dye cannot easily be metabolized by microorganism. It is essential that additional carbon source such as glucose is needed to aid microbial growth, which progressively contributes for enhanced dye decolorization (Kapdan et al. 2003; Li et al. 2010).

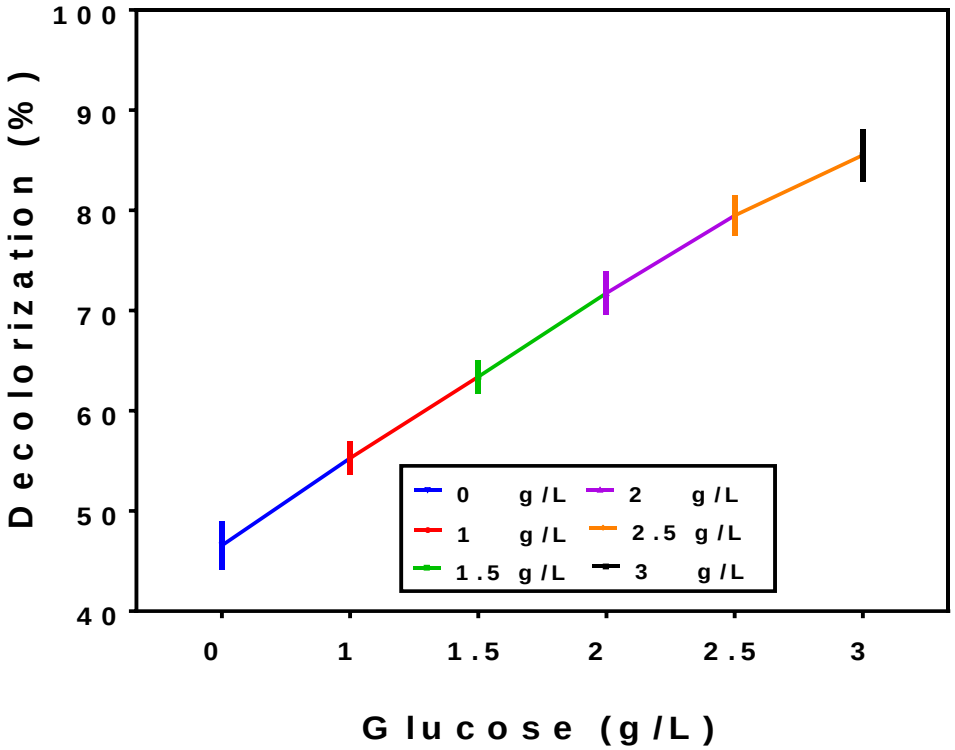


Figure 33: Effect of glucose concentration on Congo red decolorization in MFC.

HRT is a vital factor in wastewater treatment for assessing the effluent dye concentration feed rate, which is feasible to completely remove the dye. MFC was operated for decolorization at different HRT time points 12, 15, 18, 24 and 36 h. The increase in decolorization rate with increasing HRT form 12 h to 36 h confirmed that the effluent dye concentration has diminished (Fig. 34). However, time required to achieve the maximum decolorization got varied with respect to HRT. The higher decolorization of 85.06% and 88.5% were achieved in 5 and 6 days at HRT of 24 h and 36 h respectively. It is noticeable that there is no significant decolorization at 12 h and 15 h, which represents that 49.2%, and 44.3% of effluent dye remained after MFC

treatment. At HRT of 18 h, 79.4% of decolorization was achieved in 2.6 days (54 h). Though there is a slight increase in decolorization rate that was observed at 24 h and 36 h compared over 18 h, it has taken the longer period for achieving maximum decolorization. Thus, the optimal HRT considered for further investigation is 18 h. HRT and glucose concentration have shown significant effect on decolorization and power output in MFC (Li et al. 2010). The results of the present study confirmed that HRT of 18 h and glucose concentration of 2.5 g/L are optimal values for higher decolorization without inhibiting the power output performance of MFC and reiterated the importance of optimal glucose concentration and HRT for decolorization via MFC treatment, which is in line with reports of Li et al.(2010) (Li et al. 2010).

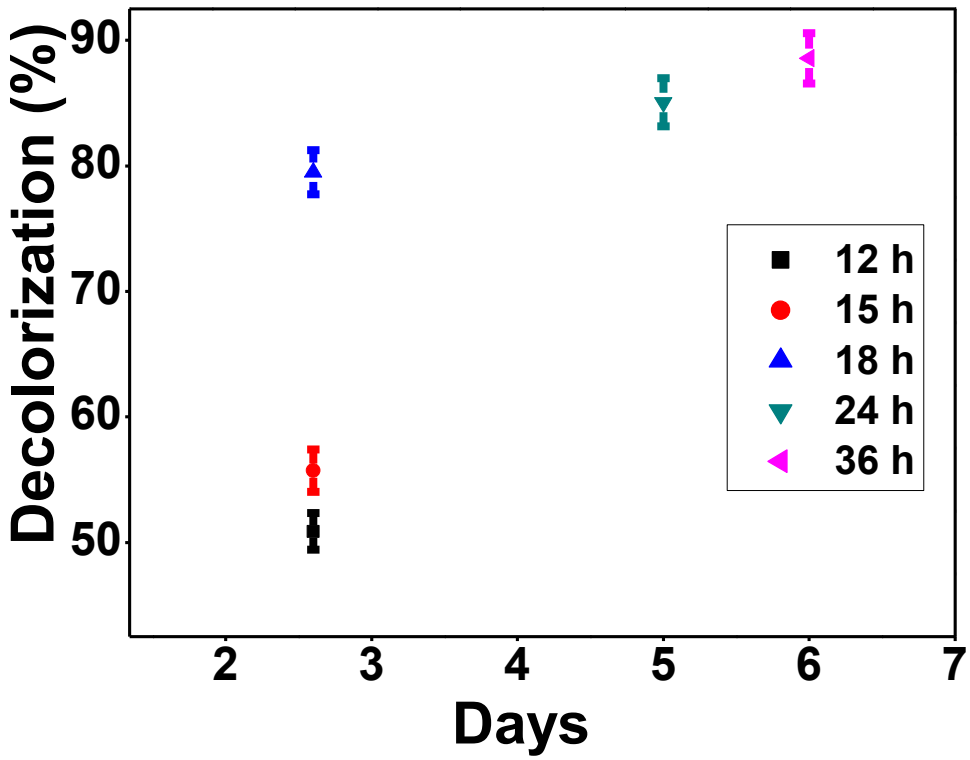


Figure 34: Effect of hydraulic retention time (HRT) on Congo red decolorization in MFC.

4.3.5 Decolorization, chemical oxygen demand (COD) reduction and UV-Visible Spectrophotometry

The dye decolorization and COD reduction in MFC at optimal parameters (dye concentration of 700 mg/L, glucose concentration- 2.5 g/L, and HRT of 18 h) was studied. The maximum decolorization of 80.95±2.08% and COD reduction of 73.96±1.76% were obtained at 54 h (Fig. 35). The UV-visible spectrophotometry analysis was performed at various time intervals and

was depicted in Figure 36. The absorbance of samples at different time points has confirmed the variation in peak intensities. The absorbance peak at 490 nm has shown decreasing trend with increase of time, which clearly confirmed that decolorization is taking place in MFC. It was reported that the absorbance peak at 490 nm represents the azo bond (-N=N-), and the decrease of this peak intensity authenticates the decolorization of CR dye (Pandey et al. 2007). In this study, the peak at 490 nm has declined with increasing time from 0 h to 54 h, which confirmed that the dye has decolorized using MFC.

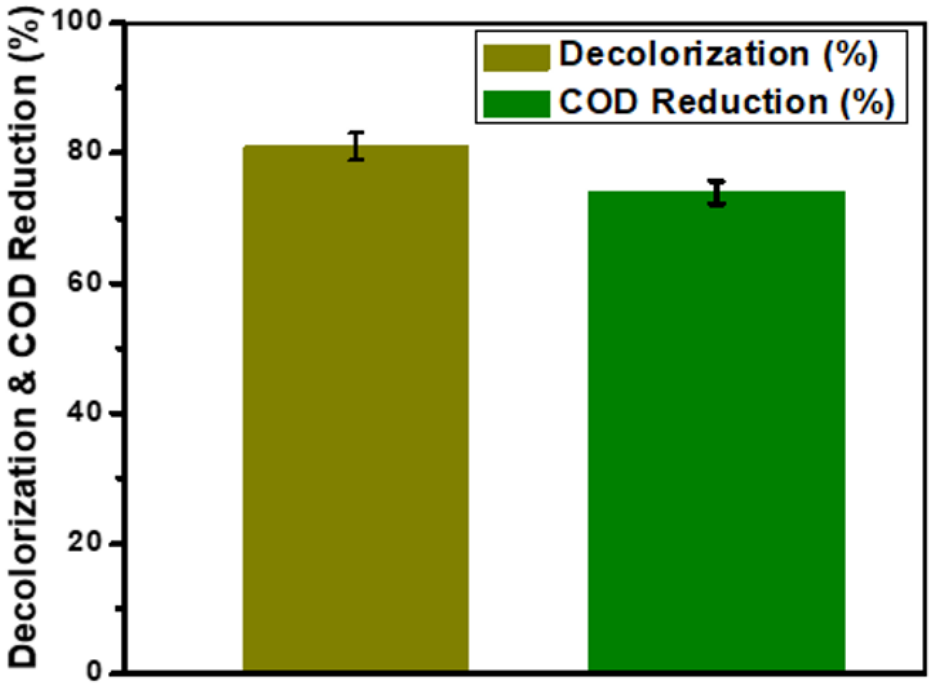


Figure 35: Dye decolorization and COD reduction in MFC after treatment.

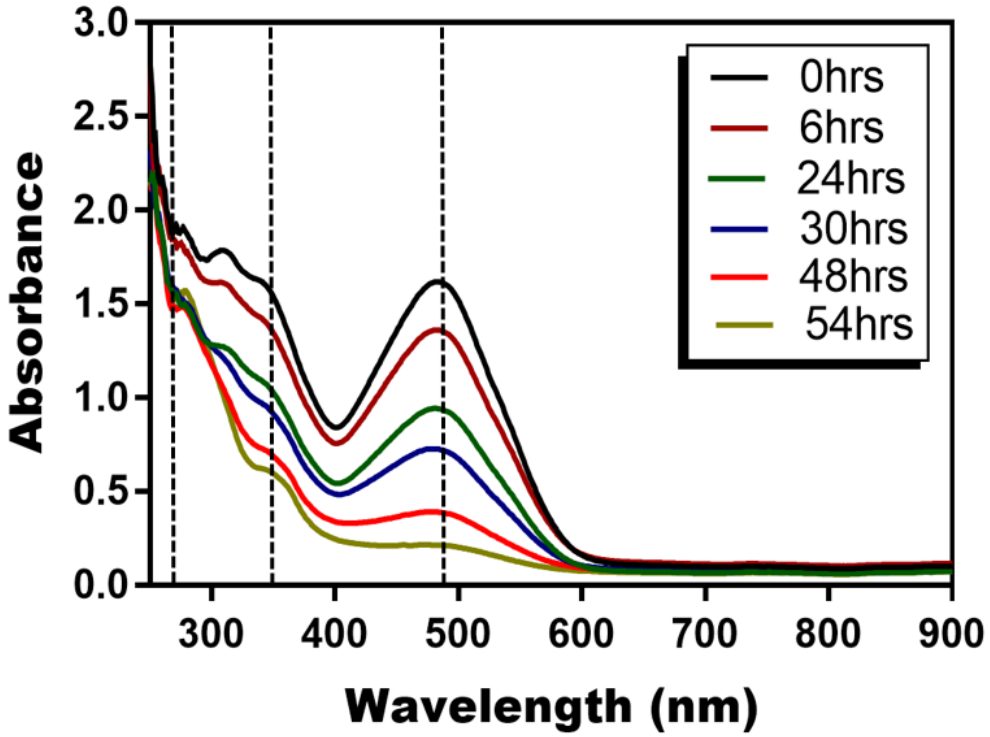


Figure 36: UV-visible spectra of dye contained samples at different time points during MFC treatment.

The absorbance peaks were also observed at 241 nm and 342 nm, which were not found in the spectra at 0 h and 12 h. The anaerobic treatment in MFC resulted in the formation of aromatic amines representing peaks at 241 nm, which formed during the decolorization process after 12 h of treatment. In other study, MFC assisted CR dye decolorization reported the absorbance peak at 241 nm, which represents the aromatic amine formation. The concluding remarks of published work on MFC assisted CR dye decolorization were that the higher dye concentration can be decolorized using MFC system, wherein the faster cleavage of azo bond resulted in aromatic amine formation has taken place due to the anaerobic treatment process (Pandey et al. 2007; Cao et al. 2010; Li et al. 2010). The present study also confirmed that the decolorization was successfully achieved with MFC treatment and aromatic amines were formed. The complete dye degradation is required to provide aerobic treatment to remove the left-over aromatic amines in the MFC treated water.

4.4 Microbial Fuel Cell and Multistage Restricted Fluidized Bed Bioreactor Integrated Approach for Congo Red Dye Degradation using Corncob Agro Residue as co-substrate

4.4.1 Dye decolorization in Microbial Fuel Cell

CR dye decolorization studies were performed in MFC and the experimental plan was to find out the optimal reducing sugars (RS) concentration and feeding strategy in fed-batch mode for the improved decolorization. The effect of concentration 5, 10, 15, and 20 g/L on CR dye decolorization under anaerobic condition was studied in MFC. The varied concentration of RS has shown significant effect on the decolorization of CR dye in the MFC. The maximum decolorization was observed at RS concentration of 10 g/L, whereas higher and lower RS concentrations (5 g/L and 20 g/L) have shown less decolorization (Fig. 37). The results of decolorization with varied RS concentration has confirmed that optimal RS concentration is quite essential to maximize the decolorization in MFC. Generally, glucose as an electron donor is being widely used co-substrate in dye decolorization using MFC. Numerous studies have reiterated that additional co-substrate such as glucose is essential for decolorization of azo dyes by reduction of azo bond in both pure or mixed colony culture mediated dye decolorization (Teli 2008; Cao et al. 2019). Instead of glucose as co-substrate, it would be worthy to replace with any agriculture residue for microbial mediated dye decolorization. The total organic content available in the biomass may contribute for additional treatment for the removal and generates other secondary metabolites during decolorization (Mishra et al. 2019). In this study, RS obtained after Corncob pretreatment was used as co-substrate to enhance CR dye decolorization and the major raised concerns of using organic biomass as co-substrate are addressed at the same time.

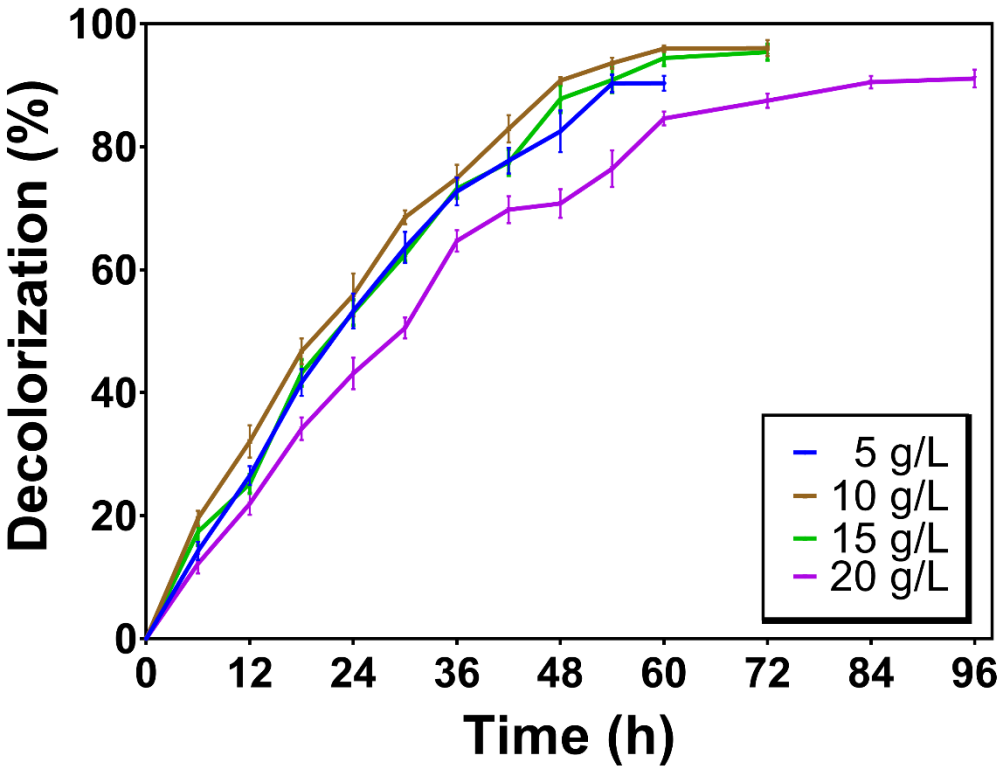


Figure 37: Effect of reducing sugar concentration on decolorization.

Dye concentration of 200 mg/L was fed into the MFC at different feeding intervals, herein 50 mg/L was fed at each time point and divided in four time points of total time. The CR dye 200 mg/L given to the reactor within 18 h has shown higher decolorization rate compared to 12 h, 15 h and 24 h. Though the maximum decolorization was noticed at 3 day of the treatment in MFC for 12 h, 15 h and 18 h, the higher decolorization was achieved only when feed was given into the reactor with18 h of feed time (Fig. 38). The dye concentration and co-substrate concentration might be influencing the microbial metabolism and decolorization mechanism, wherein the CR decolorization was influenced with feeding time and RS concentration. The CR dye concentration and RS concentration play critical role in the decolorization performance by *Bacillus subtilis.* Further, the decolorization experiments were performed at RS concentration of 10 g/L and 18 h of feeding interval. The dye decolorization and COD reduction were recorded as 95.5% and 85.6% respectively (Fig. 39).

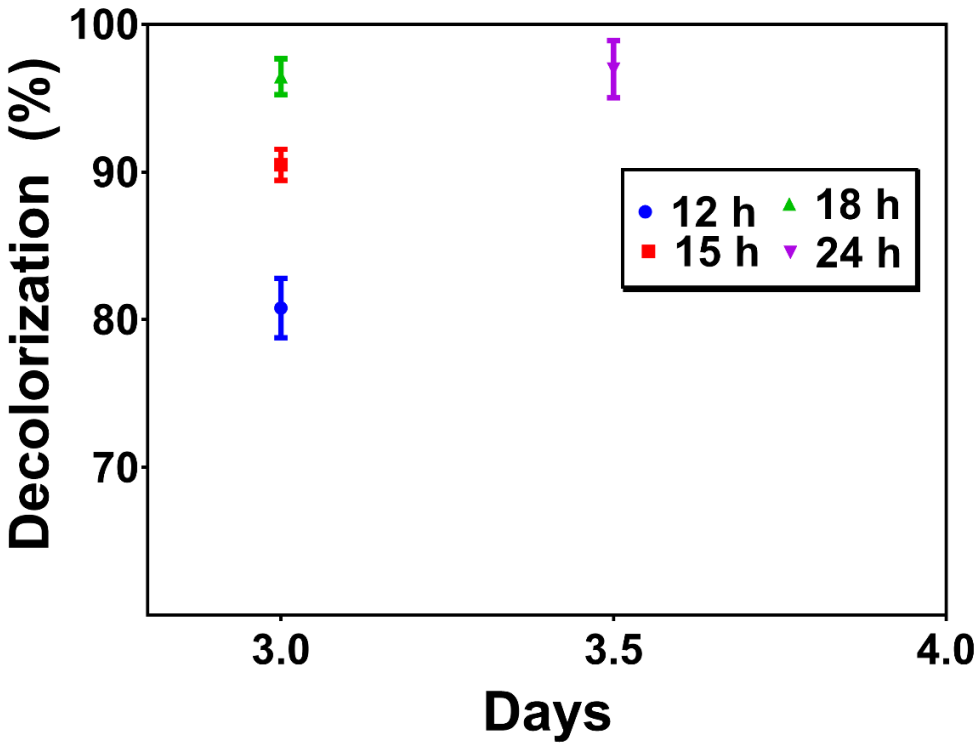


Figure 38: Effect of CR dye feeding in fed-batch mode on CR dye decolorization.

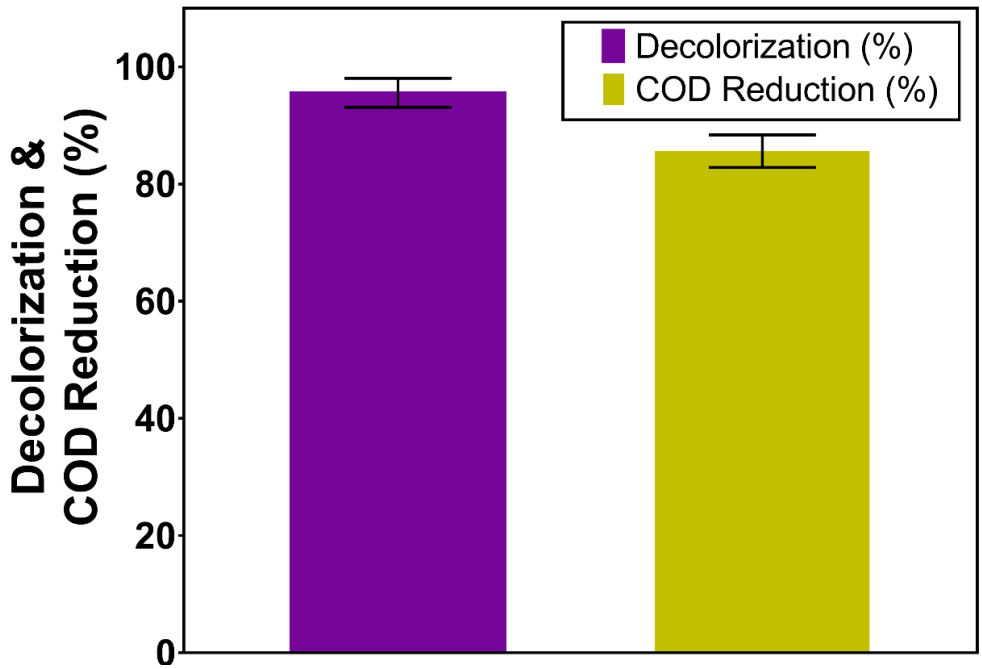


Figure 39: Dye decolorization (%) and COD reduction (%) after MFC treatment at 72 h. The optimal RS concentration of 10 g/L and feeding time of 18 h used for the treatment.

The enhanced biogenic transformation by *Bacillus subtilis* contributed for dye decolorization. Open circuit MFC has shown the maximum open circuit voltage (OCV) of 623±22 mV. The maximum power density of 29.16 mW/m^2 was obtained (Fig. 40).

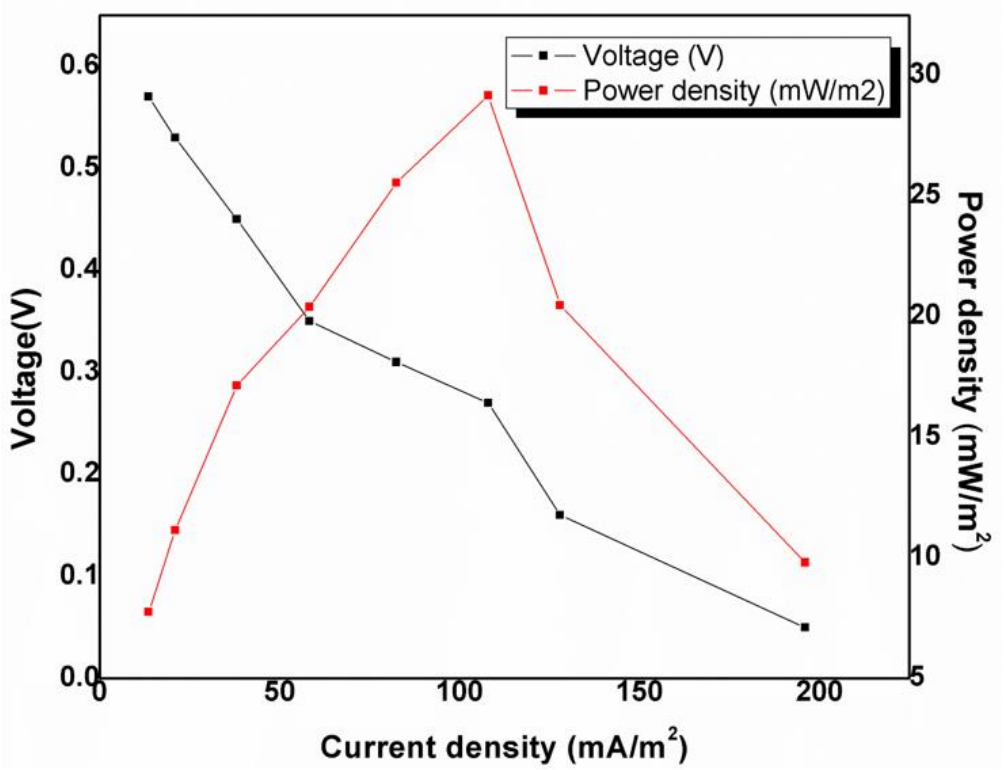


Figure 40: Polarization curve for MFC at optimized condition.

4.4.2 GC-MS analysis

The metabolites obtained after the anaerobic treatment in MFC and subsequent treatment in MRFBBR were analyzed using GC-MS analysis. To give the outline on the metabolites and degradation mechanism involved in the sequential treatment, the glimpse of metabolites and degradation phenomenon at molecular level is represented in Figure 41. The mass spectrum analysis using NIST library proposed, the metabolites formed after MFC treatment are amines, sulfides and carboxylic acid. The details of the metabolites including their chemical structure are presented in Figure 41. The various amine isomers were obtained after treatment, in which all these amines consist of benzene ring, which suggested that the azo bond was broken into simpler metabolites. 2,5-dimethoxy-4-9 methyl sulfone amphetamine appeared at the retention time (R_t) of 34.185 min, 1,2,4-Benzenetricarboxylic acid at R_t of 30.655 min, and 2-buten-1-one 1-(2-hydroxy-4,5-dimethoxyphenyl) at R_t of 27.781 min. Benzoic acid formed after treatment in MFC comes under the category of unsaturated carboxylic acid and it appeared at

R_t of 15.155 min. Other, two amine compounds 2-methylamino-1-phenylethanol at R_t of 9.945 min, 2-Butanamine N-(1-methylpeopyl) at R_t of 12.154 min also appeared. The decolorization of CR dye was observed after MFC treatment and the metabolites presented in the treated water should be removed to avoid the toxic effects on human and environment.

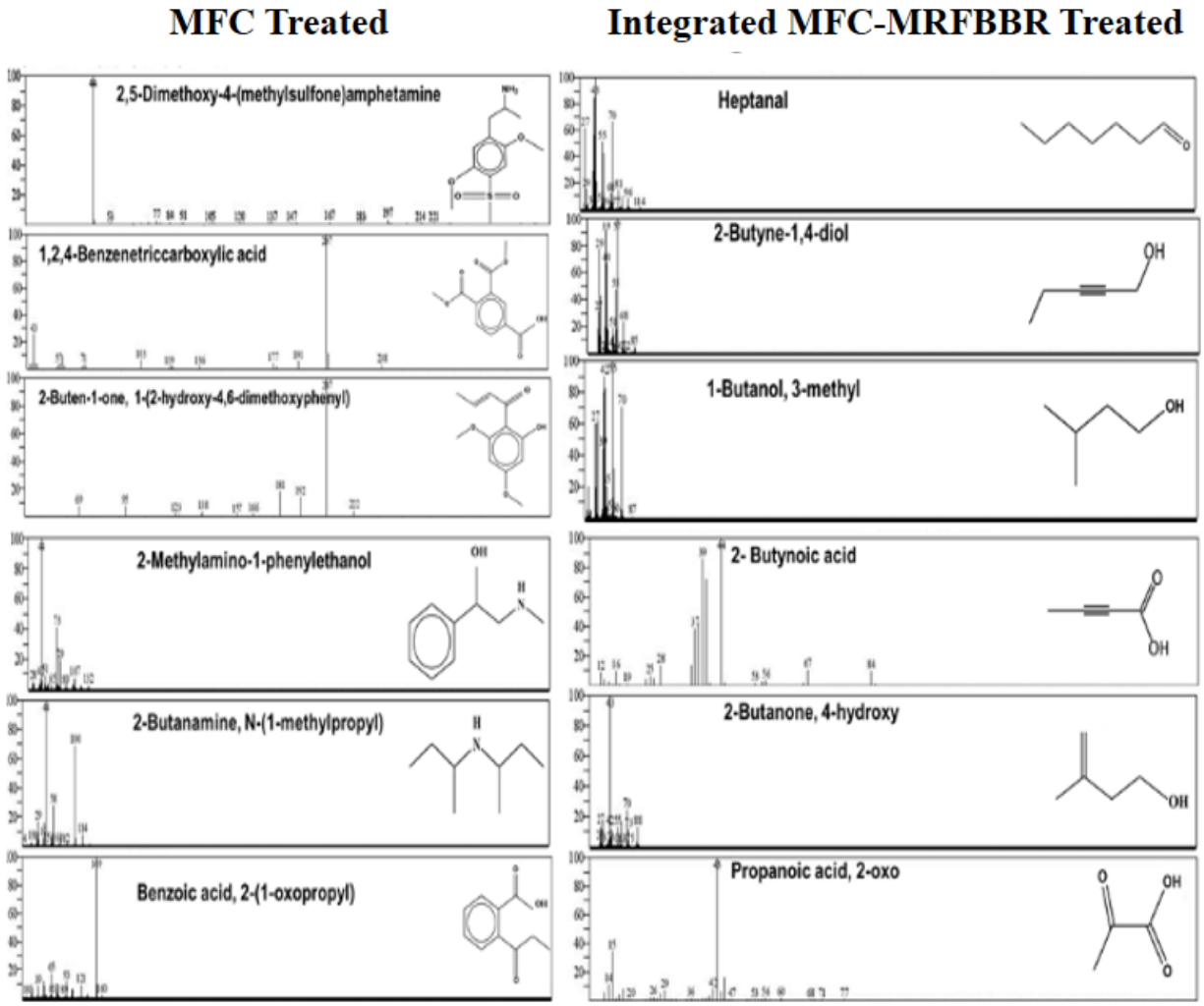


Figure 41: GC-MS peaks of metabolites obtained after treatment in MFC and integrated MFC- MRFBBR.

The anaerobic treatment for azo dye degradation was reported with similar findings, wherein the carboxylic acids, amines and sulfides were found after MFC treatment. The compounds obtained in this study might not be similar with the reported study but the similar isomeric compounds were recorded using GC-MS analysis, and further stated that these compounds are hazardous to human and environment (Das and Mishra 2019). Thus, the current study aimed to achieve the complete degradation of CR dye by treating MFC treated water further using

aerobic treatment in MRFBBR in the integrated system. The simpler compounds and breaking of these non-degradable aromatic amines for ease in biodegradation would be the potential approach to be considered. The metabolites obtained after treatment in the integrated MFC-MRFBBR were analyzed using GC-MS analysis. Interestingly, all the bio-transformed metabolites obtained after treatment in the integrated MFC-MRFBBR are simpler compounds and showed very simple chemical structures (Fig. 41). The different simpler chain compounds such as heptanal at R_t of 10.612 min, 2-butyne-1,4-diol at R_t of 6.625 min, 1-butanol 3-methyl at R_t of 24.610 min, 2-butynoic acid at R_t of 0.590 min, 2-butanone, 4-hydroxy at R_t of 1.255 min, and propanoic acid, 2-oxo at R_t of 21.805 min were found in the samples collected after treatment in the integrated MFC-MRFBBR. The simpler chain compounds confirmed using GC-MS analysis had given clarity on the molecular level breakdown during the sequential treatment in anaerobic-MFC and aerobic-MRFBBR in the integrated system and showed that metabolites formed after second stage treatment might be very less toxic compared to the dye contained water.

4.4.3 FTIR analysis after treatment in MFC and MRFBBR

FTIR analysis was performed to emphasize the metabolites formed during decolorization and degradation in the integrated MFC-MRFBBR system. CR dye was bio-transformed into different metabolites due to the sequential treatment in MFC and MRFBBR system. There is a huge variation in the spectra pattern before and after treatment, which can be seen clearly as depicted in Figure 42. CR dye FTIR spectra before treatment was considered as a control and the obtained spectra is shown in Fig 42(A). The specific peak at 3408.94 cm^{-1} for -C-H stretching of phenolic or alcoholic compound, 2917.64 and 2829.17cm^{-1} for alkanes. The azo bond -N=N- showed at the stretching peak of 1632.94 cm^{-1}. The decolorization of dye in MFC under anaerobic conditions resulted in the formation of amines, which was confirmed with major peak appearance in the spectra of MFC treated sample at 3422. 11 cm^{-1} (Fig. 42 (B)). The peak intensity got decreased after aerobic treatment which attributes for the simpler metabolites formed and the change in the peak at 1645.17 and 1619.76 cm^{-1} for carbonyl group of aldehyde and carboxylic acid (Fig. 42(B & C)). The azo bond peak has not appeared after treatment in MFC-MRFBBR integrated system and certainly indicating that the breaking of this bond leads to the formation of aromatic amines after MFC treatment. Further, these aromatic amines bio-transformed into simpler metabolites, which was confirmed with the appearance of shift in the peak to 1645.17 cm^{-1}. The aromatic amines formed after anaerobic treatment in MFC were

successfully eliminated with the aerobic treatment in MRFBBR using *Bacillus subtilis* strain and it was confirmed with the FTIR spectra.

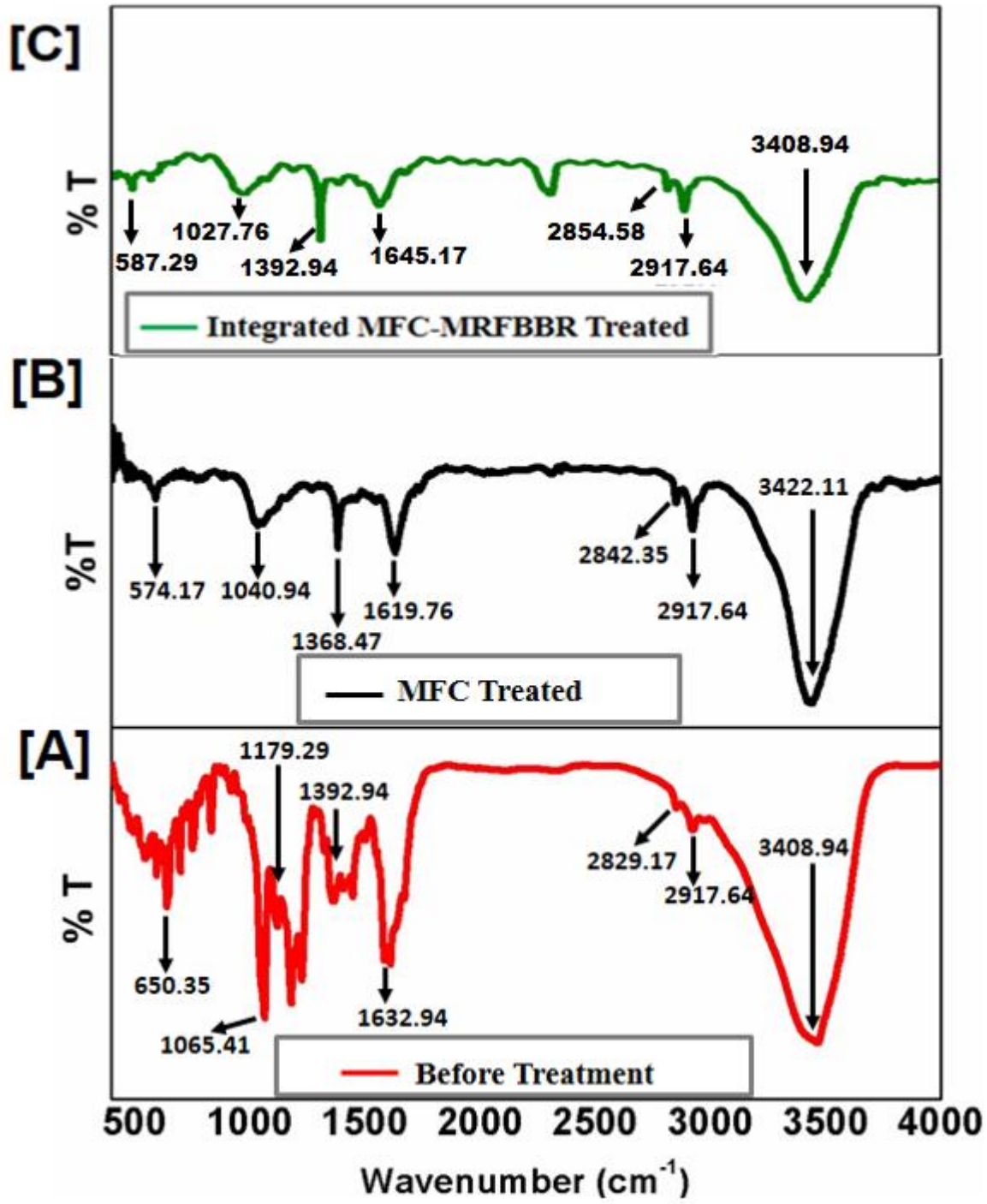


Figure 42: FTIR analysis A) Before treatment B) after anaerobic treatment in MFC C) after aerobic treatment in MFC-MRFBBR integrated system.

4.4.4 UV-Visible Spectra Analysis

UV- Visible spectra analysis was carried out for the samples obtained after treatment in MFC and integrated MFC-MRFBBR and synthetic wastewater was considered as control. The azo bond peak showed at 490 nm in the control sample i.e., before being treated, the peak has decreased rapidly after treatment in MFC and integrated MFC-MRFBBR (Fig. 43). The peak at 490 nm after completion of treatment in the integrated system is almost showing the straight-line trend, which is confirmed that degradation was achieved using the sequential treatment. It has already been proven that the azo bond -N=N- cleaved after anaerobic treatment and produces aromatic amines (Pandey et al. 2007; Li et al. 2010).

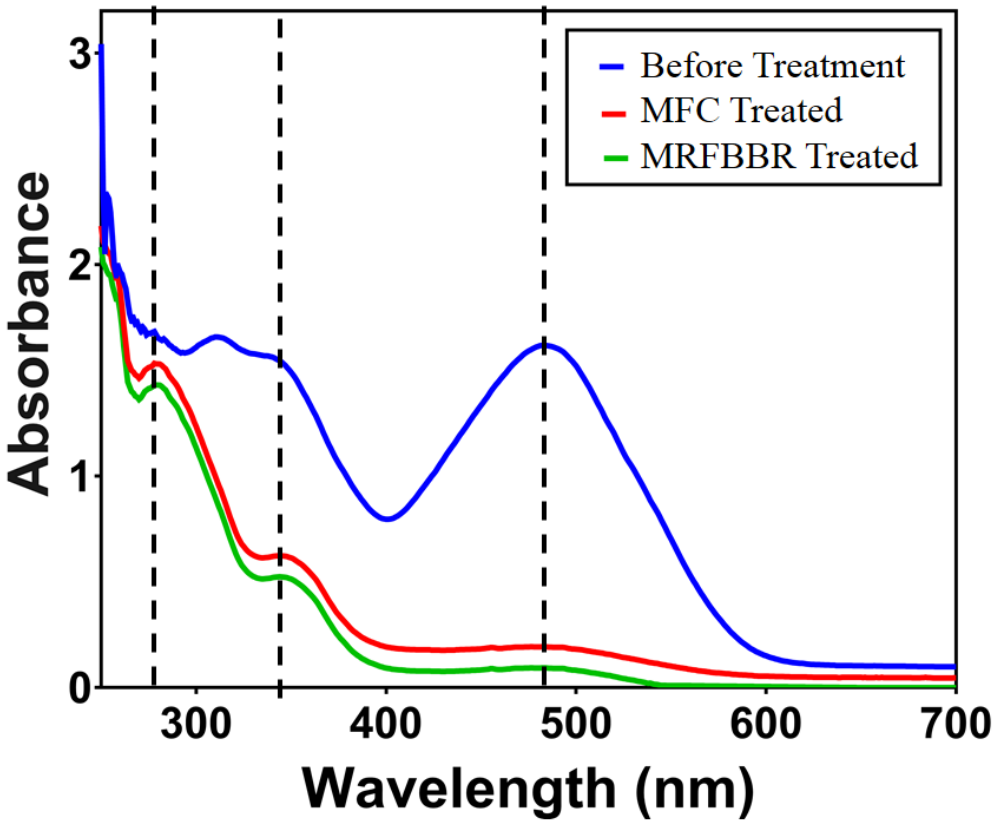


Figure 43: UV-Visible spectrophotometric analysis of untreated, MFC-treated, MRFBBR treated samples.

The destruction of aromatic amines in the MFC-MRFBBR treated samples was confirmed with the reduction of peak at 340 nm. Li et al. (2010) has reported that the peak declined after treatment in aerobic and anaerobic conditions and concluded as the degradation of aromatic amines through the cleavage of chromogenic groups (-NH_2) (Li et al. 2010). The peak at 250

nm has decreased in the MFC-MRFBBR treated sample indicating that aromatic amines are degraded. In other study, Remazol navy blue azo dye degradation using the anaerobic and aerobic treatment has showed that the peak at 340 nm has decreased after the aerobic treatment (Das and Mishra 2019), which is reiterated again in the current investigation that the decrease in peak at 340 nm of UV visible spectra after treatment in the integrated MFC-MRFBBR. This decrease in peak at 340 nm attributes for the degradation of aromatic rings. UV-Visible spectra analysis has clearly indicated that successful degradation of CR dye was achieved after aerobic treatment. The dye decolorization under anaerobic conditions in MFC has improved the decolorization efficiency and cleaved the azo bond to produce the easy degradable metabolites in further aerobic treatment. The accurate information on the metabolites formed after the treatments under anaerobic and aerobic conditions was traced out using GC-MS analysis and subsequently with FTIR and UV-Vis spectra analysis has authenticated the same. The decolorization and degradation in the sequential treatment, and subsequent analysis for understanding the metabolites obtained after treatment have confirmed a transparent solution without color having less toxicity compounds. This treatment strategy is highly effective for CR dye degradation and not hazardous to environment and humans. However, this needs to be proven using any biological system as a model for asserting that this treated water is not toxic. Instead of presenting COD reduction, it would be more validated if the toxicity effect were shown using biological system models. Thus, it was further investigated on the phytotoxic studies to demonstrate the effect of this treated water on seed germination over dye contained water.

4.4.5 Phytotoxicity study

The toxicity effect of CR dye and metabolites obtained after partial treatment is the major concern as it is a dangerous threat to plants and animals. The anaerobic treatment has been studied for azo dye degradation postulated the formation of aromatic amines and insisted the importance of further aerobic treatment owing to their toxic effect on environment (Li et al. 2010; Cao et al. 2019). The seed germination (%), shoot and root lengths (mm) were tabulated and the table clearly showed huge variation with and without treatment (Table 16). Seed germination of 40% only noticed in case of 200 mg/L concentration of dye contained water before treatment. The metabolites obtained after treatment in the integrated MFC-MRFBBR had shown significant seed germination compared over untreated and MFC treated. The seed germination percentage, root length and shoot length significantly are lower in the metabolites

obtained after MFC treatment than integrated approach. Interestingly, germination percentage, shoot length and root length recorded for the metabolites obtained after MFC-MRFBBR treatment showed less difference and appeared to be approximately all the values are close to the distilled water group. This may attribute that integrated aerobic and anaerobic treatment using MFC-MRFBBR was able to degrade the dye and produced eco-friendly metabolites, which are not toxic. The results of this phytotoxic studies confirmed that the intermediate metabolites obtained after treatment in the integrated system promoting the seed germination and growth of plant, which assured that this treatment strategy has produced non-toxic intermediates and confirmed that it is non-toxic to the animals and environment.

Table 16: Phytotoxic evaluation of CR dye and the metabolites obtained after each stage of treatment on *Vigna radiate*.

Parameters	**Distilled Water**	**CR dye contained water**	**Metabolites obtained after MFC treatment**	**Metabolites obtained after final MRFBBR treatment**
Seed Germination (%)	100	42	85	100
Shoot length (cm)	7.32±0.74	2.67±0.28*	4.74±0.73*	6.26±0.51*
Root length (cm)	2.9±0.56	0.56±0.06*	1.3±0.24*	2.62±0.41*

*One-way ANOVA was employed to analyze the data presented in the table (n=3). Significant difference in seed germination, shoot length and root lengths were observed in dye contained water, metabolites obtained after MFC and MRFBBR treatment compared over distilled water ($p^{*}<0.001$)

5. Conclusions and Scope of future work

5.1 Conclusion

In this section, the concluding remarks of thesis work have been summarized giving significant contribution in the context of the research field with emphasizing the further research scope. This thesis was aimed to provide sustainable, ecofriendly biological degradation approach for Congo red dye degradation since the available literature is warranting innovative strategies, which are cost-effective, ecofriendly treatment for dye degradation. Several studies have tried to establish efficient treatment strategies using microbial agents in bioreactors, MFCs etc. However, the lacunae are being not addressed completely so far using either bioreactor or MFC based systems for azo dye degradation. The research questionnaire using available literature and remarkable findings helped us to formulate constructively objectives of the thesis work. Overall, the main goal of thesis work is to develop MFC and bioreactor coupled system for Congo red dye degradation. The conclusions of each objective and the rationale, fundamental hypothesis is briefly discussed in this section.

The advantages of immobilization culture in terms of easy recovery of immobilized matrix along with the cultures for reuse further, effective colonization of microbes, and improved biological interaction with dye have encouraged us towards the immobilization of bacterial mediated treatment approach for Congo red removal. Despite having several concluded results to take up the immobilization approach for textile dye wastewater treatment, the importance of culture choice, advantage of pure culture including *B. subtilis*, abiotic factors influence on microbial growth and dye removal efficiency pave the way to design experiments for checking the treatment efficiency of immobilized *B. subtilis* compared to free cell assisted decolorization. The effect of process parameters like dye concentration, temperature, pH on decolorization was studied. The results of the study found that optimal conditions are favorable for enhanced decolorization and microbial growth. The optimal values of process variables like dye concentration, temperature, and pH for higher decolorization are 50 mg/L, 37 °C and 9.0, respectively. *B. subtilis* immobilization on PUF has shown significant improvement in the decolorization compared to free cell. The microbial colonization on PUF was observed using SEM studies, morphological evidences clearly convinced the profound necessity of matrix support for microbial growth, colonization. The higher cell mass which acts proportionately for

occurrence of improved biological interaction with dye molecules has favored for enhanced decolorization compared to free cell.

The preliminary results using immobilized bacterial approach for dye decolorization insisted upon to upgrade the process using bioreactors especially aerobic mode of operation was the prime concern of the work so as to use further in upcoming objective to setup the integrated anaerobic- aerobic system for Congo red degradation. Although PUF immobilization method is highly effective for decolorization, the demerits of the fluidized bed bioreactor (FBBR) for using PUF immobilized approach for decolorization conceived the idea of modulation of existing FBBR into MRFBBR. The active surface area role on decolorization performance was elucidated by changing the PUF weight. The decolorization performance of FBBR is significantly lower compared to MRFBBR approach, weight of foam and packing demonstrated the importance of optimal packing of PUF in the reactor to enrich the availability of active surface area for bacterial colonization and biological interaction with Congo red dye. It was observed that 5 g of PUF was optimal packing rate and PSSB has ensured the availability of immobilized foam throughout the reaction volume, which resulted in enhanced decolorization. The maximum dye decolorization of 92% was obtained at optimal conditions. The results of this study have affirmed the significance of MRFBBR to increase dye removal efficiency and also laid the foundation for considering the concept of localization of immobilized bio-carrier of any type if the carrier material is of low density and floats in the reactor.

As previously discussed in this section, the aim is to develop MFC based approach for anaerobic treatment. In particular, the biowaste derived electrodes without compromising the performance of MFC compared to synthetic electrodes is the emerging trend to provide cost effective, sustainable, ecofriendly systems for various applications. FRL biowaste was used to develop electrode, which was used to improve surface area, and roughness. Enhanced surface area, roughness and good distribution of FRL carbon, resulted in higher microbial colonization and thick layer of biofilm formation. Dye concentration, glucose concentration and HRT on MFC performance in terms of decolorization were optimized. The maximum decolorization, COD reduction of 80.95±2.08%, 73.96±1.76% were obtained respectively after complete treatment of MFC for 54 h at optimal dye concentration of 700 mg/L, glucose concentration of 2.5 g/L and HRT of 18 h. The decolorization was confirmed using declined intensity of peak corresponding to azo bond in UV-visible spectrometry. The results of this study confirmed the potential of FRL biowaste electrode based MFC for dye decolorization. The decolorization

using MFC has proved to be a potential application for treating the dye at higher concentration compared to the bioreactor-based systems.

Integrated MFC-MRFBBR system coupled with anaerobic and aerobic treatment for the complete degradation of CR dye using *Bacillus subtilis* was developed. Biogenic electricity generation and operating under anaerobic condition for dye decolorization are the goals of MFC. The primitive goal of the integrated system is to provide cost effective, and operation friendly setup for CR dye degradation. Given this, RS obtained after pretreatment of corncob was used as co-substrate in the integrated system, which had efficiently improved decolorization and degradation. The optimal RS concentration of 10 g/L and 18 h of feeding time resulted in higher decolorization in MFC. The aromatic amines formed after MFC treatment, which was confirmed with the FTIR, UV-Vis and GC-MS analyses. GC-MS, FTIR and UV-Vis spectra analyses have clearly confirmed that the degradation was successfully achieved in the integrated MFC-MRFBBR system. It can be concluded that this integrated system was potential enough for complete dye degradation and detoxification of dye contained wastewater.

5.1.1 Significant findings

1. PUF immobilized *Bacillus subtillis* is favorable for higher decolorzation.
2. The improved decolorization is possible only when the floating of immobilized matrix minimized and enhanced the availability of immobilized microbial bed throughout the reaction volume using the concept of restricted immobilized microbial bed.
3. Biowaste derived electrode can be used in MFC for better performance, improved degradation of dye along with minimization of the cost.
4. The Congo red dye degradation was achieved using integrated MFC-MRFBBR system, which has scientifically demonstrated the cleavage of complex dye molecule into simpler compounds due to the treatment in the integrated system.

5.2 Scope of future work

The outcomes of present study suggesting concisely, summarizes what can be done further using the established approaches. The scope of future work is to give a consolidated view how the established technologies can be upgraded for real time applications. The following bulletins are the short glimpse of future work warranted to provide treatment plants using the established MFC-MRFBBR for textile wastewater treatment application.

- ✓ PUF immobilized *Bacillus subtilis* must be studied for various other dyes for decolorization.
- ✓ Michaelis- Menton kinetics can be studied for free cell and immobilized pye culture based system to understand the dye degradation reaction.
- ✓ Optimal doping for improved conductivity of biowaste derived electrode for improved electricity generation and dye decolorization can be studied.
- ✓ MFC-MRFBBR system must be studied for various dye treatments, especially feasibility of using this approach for various textile industry wastewater treatments.
- ✓ Dye degradation pathway must be developed to understand the CR molecular breakdown.
- ✓ Pilot scale studies for real time textile wastewater treatment can be conducted.

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Nomenclature

ABRX3	Active brilliant red X3
AO7	Acid orange 7
AS-biochar	Terminalia arjuna seed-biochar
BES	Bio-electrochemical system
BOD	Biological oxygen demand
CCD	Central composite design
COD	Chemical oxygen demand
COR	Catalytic oxidation reaction
CR	Congo red
CV	Cyclic voltammetry
CW	Confectionary wastewater
CSTR	Completely stirred tank reactor
cGLP	Current good laboratory practice
cGMP	Current good manufacturing practice
DC-MFC	Dual chamber microbial fuel cell
DO	Dissolve oxygen
EDX	Energy dispersive X-ray
EP	Microbial enrichment procedure
FBBR	Fluidized bed bioreactor
FePC	Iron phthalocyanine
FRL	Ficus religiosa leaf
FTIR	Fourier transform infrared spectrometry
GC-MS	Gas chromatography-Mass spectrometry
HRT	Hydraulic retention time
ID	Internal diameter
LC-MS	Liquid chromatography-Mass spectrometry
MB	Methylene blue
MBBR	Moving bed biofilm reactor
MFC	Microbial fuel cell
MO	Methyl orange

MRFBBR	Multistage restricted fluidized bed bioreactor
MSM	Minimal salt media
NB	Nutrient broth
OCV	Open circuit voltage
OD_{600}	Optical density at 600nm
OD	Outer diameter
PBR	Packed bed reactor
PBBR	Packed bed bioreactor
PEM	Proton exchange membrane
PSSB	Porous stainless steel box
PUF	Polyurethane foam
PUF-PP	Polyurethane foam-polypropylene
RS	Reducing sugar
RNB	Remazol navy blue
RSM	Response surface methology
RTW	Real textile wastewater
SEM	Scanning electron microscopy
SS316	Stainless steel 316
TDS	Total dissolve oxygen
TOC	Total organic carbon
TSS	Total suspended solid
USAB	Upflow anaerobic sludge blanket bioraector
UV-VIS	Ultra violet-Visible spectrometry
WB	Wheat bran

Publications and conferences

Publications

1) **Shalini**, Y Pydi Setty, (2019) "Multistage fluidized bed bioreactor for dye degradation using immobilized polyurethane foam: A Novel Approach" *Biochemical Engineering Journal*: 152:107368.
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2) **Shalini**, Y Pydi Setty, (2020) "Microbial Fuel Cell assisted Congo Red Dye Decolorization using Biowaste Derived Anode Material" *Asia pacific journal of chemical engineering*: e2558.
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1) **Shalini**, Y Pydi Setty (2019) "Screening of potential bacteria from soil sample for methylene blue dye degradation using 16S rRNA sequencing" INCEEE-2019, National Institute of Technology, Warangal, India.
2) **Shalini**, Y Pydi Setty (2019) "Ficus religiosa as a novel anode material for Congo Red dye decolorization using *Bacillus subtilis*" ICMHCEE-2019, National Institute of Technology, Trichy, India.