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Characterization of Reaction Parameters in the Bioremediation of Congo red and Crystal Violet Dyes Using Mixed Cultures of E.Coli and Bacillus Subtilis.

Sony George[1, A)], Jyothi K Nair[2b)], Dr.Anju K A,[3c)]Abhishek S[4,D)]
[1, 2,3] *Department of Food Technology, Saintgits College of Engineering. Kerala, 686532, India*
[3] *Department of Food Technology, TKM Institute of Technology, Kerala, 691505, India*

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ABSTRACT: Textile dyeing waste effluents are rich in unconscionable dyes which are polluting waters and water bodies due to their color and by the formation of carcinogenic or toxic intermediates like aromatic amines from azo dyes. The Conventional treatment systems based on physical or chemical treatment methods are highly high-priced and may consume high amounts of energy and chemicals. Novel biotechnologies have been proposed and has been recently developed. A large volume of both aerobic and anaerobic processes has been evolved at laboratory process to purify dyestuff. The pilot-scale plants are made to serve the purpose. The bio sorption shows is used as widely acceptable and proven technology for decolorizing textile effluents. In this context, we review applied and fundamental aspects of biological treatment of textile Congo red and crystal violet dyes, which has complex and stable molecular structure with different diazo aromatic groups and is widely consumed in the textile industry as an anionic dye. The basic aim of this envisioned study was to investigate the degradation of Congo red and crystal violet in laboratory solution which had the similar chemical properties of the rinse waters of textile manufacturing dye-houses and the samples with Congo red and crystal violet in the wastewater by bioremediation and to optimize the reaction parameters such as pH, time and glucose concentration using a mixed culture containing E.coli & Bacillus subtilis bacteria
Keywords: Bioremediation, Azo dyes, Congo red, Crystal violet, E.coli, Bacillus subtilis. The results show that the proposed consortium has great potential in the decolorization of azo dyes and studied the effect of different reaction parameters. The reaction parameters such as pH, time, and glucose concentration have a recommendable effect in the decolorization of both the dyes.

I. INTRODUCTION

Azo dyes is chemically (-N= N- group) holds the major percentage of manmade dyes with large differentiation of color and different structures. These dyes comprises for about 60-70% of dyes employed in the market. The global production of these dyes is calculated as 450, kilotons/year with at least 50 kilotons/year lost as effluent through its applications. During manufacturing 250 % dyes are discarded as waste effluents.Congo red (sodium salt of benzidine diazo bis1naphtyl amine 4sulfonic acid) are proven as cancer simulating diazo dye and its main application is the coloration of paper and its byproducts. Enormous research has been identified in the pollution related problems in connection with the discharge of this diazo dye effluent from the processing industries. It has been well documented and proven that the safe and ecofriendly method for azo dye is biodegradation associated with aerobic treatment. Many organisms namely Pseudomonas, Bacillus, E.coli, Enterobacter, have been studied for their ability to decolonization of Congo red dye and crystal violet dyes. Since the magnitude of the problem is high and needs a recommendable pure and ecofriendly oriented microbial solution. Decolorizing ability could evolve new indigenous strains to be used as bioremediation tools for removal of azo dyes. Bioremediation are often outlined as any method that uses microorganisms or their enzymes to come the setting altered by contaminants to its original condition. Bioremediation technologies are divided as in place or ex situ. Bioremediation done in site involves mainly treating the effluent material

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at the same location and ex situ operation will do the removal of the effluent material at a remote location. The following bioremediation technologies are the examples which includes land farming, bioventing, land farming, bioreactor, bio augmentation, composting, and bio stimulation. The bioremediation is a processes that make use natural available resources to control pollution problems caused by xenobiotics. [1].

All major mechanistic studies ended up azo dyes since they constitute major portion of textile dyes. Azo dyes are artificial dyes and unbounding of azo bonds in anaerobic conditions is due to reduction processes initiated by redox-active compounds like Quinone-type compounds [2] and cofactors like NADH [3] or cleaved inorganic groups like Fe^{2+} [4] or H_2S are formed due to the action of certain bacteria as their end products.

The specificities of certain enzymes has been pronounced [5] for the active cleavage of azo dyes. The mechanism [6] azo-dye decomposition which is of laccase mediated continues through two consecutive electron reductions and it resulted in the formation of resonance stabilized cation. Meanwhile breakage of this foresaid dye molecule may end up in the release of a proton and a N_2 molecule. It may yield aromatics and transient hydro peroxides, respectively [7].It's been discussed that the laccase-catalyst make azo dye effectual for the hydrolysis and nitrogen out thrown in their molecular structure and uses the mechanism which make us peroxidase action on different azo dyes [8, 9].

A different ranges of bacteria includes Enterococcus sp., Streptococcus faecalis, Proteus sp., Bacillus cereus, Bacillus subtilis, Pseudomonas sp., [11] and it includes different varieties of helminths [12] have proved the ability to reduce azo dyes.Azo reduction are stimulated by co-substrates and they act as reduction equivalents and depending upon the variety of organisms used nutrients provided to acts as co-substrates. The glucose as a co-substrate is studied to be effective in the decolorization of Mordant Yellow 3 and Reactive Red 22 by an anaerobic bacterial consortium [13, 14]. Further studies shows that yeast extract, low Molecular weight carboxylic acids like propionate, acetate, butyrate [15] and highly complex co-substrates like cassava starch are effective co-substrate in the reduction of azo dyes [16].various studies have done in the complete reduction azo dye under oxygen withdrawn conditions [17]. Aromatic amines which are found in the human intestines [18] and are regarded as potentially carcinogens [19] and hence it proved to be substantial risks for human health [20].Now a days emerging technology is the use of mixed of aerobic and anaerobic processes using mixed cultures [21-26].Majority of all design put forward the idea of using of either cultures of glycogen or polyphosphate producing microbe as mixed cultures from different activated sludge units.[27,28].During methanogenic process to treat granular sludge the treatment to oxygen, ethanol behaves enact facultative aerobic microorganisms to respire in the colonized material to prevent the accumulation of oxygen there by azo and diazo dyes are mineralized by the activity of methanogenic colonies in the material. Thus it lead to the construction of aerobic/ anaerobic reactors with full potential to azo-dye mineralization [29].Different examples can be depicted of well-developed bacterial systems for the treatment of textile dyestuff is in practice [30]. So many different attempts have been made to implement aerobic/ anaerobic treatment units in textile market to oxidize aromatic amines as well as to oxidize dyes by bacteria.

Lignin-degrading Streptomyces sp. and Flavobacterium sp. shows the property of oxygen attack with the release of peroxidase. [31].B.subtilis, Pseudomonas pseudomallei, Mycobacterium, Rhodococcus and different Corynebacterium, have proven too effective in degrade triphenylmethane dyes as well It's been concluded that principle dye degradation is possibly using aerobic cultures.

Congo red is the sodium salt of 3, 3'-([1, 1'-biphenyl]-4, 4'-diyl) bis (4-aminonaphthalene-1-sulfonic acid) (Formula: $C_{32}H_{22}N_6Na_2O_6S_2$; Molecular weight: 696.66 g/mol).Figure.1shows the chemical structure of the Congo red dye. It's a secondary diazo dye, which is water soluble, and yielding a high red colloidal solution. It's soluble in organic solvents like ethanol and has great affinity to cellulose fibers. Its use in cellulose industries, cotton textile wood pulp& paper has long been abandoned, primarily because of its ability to change color and its toxicity. Congo red has recommendable spectrophotometric properties and it's identified by its peak around 498 nm in a UV-visible absorption spectrum and the molar extinction coefficient of congo red dye is found to be 45000 [L]/[mol].[cm].The fluorescent activity it shows when binds to amyloid fibrils, and identified as sensitive identification tool for amyloidosis.

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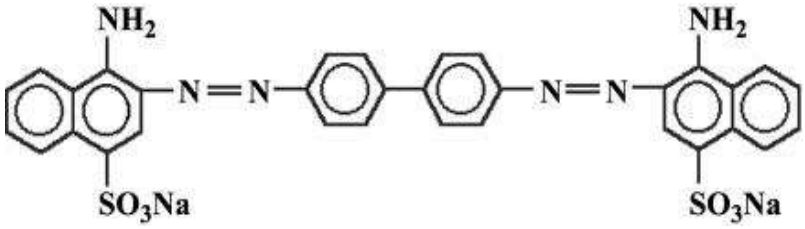

FIGURE 1. Chemical structure of the Congo red dye.

Crystal violet or Gentian violet (also known as Methyl Violet 10B, hexamethyl pararosaniline chloride is a triarylmethane dye and used as a histological stain and for the classification of bacteria using Gram's Method. The structure of the dye is shown in Figure.2.It is well known for its antifungal, antibacterial and anthelmintic properties and a recommendable antiseptic. Crystal violet has recommendable spectrophotometric properties and it's identified by its peak around 590 nm in a UV-visible absorption spectrum and the molar extinction coefficient is about 87,000 $M^{-1}cm^{-1.}$ It exhibits different colors at different pH like green with absorption maxima at 420 nm and while in a strongly acidic solution (pH of -1), it shows yellow with an absorption maximum at 420 nm.

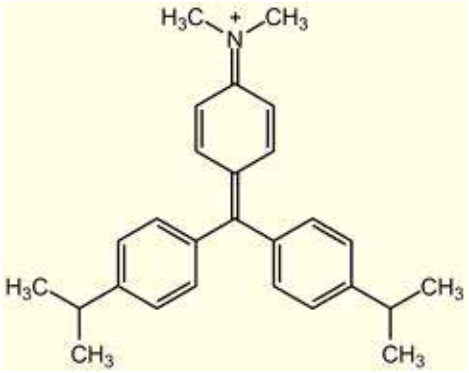

FIGURE 2. Chemical structure of the Crystal Violet

Escherichia coli (commonly abbreviated E.coli) is a rod-shaped, Gram-negative, bacterium found in the intestine of warm-blooded organisms as shown in Figure.3. E. coli strains are harmless, but some serotypes may cause serious food poisoning in humans. The harmless strains are found in the flora of the gut, and it's beneficial to the hosts by the production of vitamin K_2 and it prevents the formation of pathogenic bacteria within the intestine. It is used as an ideal indicator organisms to test environmental samples for fecal contamination. E.coli widely used in the fields of biotechnology and microbiology, as a host organism for the majority of work related with recombinant DNA.

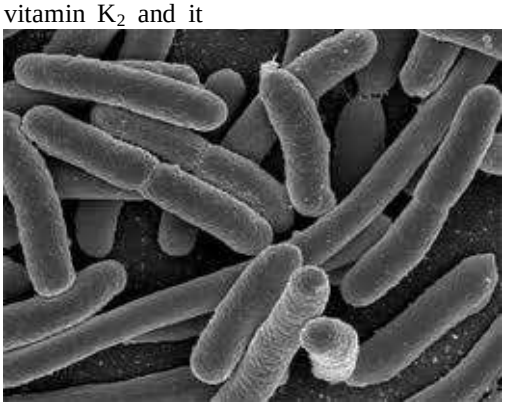

FIGURE 3. Structure of E.coli

Bacillus subtilis as shown in Figure.4 is a Gram-positive, rod-shaped bacteria and a member of the division firmicutes.It can be facultative anaerobes or it shows positive for enzyme catalase.

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Bacillus group includes both pathogenic species and free-living environmental conditions. Bacillus group, produce oval endospores which can be inactive for a long period periods.

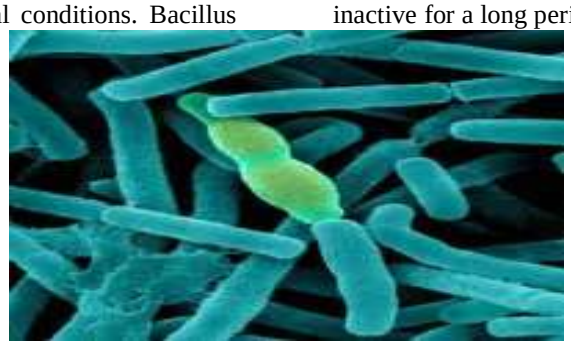

FIGURE 4. Structure of Bacillus subtilis

The present study was intended to study the effectiveness of a mixed culture contains E.coli & Bacillus subtilis and their ability to decolorize azo dyes aerobically and optimize the pH and temperature, glucose concentration required for effective decolorization. The brilliance effect of this azo dyes even at their lower concentrations make it undesirable in industry. The limit of this dyes are stipulated as about 1 ppm as per US regulations and dye concentration reduction of 98 % is needed. The structure of the azo dyes are able resist fading on exposure to light and they prove to be resistant towards microbial degradation. The studies shows that azo dyes, which accumulates about 60% of textile dyes ensure adverse effects on the growth methanogenic bacterial cultures. The toxicity of is contributed due to the presence of azo groups and results in reductive cleavage products. The chromophore group present in the above said azo dyes can be cleavage or reduced using the anaerobic bacteria present in the human intestine. It is been studied and shown that a number of microorganisms and even helminthes have the ability to degrade azo dyes. Due to the ability of the azo dyes to damage the microorganisms in the municipal waste treat treatment plants there is urge for new technologies for to azo dye concentration. The use of Microbial or enzymatic dye degradation as a substitute can be developed as an alternative for the treatment of effluent and therefore the recycling of waste water from different dye industries. The enzymatic cleavage has great advantage that it selectively attacks dye molecule by leaving valuable dye additives and fibers intact so that they can be reused. Microorganism and isolated enzymes shows great reduction ability of effluent from textile industries and their selectiveness make them to reuse as like in wood and pulp industry.

II. MATERIALS AND METHODS

2.1 Chemicals, Dyes, Media

The dyes used for the study are Congo red **(**CAS number 573-58-0) and crystal violet (CAS number 548-62-9).The main chemicals used for the medium solution contains Glucose, Yeast extract, Ammonium chloride(NH_4Cl),Potassium dihydrogen phosphate (KH_2PO_4),Dipotassium hydrogen phosphate (K_2HPO_4), Hydrated magnesium chloride ($MgCl_2.6H_2O$).Glucose is acting as a carbon source. Preparation of chemicals for 10% medium is shown in Table.1

Component	g/l
Carbon source	10
Dyes	As required
Yeast extract	0.34
NH_4Cl	0.84

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KH_2PO_4	0.14
K_2HPO_4	0.24
$MgCl_2.6H_2O$	0.084

TABLE.1 Composition of components for Media preparation in g/l

2.2 Preparation of Inoculum: Mixed Culture

Inoculum for the decolorization studies is prepared by adding E.coli and Bacillus subtilis to 5 ml glucose media in laminar chamber and it's incubated in an incubator. After 24 hours, 5ml of mixed culture is shifted to 50 ml glucose media, and keep it in the incubator. From this media 20 ml of each culture is shifted to two 500 ml conical flasks for making inoculum of 200 ml make up with 180 ml glucose media in each flask and this inoculum is used for the decolorization studies.Figure.5 shows that laboratory prepared inoculum for the decolorization studies.

FIGURE 5.Prepared inoculum for decolorization studies

2.3 Preparation of Dye Solution

1000 ppm of dye stock solution is prepared in distilled water by adding 1 g of each dye in 1000 ml of water. From the stock solution different concentrations like 25 ppm, 50 ppm, 100 ppm, 150 ppm, 200 ppm is prepared by adding dye stock solution with water in different proportions.

2.4 Experimental Method

All the experiments were performed in different conditions as reactors, consisting of a 500 mL flask containing 25mL of mixed inoculum and dye. The reactors contains different glucose concentration as 2g/l,4g/l,8g/l,10g/different pH and different dye concentrations of 25 ppm,50 ppm,100ppm,150ppm,200ppm.The reactors were incubated in rotary shaker at room temperature. Four absorbance readings were taken at 0 hr, 24 hr, 48 hr, and 72hr.Each culture tested and each reactors are studied individually for their decolorization effect in order to ensure the efficiency of the mixed culture to decolorize dyes. Biomass production in the reactors are finding out by UV spectrophotometer. Biomass concentration is finding out by reading the absorbance value at 600 nm.

2.5 Instruments Used

- UV-Visible spectrophotometer, shimanzdu, Japan.
- Centrifuge, Hettich, Zentrifugen, Universal 320 R, Germany.
- Autoclave, Testmaster, Kolkata, India.
- Shaker, Metrex scientific instruments, New Delhi, India

2.6 Decolorization Measurement

For determination of Congo red and crystal violet color removal, 2mL of culture were used for evaluation at different periods (0, 24, 28, 72 hr),centrifuged at 1200 rpm for 10 min to discard the bacterial cells, and the resultant supernatant was examined by using uv spectrophotometer
(Shimadzu UV-2401 PC model, Kyoto, Japan) at $t_{\lambda max}$ of 498 nm for Congo red and 503 nm, for crystal violet. The percentage of removal of color was calculated as following:
Decolorization (%) = [ ] × 100] (1)

III. RESULTS AND DISCUSSION

The present study investigates the feasibility of efficiency of a mixed culture containing E.coli and Bacillus subtilis in the

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removal Congo red dye and crystal violet dye. The study involves the optimization of different parameters and hence to find out the most suitable condition for the high percentage removal of the dyes. The resultant graphs showing the percentage removal is shown below

3.1 Removal of Congo red dye at different glucose and dye concentration

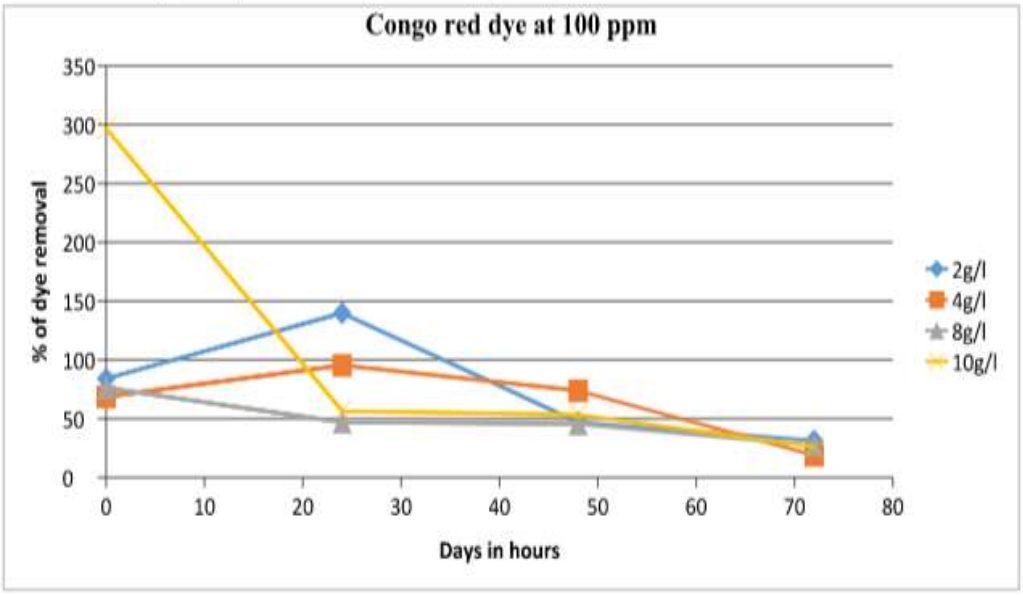

FIGURE 6. Effect of dye concentrations and glucose concentrations in the removal of Congo red dye

Figure 6 gives the effect of glucose concentration on removal of Congo red dye. Glucose concentration have positive effect on bioremediation and the present results shows that even at 2 g/L glucose concentration, 60-70% removal of the dye color is achieved. Congo red dye concentrations were kept at 25, 50,100 ppm and distinguishable results have been identified in all cases. The cleavage of -N=N- has identifiable in differing both dye concentrations and glucose concentrations

3.2 Effect of Initial Dye Concentration on the removal of Congo red dye

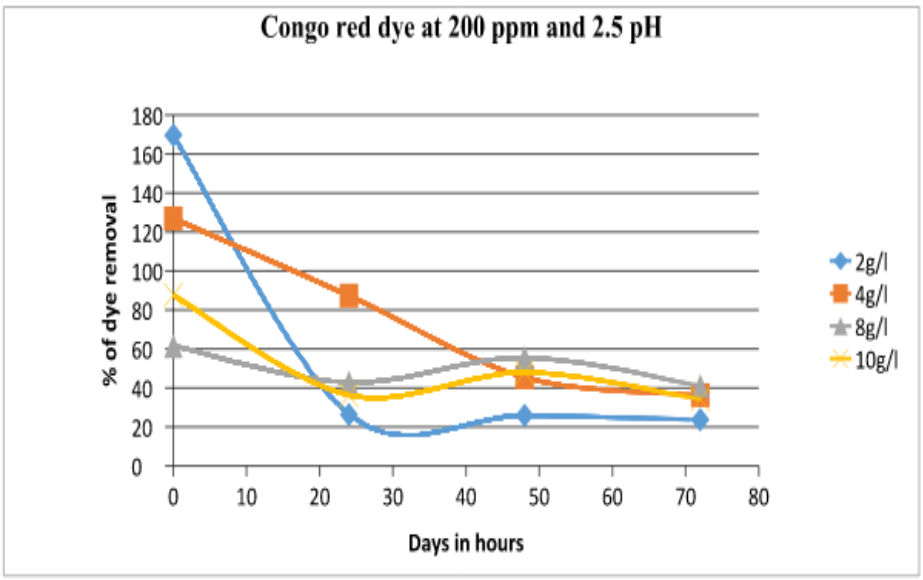

FIGURE.7 Effect of Initial Dye Concentration on the removal of Congo red dye at same P^H

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Dye concentrations were varied to see the effect of initial concentrations. The increase in initial concentrations have somewhat positive effect on dye degradation. Figure 7 shows around 90% degradation is achieved for 200 ppm initial concentration of Congo red dye, while it reduced for lower dye concentrations.

3.3 Comparison of % of Congo red dye Removal at same Glucose Concentration and different dye Concentration and PH

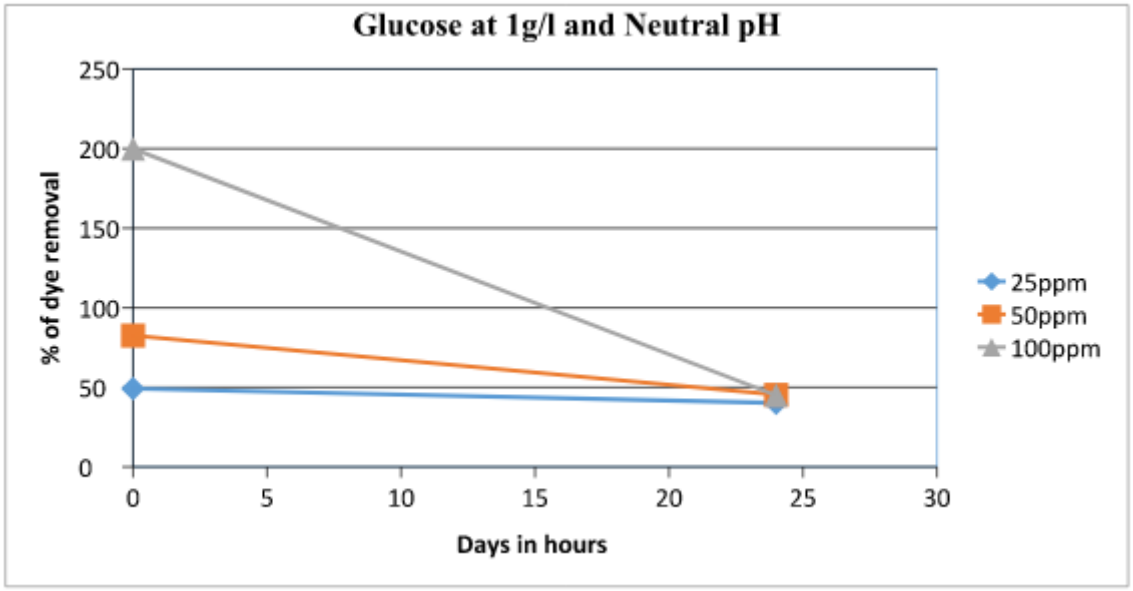

FIGURE.8 Effect of same glucose Concentration and different P^H on the removal of Congo red dye

The Dye removal efficiency of the mixed culture has been studied at constant glucose concentration of 1g/l and varying the dye concentration and P^H as presented in Figure 8 .It is been observed that Congo red dye removal is more effective at Neutral pH as compared to lower P^H. Neutral P^H may stimulate the growth and multiplication of the mixed culture and enhance the cleavage of the azo bonds of the dyes.

3.4 Removal of Crystal Violet dye at different Glucose and dye concentrations

Crystal violet studies was also undertaken to study the effect of different parameters on bioremediation of crystal violet includes pH, varying dye concentrations and for different glucose concentration. It can be seen that there is no clear trend as far as pH is concerned. In some cases, there is 70% degradation while it was as low as 40% in other cases.

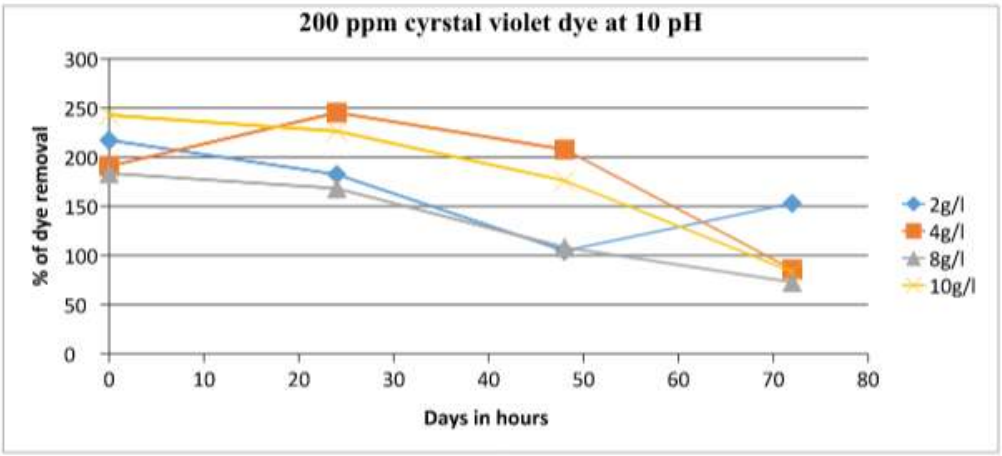

FIGURE.9 Effect of glucose Concentration and different P^H on the removal of Crystal violet dye

Effect of initial concentration has significant reduction in the dye concentrations. It is apparent that higher initial concentration has given lower reductions. High concentration adds to the toxic components of the system. There is a possibility that biomass gets affected in such high toxic exposure and hence become less efficient. This results in lower degradation. Figure 9 shows the percentage of dye removal of crystal violet with respect to different reaction parameters

IV. CONCLUSION

A large of innovative techniques has been formulated for the active enzymatic and microbial treatment of effluents containing having high concentrations which is unacceptable by law and regulations. All the technologies uphold the peculiarity of effluent composition and potential of reuse and its shows that enzymatic processes have high ability to decolorization of effluent dyes and their reuse in dyeing baths. The enzymatic cleavage has great advantage that it selectively attacks dye molecule by leaving valuable dye additives and fibers intact so that they can be reused. The commendable drawback of the enzymatic based processes is that it's the inadequacy of a single enzyme for the treatment of similar dyes and it will be addressed in in the coming decades by using different enzyme at single time. The mixed consortium of microorganisms shows surprisingly great ability in removing textile dye effluents. In the above done experiments it has been shown that at all conditions mixed culture containing E.coli and Bacillus subtilis shown good percentage of decolorizing of dyes Congo red and crystal violet. In the case of crystal violet dye the most decolorizing has been shown in the case of pH 4 and dye concentration 50 ppm shows high percentage decolorization.in this condition at all concentration dye shows a decolorization from 60% to 85%.in the case of Congo red at all conditions it shows a good percentage of decolorization from 60 % to 80%. So from this all we conclude that this mixed culture can be used as an alternative for the bioremediation for the industrial dyes which are discharge to the water resources, and pollute them worst in the cheapest way.

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