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Dye Biodegradation Using Alkalophilic Consortia in Anaerobic-Aerobic Bioprocess

By

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LIST OF ABBREVIATIONS

BOD	Biological Oxygen Demand
COD	Chemical Oxygen Demand
MSM	Mineral Salts Medium
RR 184	Reactive Red 184

ABSTRACT

The decolorization and Chemical Oxygen Demand (COD) removal efficiency of consortia of alkalophilic microorganisms was evaluated in a continuous anaerobic/aerobic reactor system. The biodegradation products of RR 184 in the continuous anaerobic/aerobic reactor were studied by employing different analytical tools such as Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC). When the synthetic dye wastewater was subjected to a two stage reactor, a 96.0 % color removal of RR 184 was attained after the anaerobic treatment. However, after the aerobic treatment, overall decolorization efficiency was 100 % as compared to the anaerobic stage. On the other hand, the removal efficiency of Chemical Oxygen Demand (COD) was greater after the aerobic treatment (66.6%) than the anaerobic treatment (94.26 %). The band of the Thin Layer Chromatography shows fragments of dye decolorization products. On the other hand, the HPLC Chromatograms show that the dye decolorization products that are produced at the anaerobic stage were degraded. From HPLC chromatograms, three new peaks were obtained and one of it was degraded completely and the retention time and peak areas of the other two peaks was decreased. From the consortia, dye decolorizing microorganisms were isolated and tested for their decolorization efficiency in batch test. The isolates from the anaerobic/aerobic reactor were inoculated into a sterilized decolorized media with the addition of glucose as a carbon source to a final concentration of 0.1 % and the isolates were incubated under aerobic condition. After 12 h the isolates growth (OD) was measured and all the isolates tested were able to grow using metabolites of the decolorized media as a sole nitrogen source.

Key Words: biodegradation, Reactive Red 184, dye biodegradation products, alkalophilic microorganisms

1. INTRODUCTION

Recently, pollution problems due to the discharge of effluents from a textile industry have increased tremendously. Approximately 75% of the dyes that are discharged by textile processing industries belong to the classes of reactive (36%), acid (25%) and direct (15%) dyes (Anjali, *et al.*, 2007). Among these classes of dyes, the azo dyes which are characterized by aromatic moieties linked together by azo bond (-N=N-) chromophores are the most important chemical classes of synthetic dyes and pigments, representing between 60% and 80% of the organic dyes used in industries such as textile, leather, plastic, cosmetic and food industries (Vandevivere, *et al.*, 1998).

The removal of dyes from a textile effluent is desired not only for aesthetic reasons, but also many azo dyes and their breakdown products are toxic to aquatic life (Chung and Stevens, 1993) and mutagenic to humans (Brown and Devito, 1993; Weisburger, 2002). Furthermore, their discharge into surface water obstructs light penetration and oxygen transfer into water bodies, hence affecting aquatic life (Asad, *et al.*, 2007). Since synthetic reactive azo dyes are made soluble in water and stable both chemically and photolytically by design, they are persistent and recalcitrant in the environment (Ramalho, *et al.*, 2005). Therefore, textile effluents containing dyes must be treated before their discharge into the environment.

Although, it is difficult to treat textile industry effluents due to their high BOD, COD, heat, color, pH and the presence of metal ions (Dos Santos, 2005), now a days, different systems are developed and applied to treat textile waste water before they are being discharged into water bodies. Currently, physical, chemical and biological methods are employed to decolorize dyes (Hao, *et al.*, 2000).

Table 1. Composition of cotton textile mill waste (Babu, *et al.*, 2007)

Characteristics	Values
pH	9.8-11.8
Total alkalinity	17.22 mg/l as CaCO ₃
BOD	760-900 mg/l
COD	1400-1700 mg/l
Total solids	6000-7000 mg/l
Total chromium	10-13 mg/l

As compared to the physical and chemical methods, which are characterized by limited versatility, high operation cost and energy requirement, generation of a large amount of sludge and secondary pollution due to excessive chemical usage, biological treatment methods of textile industry waste water using biological processes are advantageous because they are relatively inexpensive, environmental friendly and generate less waste (Pandey, *et al.*, 2007; Ramya, *et al.*, 2007).

The complete mineralization of dyes by a microorganism involves two major processes, reductive cleavage of the dyes' azo linkages under anaerobic conditions which results in the formation of generally a colorless but potentially hazardous aromatic amines and degradation of the aromatic amines aerobically. However; the most logical and economical process for the complete mineralization of dyes is achieved by combining the anaerobic and aerobic stages together (Van der Zee & Villaverde, 2005).

Currently, many researches are carried out to decolorize the color of the dye in textile waste water with an anaerobic microorganism under anaerobic condition. But azo dye reduction under anaerobic condition leads to the accumulation of toxic aromatic amines which are resistant to anaerobic degradation (Franciscon, *et al.*, 2009). These dye metabolites are only degraded aerobically.

Field *et al.*, (1995) showed that the aerobic stage of the combined anaerobic/aerobic treatment of dye wastes eliminated the additional COD, attributed to the removal of aromatic amines, which are anaerobically recalcitrant. As a consequence, anaerobic/aerobic sequential processes could

prove for the reduction of both color and organic carbon. Therefore, sequential anaerobic/aerobic treatment can be used to decompose toxic and carcinogenic compounds efficiently (Sponza and Isik, 2005).

In the case of Ethiopia, the wastes of textile industries are released into the nearby water body without proper treatment. This will have a great impact on the environment as well as human life. In some industries pretreatment were done aerobically before being discharged. But aerobic treatment of textile effluents containing azo dyes by an activated sludge is resistant to biodegradation. Hence, it is important to develop a system that can treat the dye containing wastewater of a textile industry. This can be achieved by using consortia of alkalophilic microorganisms in a sequential anaerobic/aerobic system.

1.1. Objectives of the study

General Objective

The overall objective of this study was to study biodegradation of dyes and analyze decolorization products in a sequential anaerobic- aerobic system using consortia of alkalophilic microorganisms.

Specific objectives

1. To isolate microorganisms from a sequential anaerobic-aerobic reactors enriched with Reactive Red dye
2. To evaluate the efficiency of the isolate isolated from the anoxic/oxic reactor system in dye decolorization
3. To study the degradation of aromatic amines by pure isolates isolated from the aerobic reactor.
4. To analyze qualitatively the products that are produced as a result of dye reduction
5. To evaluate the efficiency of the continuous anaerobic/aerobic reactor system in dye decolorization

2. LITERATURE REVIEW

2.1. The nature of textile wastewater

In textile industries synthetic dyes from residual dyebaths are directly released to waste streams. All dyes that are used for dyeing process do not bind to the fabric. Depending on the class of the dye, it is estimated that from 5% for basic dyes to 50% for the reactive dyes of the applied dye can be lost in effluents during textile dyeing processes (Easton, 1995), leading to severe contamination of surface and ground waters in the vicinity of dyeing industries (Ganesh *et al.*, 1994; O'Neill *et al.*, 1999).

Table 2. Estimated degree of fixation for different dye/fibre combinations (Easton, 1995).

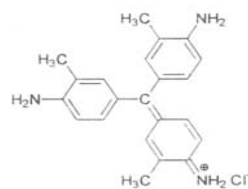
Dye class	Fiber	Degree of fixation (%)	Loss to effluent (%)
Acid	Polyamide	80-95	5-20
Basic	Acrylic	95-100	0-5
Direct	Cellulose	70-95	5-30
Disperse	Polyester	90-100	0-10
Metal – complex	Wool	90-98	2-10
Reactive	Cellulose	50-90	10-50
Sulfur	Cellulose	60-90	10-40
Vat	Cellulose	80-95	5-20

Table 3. Some important commercial azo dyes (Hao, *et al.*, 1999)

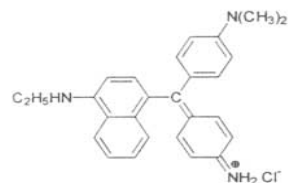
Generic Name (C.I. No.)	Structure
Acid Dyes	
Acid Yellow 36 (13065)	
Acid Violet 34 (61710)	
Direct Dyes	
Direct Blue 150 (35110)	
Direct Brown 44 (35005)	
Disperse Dyes	
Disperse Yellow 3 (11855)	
Disperse Blue 19 (61110)	
Vat Dyes	
Vat Yellow 3 (61725)	
Vat Orange 9 (59700)	

Basic Dyes

Basic Violet 2 (42520)

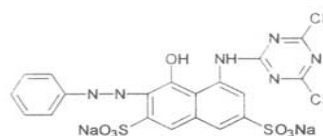


Basic Blue 11 (44040)

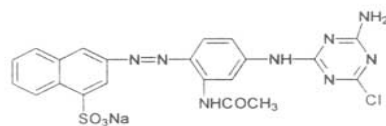


Reactive Dyes

Reactive Red 2

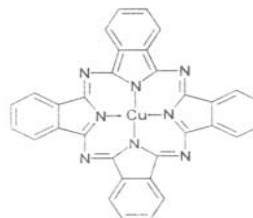


Reactive Yellow 3



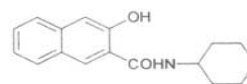
Pigments

Pigment Blue 15 (74160)
(copper phthalocyanine)

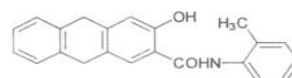


Azoic Coupling Component

Azoic Coupling Component 2
(37505)



Azoic Coupling Component 36
(37585)



2.2. Dye removal techniques

The two major dye removal methods of textile wastewater are generally grouped into physico-chemical and biological. The physico-chemical method includes processes such as membrane filtration, coagulation/flocculation, precipitation, flotation, adsorption, ion exchange, ion pair extraction, ultrasonic mineralization, electrolysis, chemical reduction and advanced chemical oxidation (Francisco, *et al.*, 2009). Biological techniques include biosorption and biodegradation in aerobic, anaerobic, anoxic or combined anaerobic/aerobic treatment processes (Van der Zee, 2002).

Due to the complex nature of textile wastewater, several factors determine the technical and economic feasibility of each single dye removal technique as dye type, wastewater composition, dose and costs of required chemicals, operation costs (energy and material), environmental fate and handling costs of generated waste products (Andrea, 2005).

2.2.1. Physico – chemical methods

Physico-chemical methods, such as membrane filtration, coagulation, flocculation and advanced oxidation, adsorption, can be very effective for the removal of the color in wastewaters (Andrea, 2005). Nanofiltration and reverse osmosis, using membranes are used to treat textile effluents. Membrane filtration is a quick method with low spatial requirement and the permeate can be reused. The disadvantage of membrane techniques are membrane fouling, frequent cleaning and regular replacement of the modules.

Coagulation/flocculation is often applied in the treatment of textile-processing wastewater to partly remove Chemical Oxygen Demand (COD) and color from the raw wastewater before further treatment (Van der Zee, 2002). The principle of the process is the addition of a coagulant followed by a generally rapid chemical association between the coagulant and the pollutants. The formed coagulates subsequently precipitate or are to be removed from the water phase by flotation. Coagulation/flocculation with inorganic chemicals generates considerable volumes of useless or even toxic sludge that must be incinerated or handled otherwise.

The dyes from textile wastewater can also be removed by adsorption. This technique yields waste sludge that should be disposed of or regenerated. As compared to other adsorbents such as wood charcoal bacterial biomass modified or non-modified cellulose and others, activated carbon is capable of adsorbing many different dyes with high adsorption capacity (Liversidge, *et al.*, 1997) but it is expensive and the costs of regeneration are high because desorption of the dye molecules is not easily achieved. Low-cost adsorbents can be used but the surface charge of the adsorbent and the pH play a crucial role in color removal efficiency (Dos Santos, 2005; Vandevivere, *et al.*, 1998). It should also be taken into account that these processes merely transfer the pollutants from one phase to another.

Advanced oxidation processes (AOPs) that have been most widely studied are ozonation, UV/H₂O₂, Fenton's reagent (Fe²⁺/H₂O₂) and UV/TiO₂ (Aplin, and Wait, 2000). The concept behind an AOP is that exposure of a strong oxidizing agent exposed to UV light generates hydroxyl free radicals (Ince and Gonenc, 1997), which are even stronger oxidants. Advanced oxidation processes (AOPs) are therefore mostly based on the generation of highly reactive radical species (especially the hydroxyl radical HO₂) that can react with a wide range of compounds, also with compounds that are otherwise difficult to degrade, e.g. dye molecules.

Advanced Oxidative Processes (AOPs) can attack organic compounds 10⁶–10⁹ times faster than oxidizing agents like ozone or hydrogen peroxide (Me´ndez-Paz, 2005). Although photocatalytic oxidation with H₂O₂ (H₂O₂/UV) would be more attractive from an economic point of view, photocatalytic processes are limited to post-treatment units because of the low penetration of UV irradiation in highly colored wastewaters (Vandevivere *et al.*, 1998).

Other physico-chemical technologies, such as membrane filtration or activated carbon adsorption, are expensive and commercially unattractive (Arslan *et al.*, 2002). Low-cost adsorbents can be used but the surface charge of the adsorbent and the pH play a crucial role in color removal efficiency (Carvalho, 2007). It should also be taken into account that these processes merely transfer the pollutants from one phase to another.

2.2.2. Biological Methods

Unlike the physico-chemical method, biological processes provide a low cost and efficient alternative for simultaneous color and organic matter removal. However, complete dye degradation in wastewater treatment plants based only on aerobic processes is difficult to achieve because the main mechanism responsible for color removal is adsorption onto the sludges (Pagga and Brown, 1986).

2.2.2.1. Anaerobic azo dye reduction

The ability of bacteria to metabolize azo dyes has been investigated by a number of research groups (McMullan *et al.* 2001 Pandey, *et al.*, 2007). Under anaerobic conditions, many bacteria reduce azo dyes by the activity of unspecific, soluble, cytoplasmatic reductase, known as azo reductases.

Bacterial azo dye biodegradation under anaerobic condition requires an organic carbon/energy source such as glucose, starch, acetate, ethanol and more complex ones, such as whey and tapioca, have been used for dye decolorization under methanogenic conditions (Talarposhti *et al.*, 2001). In anaerobic azo dye biodegradation the dye act as an acceptor of electrons supplied by carriers of the electron transport chain. In addition to bacteria, fungal and algal biodegradation of dyes under anaerobic condition was also investigated by different groups of researchers (Stolz, 2001; Robinson, *et al.*, 2001).

2.2.2.2. Aerobic biodegradation of azo dyes

Several bacterial strains that can aerobically decolorize azo dyes have been isolated during the past few years. Many of these strains require organic carbon sources, as they cannot utilize dye as the growth substrate (Stolz, 2001).

There are only very few bacteria that are able to grow on azo compounds as the sole carbon source. These bacteria cleave $-N=N-$ bonds reductively and utilize amines as the source of carbon and energy for their growth. Such organisms are specific towards their substrate. Examples of bacterial strains with this trait are *Xenophilus azovorans* KF 46 (previously

Pseudomonas sp. KF46) and *Pigmentiphaga kullae* K24 (previously *Pseudomonas sp.* K24), which can grow aerobically on carboxy-orange I and carboxy-orange II, respectively (Pandey, *et al.*, 2007).

Table 4. Technologies used for decolorization (Hao, *et al.*, 1999)

Types	processes	Examples
Physical	Membrane filtration	Nanofiltration Electroflotation
Electrochemical	Oxidation/reduction	Electro-coagulation, electro-oxidation, electro-flotation
Chemical	Coagulation/precipitation	Iron/aluminium/lime with or without polymer
	Chlorination/ozonation	Cl ₂ , NaOCl, ozone
	adsorption	Carbon or other low cost materials (e.g., bagasse pith, teak wood bark, rice husk, cotton waste, coal, hair, and bentonite clay, silica)
	Wet air oxidation	High temperatures and pressures
	Fenton reagent oxidation	H ₂ O ₂ /Fe(II)
	Reduction	Na ₂ S ₂ O ₄
	Ion exchange	Anion exchange resin
	Ion pair extraction	Amines reacting with sulphonic groups forming hydrophobic pairs of ions and accumulated in an organic medium
photo catalytic	UV; H ₂ O ₂	UV/ H ₂ O ₂ , UV/O ₃ , UV/TiO ₂
Biological	Anaerobic/anoxic/aerobic	Activated sludge, fungi

2.3. Degradation of aromatic amines

Aromatic compounds possess large negative resonance energy, resulting in thermodynamic stability. Microorganisms, particularly bacteria, have evolved enzyme systems that degrade the benzene structure under aerobic and anoxic conditions (Schink *et al.*, 2000). In aerobic metabolism, the initial reactions involve the replacement of other functional groups of the aromatic ring with hydroxyl groups, followed by cleavage by incorporating two oxygen atoms. These reactions are catalysed by hydroxylases and oxygenases. Under anoxic conditions, dearomatization is achieved by ring reduction and also includes other unique reactions such as carboxylation, reductive dehydroxylation and addition reactions, which are absent in the aerobic metabolism (Pandey, *et al.*, 2007).

2.3.1. Anaerobic biodegradation of aromatic amines

Decolorization of azo dyes in anaerobic environments leads to the formation of aromatic amines, many of which were assumed to resist further degradation under these conditions (Stolz, 2001).

Nevertheless, mineralization of few simple aromatic amines has been reported under methanogenic conditions. They include the three isomers of aminobenzoate, 2- and 4-aminophenols, 2, 4-dihydroxyaniline and 5-aminosalicylic acid (5-ASA). (Yemashova *et al.*, 2004). Many reports have shown that sulfonated aromatic amines (SAA) are nonbiodegradable under methanogenic conditions (Tan *et al.*, 2005).

2.4.4.2. Aerobic biodegradation of aromatic amines

Aromatic amines formed from azo dye reduction, have been reported to be more easily degraded under aerobic conditions (Ekici *et al.*, 2001). 4-aminophenol (4-AP) and 5-ASA tend to autoxidise in the presence of oxygen (Stolz *et al.*, 1992; Tan *et al.*, 1999). However, the autoxidation rate for 4-AP was in orders of magnitude greater than that of 5-ASA. Hence biodegradation of 5-ASA was possible, whereas 4-AP removal was mainly due to autoxidation under aerobic conditions (Tan *et al.*, 1999).

A group of aromatic amines difficult to degrade even under aerobic conditions are represented by aryl sulfonates, aminobenzene (ABS) and aminonaphthyl sulfonates (ANS), which are the constituents of many azo dyes. Among the three isomers of ABS, 4-ABS appears to be more susceptible to biodegradation than 2- and 3-ABS.

2.4. Mechanism of azo dye reduction

The first step in the bacterial degradation of azo dyes, in either anaerobic or aerobic conditions, is the reduction of the $-N=N-$ bond. This reduction may involve different mechanisms, such as enzymes, low molecular weight redox mediators, chemical reduction by biogenic reductants like sulfide, or a combination of these (Fig.1). Additionally, the location of the reactions can be either intracellular or extracellular.

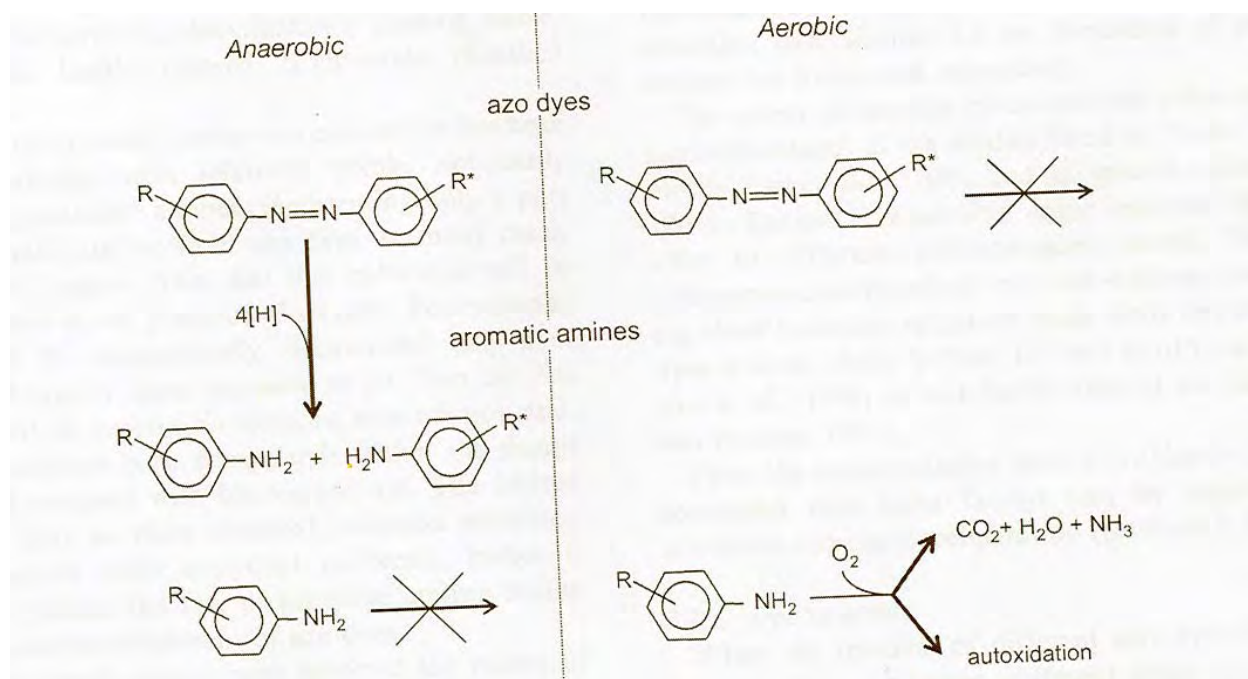


Figure 1. General Overview of the fate azo dyes and aromatic amines during anaerobic/aerobic treatment (Van der Zee, et al., 2005)

2.4.1. Direct enzymatic azo dye reduction

This mechanism involves enzyme-mediated transfer of reducing equivalents, generated from the oxidation of the substrate/coenzyme to azo dyes. These enzymes can be specific, catalyzing only azo dye reduction, or nonspecific. Due to their nonspecific nature, these enzymes are able to reduce azo dyes.

The presence of azoreductases in anaerobic bacteria that decolorized sulfonated azo dyes during growth on solid or complex media was first reported by (Rafii *et al.*, 1990). These strains belonged mainly to the genera *Clostridium* and *Eubacterium*. Azoreductases from these strains were oxygen sensitive and were produced constitutively and released extracellularly. Later investigations made with *C. perfringens* showed that azo dye reduction is catalyzed by an enzyme presumed to be flavin adenine dinucleotide dehydrogenase, which can also reduce nitro aromatic compounds (Rafii, 1995). Another mechanism of dye decolorization could involve cytosolic flavin-dependent reductases, which transfer electrons via soluble flavins to azo dyes.

2.4.2. Mediated biological azo dye reduction

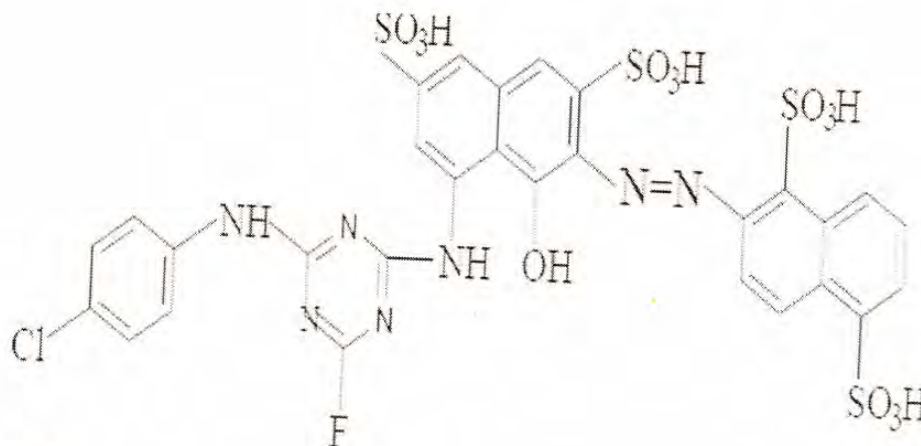
These days, many reports are released on the role of redox mediators in azo bond reduction by bacteria under anaerobic conditions (Van der Zee *et al.*, 2001; Dos Santos *et al.*, 2003). 1-amino 2-naphthol, one of the constituent amines of the azo dye, AO7, increased its decolorization rate, possibly by mediating the transfer of reducing equivalents (Mendez-Paz, *et al.*, 2005). The addition of synthetic electron carriers such as anthraquinone-2, 6-disulphonate could also greatly enhance the decolorization of many azo dyes (Van der Zee, *et al.*, 2001).

Other bacterial cultures generating redox intermediates during the aerobic degradation of aromatic compounds can also lead to the enhancement of dye decolorization in anaerobic conditions (Keck *et al.*, 1997).

3. MATERIALS AND METHODS

3.1. Dyes and Chemicals

A reactive red 184 (RR184) azo dye (Fig. 2) were kindly supplied by Akaki textile industry. The dye was selected on the basis of structural diversity and frequent use in textile industries.



A] Reactive Red 184

Figure 2. Structure of Reactive Red 184

3.2. Composition of synthetic wastewater

The basic composition of a synthetic dye wastewater medium was (g L^{-1}): Na_2HPO_4 (3.6), KH_2PO_4 (1.0), $(\text{NH}_4)_2\text{SO}_4$ (1.0), MgSO_4 (1.0), CaCl_2 (0.1), $\text{FeC}_6\text{H}_5\text{O}_7$ (0.01), and 10 ml of trace element solution per liter was used for all the studies. The trace element solution used was of the following composition (mg L^{-1}): $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (10.0), $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (3.0), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (1.0), $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ (2.0), $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (3.0), H_3BO_3 (30.0) and $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (1.0). The medium was supplemented with 10 mg/L. Soluble starch and yeast extract were added to the synthetic dye media to a final concentration of 0.5% (w/v) and 0.01% (w/v) respectively and sterilized along together with the media. Sodium carbonate, Na_2CO_3 , (25 % w/v) was separately sterilized and added to the media in order to maintain an alkaline pH.

3.3. Configuration of the continuous reactor system

The basic configuration of the continuous reactor system consists of anaerobic and aerobic reactors respectively (Fig. 3). In the continuous reactor system, the synthetic wastewater containing a reactive Red 184 azo dye (0.01 g/L) were continuously pumped with a flow rate of 1 ml/min into an anaerobic reactor consisting of anaerobic consortia of alkalophilic mud samples (10 % w/v) which are collected from Ethiopian Soda Lakes (Chitu and Abijatta). The retention time of reactive Red 184 azo dye in the anaerobic reactor was 2 h. The treated dye along together with some consortia of alkalophilic mud samples were then pumped into the aerobic reactor with a flow rate of 1 ml/min. The aerobic reactor was continuously supplied with air by using a pump. The continuous anaerobic/aerobic reactor also consists of three sampling ports (before the anaerobic reactor, after the anaerobic reactor and after the aerobic reactor) for the High performance Liquid Chromatography (HPLC), Thin Layer Chromatography (TLC) and Uv-Visible Spectrophotometry analysis.

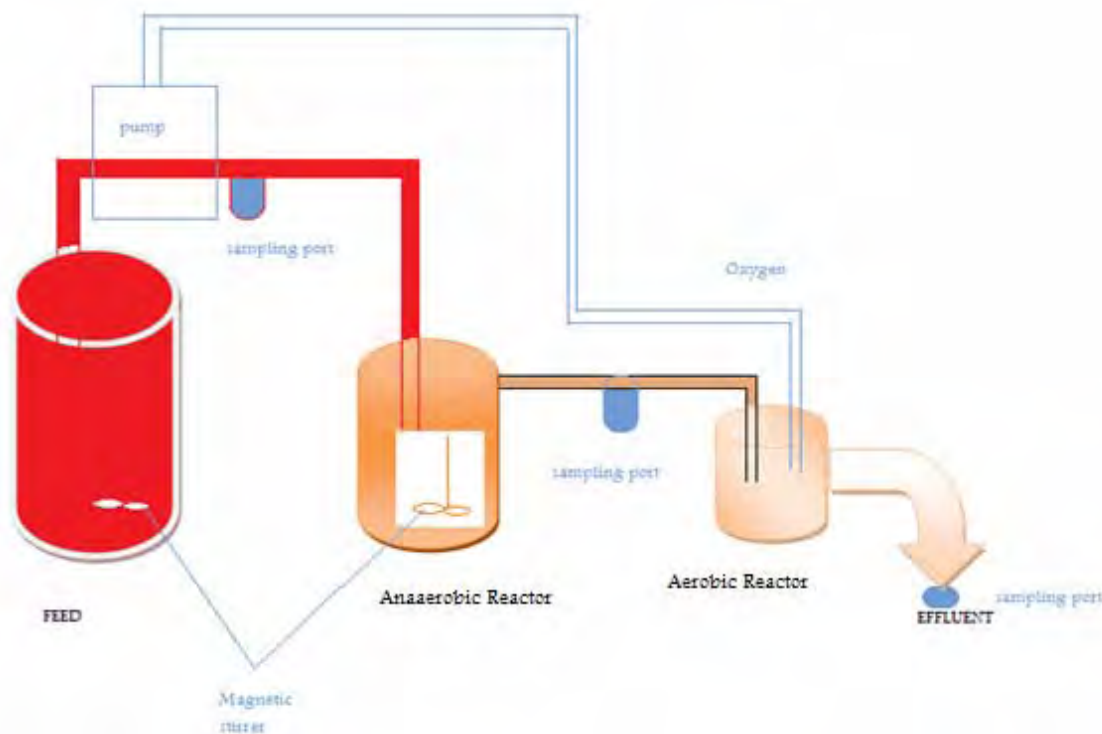


Figure 3. Configuration of the continuous anaerobic/aerobic reactor system

3.4. Decolorization and growth measurement

In order to measure decolorization, sampling was done for every 24 h for 5 days from the three sampling sites of the continuous reactor (before the anaerobic reactor, after anaerobic treatment and after aerobic treatment). Then the samples were centrifuged at 6000 rpm for 10 min and were analyzed by Uv-Vis spectrophotometry. Similarly, sampling was also done for every 24 h from the anaerobic batch decolorization test by isolates (S1, B3 and N3) from the continuous reactor, clarified and analyzed by Uv-Vis spectrophotometry. For both tests, an uninoculated culture media with and without added dyes were used as a control and blank respectively.

Decolorization efficiency of the sediment consortia in the anaerobic/aerobic reactor and the different isolates (S1, B3 and N3) in an anaerobic batch decolorization was expressed as

$$\text{Decolorization (\%)} = (A_0 - A) / A_0 \times 100,$$

Where, A_0 = the initial absorbance and

A = the absorbance after decolorization at the λ_{\max} (nm) of the dye under study.

The average decolorization rate (μgh^{-1}) was calculated as

$$C \times \%D \times 1000 / (100 \times t)$$

Where C is the initial dye concentration and $\%D$ is the dye decolorization (%) after time t (h)

The bacterial growth or turbidity after decolorization of Reactive Red 184 under batch test by three isolates isolated from the continuous reactor was calculated by determining the difference between the absorbance of the culture samples before and after centrifugation at 600 nm.

$$\text{Turbidity} = \text{OD}_{(\text{before centrifugation})} - \text{OD}_{(\text{after centrifugation})}$$

3.5. Decolorization under different culture conditions

Decolorization under different culture conditions was studied by changing carbon source, nitrogen source and dye concentration. In effect different decolorization efficiency was obtained at different conditions. The effect of aeration was examined under three culture conditions, namely, static (no shaking), agitated (aerobic) and anaerobic. In the case of aerobic decolorization batch tests using the three isolates (S1, B3 and N5), the culture flasks were shaken on a rotary shaker running at 120 rpm/min. In the anaerobic batch test, flasks containing decolorizing medium were sealed with rubber septa and incubated under anaerobic condition and in the static condition flasks were placed in the incubator directly

3.6. Decolorization by active and inactive cells

After the dye was decolorized in an anaerobic batch test by the isolate S1, the decolorized medium was autoclaved, centrifuged at 6000 rpm for 10 min, incubated with the dye and their decolorization activity was monitored by Uv-Vis spectrophotometry.

3.7. Isolation of bacterial strains from the continuous reactor

Isolation was made from the continuous reactor system that was acclimatized for a month with alkalophilic consortia. Serial dilutions (10^{-1} to 10^{-6}) of the samples collected from both anaerobic and aerobic reactors were inoculated into a mineral salt agar medium (MSM) by the spread plate technique. Isolates were inoculated into a synthetic dye media containing 10 mg/L and incubated at 30 °C for 5 days. The isolates were tested for their decolorization efficiency under anaerobic batch conditions and the strain that achieved the best decolorization efficiency was selected for further study.

3.8. Analysis

3.8.1. Color measurement

Color in the influent and effluent samples of the anaerobic/aerobic reactors and color in the anaerobic batch treatment with the selected isolate, S1, was measured with an Optima Photomech 301-D UV-Vis spectrophotometer at the maximum visible absorbance wavelength of the dye. In both cases samples were centrifuged at 6000 rpm for 10 min and absorbance values of supernatants were recorded for color measurements. In order to measure the real color removal, the absorbance values of the treated media at λ_{\max} was calibrated with the control samples containing no dye (Awoke, 2008).

3.8.2. Biodegradation assay via TLC

After complete decolorization by the efficient decolorizer isolate (S1) which was selected from the isolates, the decolorized medium was centrifuged at 6000 g for 10 min. And the supernatant extracted with chloroform after alkalization to pH 8 to extract the biotransformed products. Then the extracted product was evaporated in a rotary evaporator. The concentrated extract was dissolved in 1 ml chloroform and used for thin layer chromatography (TLC). The mobile phase for the organic and the aqueous extracts was petroleum ether: chloroform: methanol (4:1:1). The bands of decolorization metabolite were observed under UV light.

3.8.3. HPLC analysis of decolorization metabolites

To determine the dye fragments produced upon decolorization in a continuous reactor system using microbial consortia the treated samples were used directly for HPLC analysis. HPLC analysis was carried out On a Ceccil model Adept CE 4900 chromatograph equipped with a Cecil model CE 4200 UV detector, a column model CE 4601, and a lichrosorb C-18 column with a 4.6 mm inside diameter and 25 cm height.

In the continuous reactor, sampling was done before the anaerobic treatment, after anaerobic treatment and aerobic treatments and centrifuged at 6000 g for 10 min, clarified by 0.45 filters, and analyzed with HPLC. In the batch test, the decolorized media under anaerobic condition with the efficient isolate were centrifuged, pH adjusted to 7.5, autoclaved, supplemented with glucose to a final concentration of 0.1% and inoculated with three isolate aerobically that are capable of decolorizing under aerobic, incapable of decolorizing under aerobic and the efficient isolate that is capable of decolorizing under anaerobic condition. Samples before autoclaving (after centrifugation) and incubation with the three isolates were centrifuged at 6000 rpm for 10 min, clarified by 0.45 filters, and analyzed with HPLC.

In the analysis of dye fragments, a mobile phase composed of a phosphate buffer solution (0.7 g/L Na₂HPO₄, 0.58g/L NH₄H₂PO₄) and methanol with a flow rate of 1 ml min⁻¹ were used. The elutes were monitored by the UV absorption at 220 nm and 217 nm. To determine the dye fragments produced upon decolorization, the treated samples were used directly for HPLC analysis.

3.8.4. COD, Total Nitrogen and NH₄⁺ measurements

In the continuous reactor, samples before the anaerobic treatment, after anaerobic treatment and after aerobic treatment were taken and analyzed for chemical oxygen demand (COD), Total nitrogen, ammonia and nitrate. Chemical Oxygen Demand, Total Nitrogen and NH₄⁺ measurement were done according to the standard methods of HACH instruction manual and instruments.

The percent removal efficiency of Chemical Oxygen Demand (COD) and Total Nitrogen (TN), was calculated as:

$$\% \text{ Removal efficiency} = \frac{C_i - C_f}{C_i} \times 100,$$

Where, C_i = the initial concentration of the feed

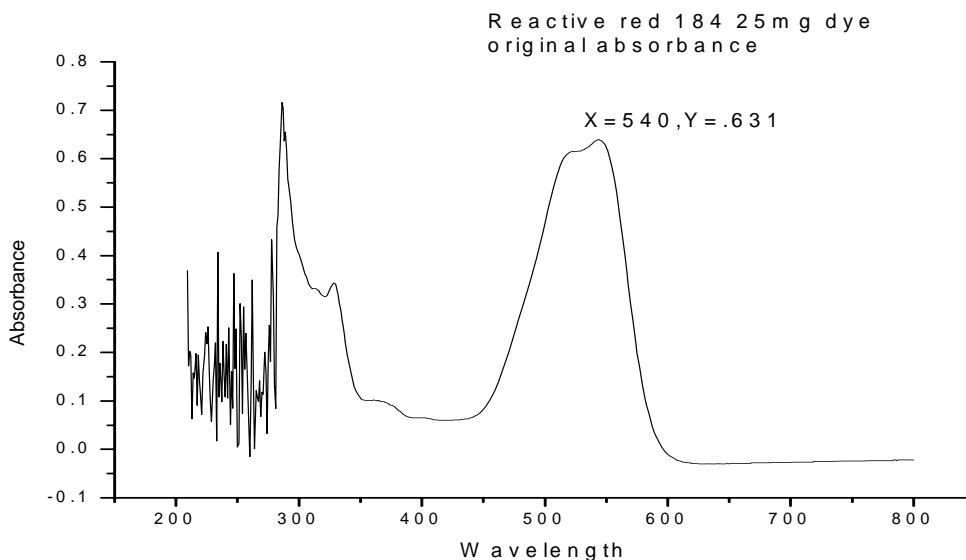
C_f = final concentration after treatment

4. RESULTS

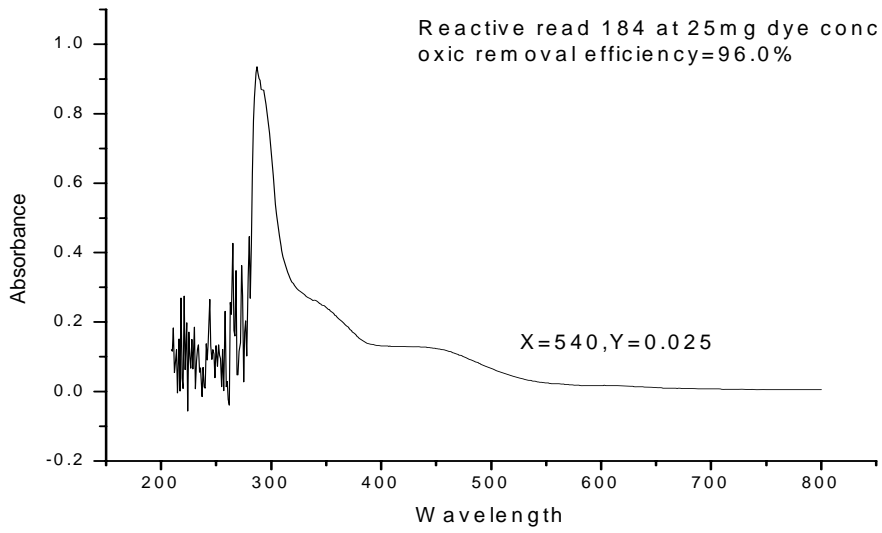
4.1. Color measurement from the continuous reactor

From Figure 4a, the maximum absorbance of the dye ($\lambda_{\max} = 540 \text{ nm}$) was 0.631. After the anaerobic stage (Fig. 4b), the maximum absorbance of the dye was found to be 0.025. The color removal efficiency of the reactor was approximately 96.0%. After the dye was treated in an anaerobic condition, it was pumped into a second stage known as the aerobic reactor. From figure 4c, the absorbance of the dye after aerobic treatment or the effluent was found to be 0.0. And the color removal efficiency of reactive Red 184 at λ_{\max} was 100 %.

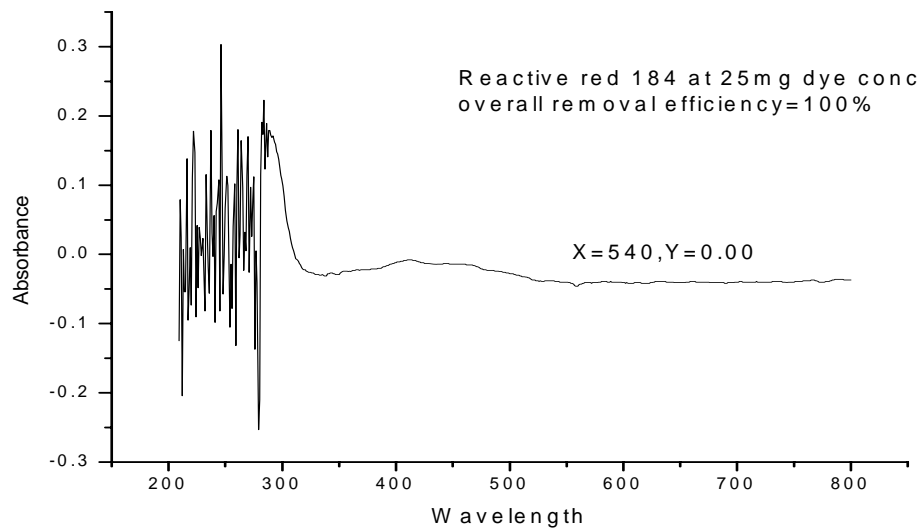
Thus the major color removal was attained at the anaerobic stage than the aerobic stage. After biodegradation of the dye, the absorbance peaks in the visible region disappeared while the absorption peak in the UV range did not diminish, indicating complete decolorization.



a.



b.



c.

Figure 4. Color removal efficiency of a) dye b) anaerobic effluent c) aerobic effluent

Table 4. Percent decolorization of the RR 184

Parameter	Absorbance	Decolorization (%)
Ineffluent (dye)	0.631	0
anaerobic effluent	0.025	96
Overall	0	100

4.2. HPLC analysis of biodegradation products

At 220 nm, HPLC analysis of the parent compound, the dye (Fig. 5a) shows the presence of two peaks with retention times 0.62 and 1.35 minute. The peak area of the peak with retention time 0.62 was 47.6 mv. For the second peak with retention time 1.35, the area of the peak was 23.3 mv.

At the same wavelength, the dye after anaerobic treatment was analyzed by HPLC and it gives three new peaks with different retention times than the chromatograms of the parent molecule (Fig. 5b). The retention times of the peaks were, 0.46, 0.58 and 1.23 minute. The peak areas of the three peaks were 121, 245 and 46 mv respectively. The appearance of these three new peaks with different retention times after anaerobic treatment can be attributed by the cleavage of the azo bond by the consortia into aromatic compounds. It should be noted that due to the unavailability of authentic standards, the chromatographic peaks appearing in samples taken after the anaerobic system could not be identified or quantified. Therefore, it is reasonable to consider that the three peaks contain aromatic amines originating from total reduction of Reactive Red azo dye.

Further HPLC analysis of the effluent after aerobic treatment at 220 nm, gives three peaks with retention times, 0.46, 0.58 and 1.25 minute (Fig. 5c). The areas of the three peaks were 12, 61, 52 mv respectively. At the end of aerobic phase, the HPLC analysis seems to indicate that the decolorization metabolites produced during the anaerobic phase were removed in the subsequent aerobic phase. Figure 5c shows that when the products produced during the anaerobic stage were metabolized aerobically, a less aromatic compound was formed. This is because the product peak area of the peaks decreased tremendously.

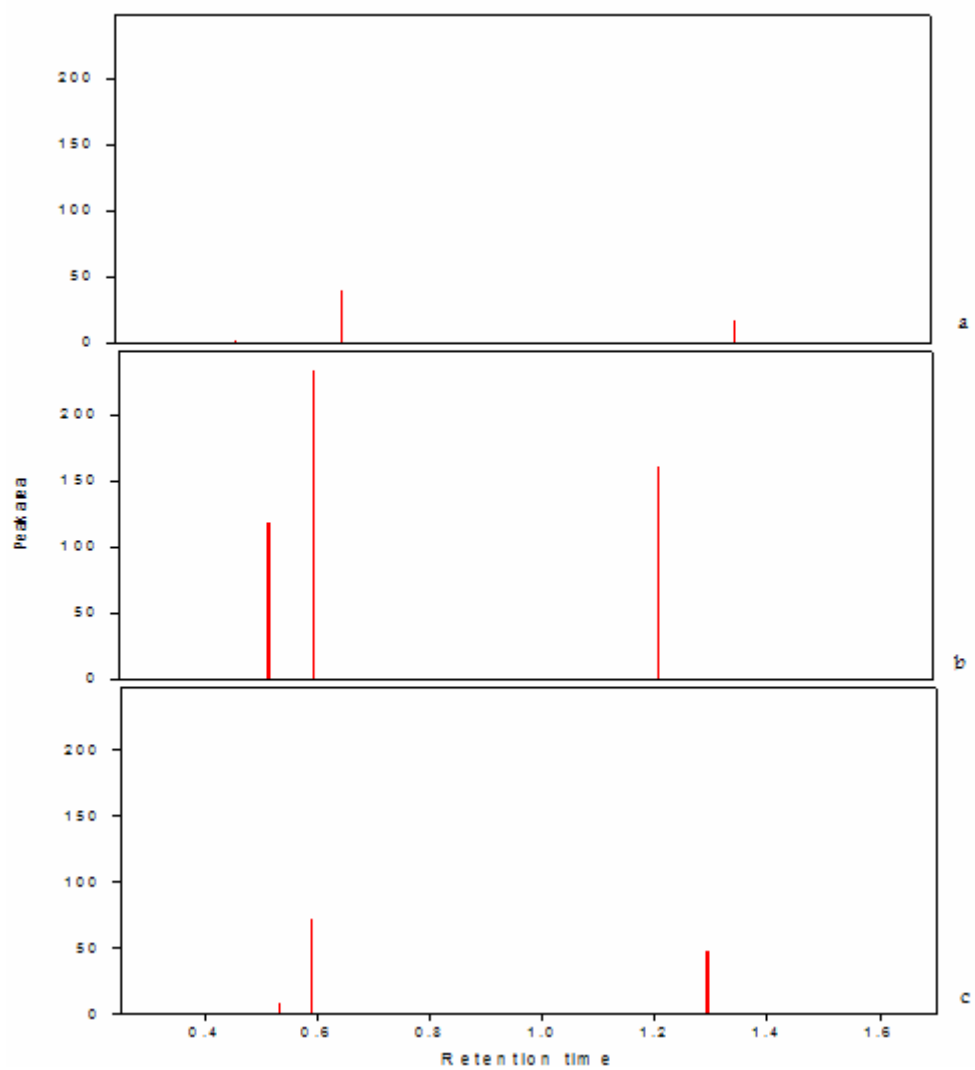


Figure 5. HPLC chromatograms of the a) dye, b) anaerobic effluent c) aerobic effluent at 220 nm

4.3. Thin layer chromatography/TLC

The TLC chromatograms under UV light showed that the decolorized sample had two bands indicating that Reactive Red 184 was cleaved into two fragments (data not shown).

4.4. Decolorization of RR184 by active and inactive Cells

In the study of active versus inactivated cells, half of the decolorized media by the isolate S1 in an anaerobic batch experiment were autoclaved and half of the remaining decolorized media were left without being autoclaved. In the study it is only active (non autoclaved) cells were able to decolorize the dye whereas inactivated cells are unable to do so (figure 6). This shows that decolorization was primarily taking place by degradation than adsorption.



Figure 6. The effect of active versus inactivated cells on RR 184 decolorization

4.5. COD, Total Nitrogen, and NH_4^+ measurements

As it was indicated in table 4, treatment of synthetic dye wastewater with a continuous anaerobic/aerobic process showed that evolution of NH_4^+ in the subsequent aerobic process increases from the anaerobic reactor (25.6 mg/L) to the aerobic reactor (54 mg/L). The pH after the anaerobic treatment decreases to 8.92 and it increases to 9.42 after aerobic treatment (Table 5).

Table 5. The influent and effluent parameters of anaerobic/aerobic system

Parameter	Sample		
	feed	anaerobic effluent	aerobic effluent
COD (mg/L)	1290	430	74
Total Nitrogen (mg/L)	104.8	52.2	23.2
Absorbance at λ_{max} (nm)	540	220	217
$\text{NH}_3\text{-N}$ (mg/L)	0	25.6	54
pH	9.97	8.92	9.42

As figure 7 shows, the Chemical Oxygen Demand (COD) and Total Nitrogen (TN) removal efficiency after anaerobic treatment were 66.6% and % 50.2 % respectively. And the overall COD and Total nitrogen removal efficiency after aerobic treatment were 94.26 % and 77.9 % respectively.

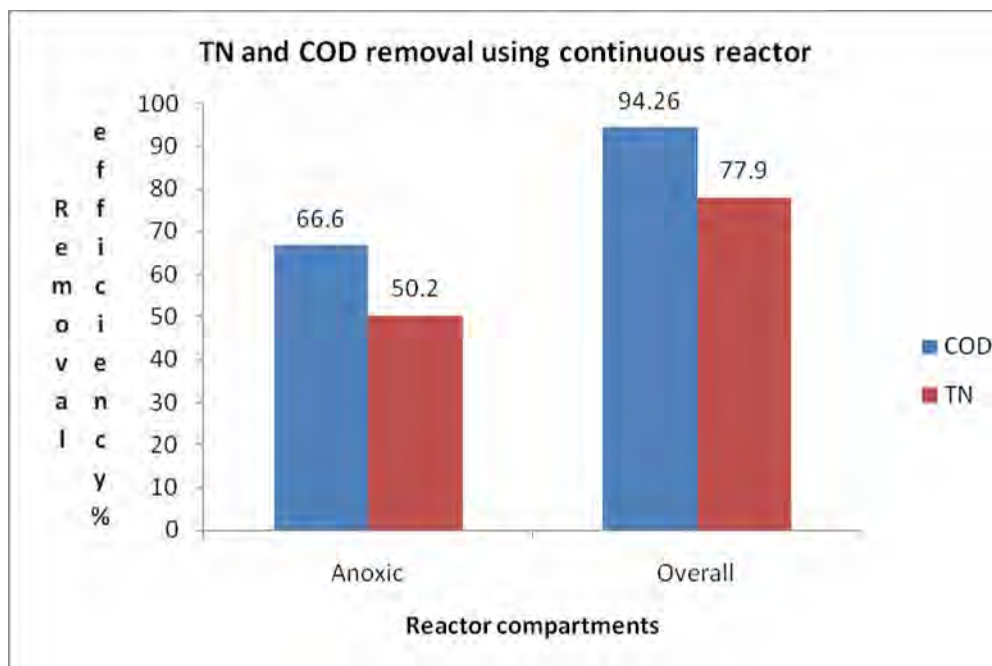


Figure 7. Comparison of COD and Total Nitrogen Removal in an anaerobic and Overall.

4.6. Isolation of isolates from the continuous reactor

isolate S1, which is isolated from the anaerobic reactor were tested for its decolorization efficiency, as a function of time, with various concentration of dyes ranging from 1 mg/L, 2.5 mg/L and 5 mg/L and it shows a pronounced efficiency in decolorizing reactive Red 184 in 48 h (Fig. 4).



Figure 8. RR 184 decolorization using concentrations 1mg/L, 2.5 mg/L and 5mg/L using S1 isolate.

Similarly, the decolorization efficiency of B3 and N5, which were isolated from the aerobic reactor, were tested under aerobic and anaerobic conditions, respectively. Isolate B3 decolorized the dye within four days of incubation in a shaker (data not shown). When the isolate N5 was incubated with the dye under aerobic condition, its decolorization efficiency under this condition was almost negligible. Unlike isolate B3, it was capable of decolorizing the dye in 48 hours of incubation.

In order to test whether the three isolates are able to grow on the decolorized media using it as a sole nitrogen source or not the decolorized medium by isolate S1 under anaerobic condition were centrifuged at 600 g for 10 min and the pH of the supernatant was adjusted to 7.5. And then the

supernatant was autoclaved and placed in to a 50 ml flask. Since the cells already utilized the carbon source during decolorization, glucose was separately autoclaved and added to each flask to a final concentration 0.1 %. The three flasks were inoculated with the three isolates and incubated under aerobic condition. The sole nitrogen source for the isolates was the dye decolorization metabolite, which mainly consist of aromatic amines. Samples were taken every 12 hours and the cell growth (turbidity) was measured at 600 nm (Fig. 5). All the three isolates are able to grow.

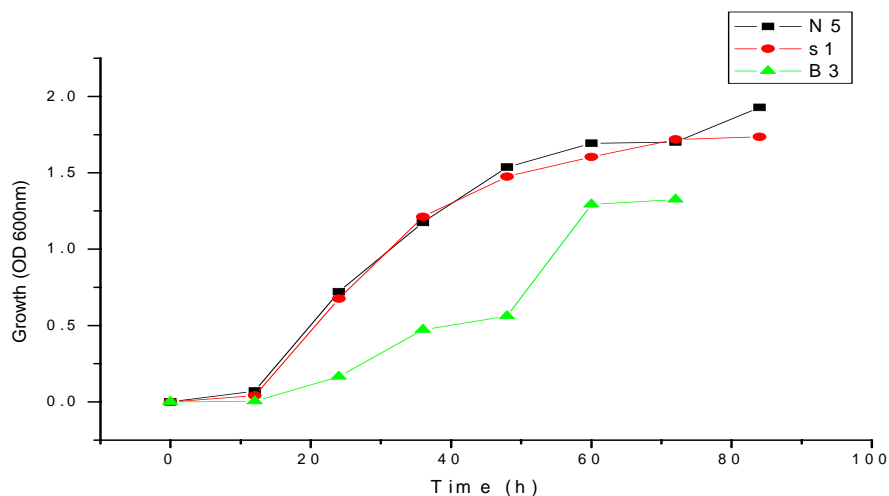


Figure 9. Growth profile of three isolates, N5, S1 and B3 on decolorization product of isolate S1 under aerobic condition.

5. DISCUSSION

In the present study, consortia of alkalophilic microorganisms from Ethiopian Soda Lakes (Chitu and Abijata) are used to decolorize RR 184 in a continuous anaerobic/aerobic reactor system. In the system 100 percent color removal was obtained at the end of the treatment (Table 4).

As the dye was decolorized, the azo bond of Reactive Red 184 serves as a final electron acceptor and cleaved into aromatic amines (Chung and Stevens, 1993). Although color removal is greater at the anaerobic stage, the presence of the anaerobic stage alone is not effective for complete decolorization. If dye reduction is not taking place at the anaerobic stage, it will leave the aerobic stage intact (Van der Zee and Villaverde, 2005). Therefore; for the removal of color as well as for removal of the products that are produced as a result of dye decolorization at the anaerobic stage, a continuous anaerobic/aerobic system is crucial.

In the study, greater color removal efficiency was achieved at the anaerobic stage than the aerobic stage. The color removal efficiency after the anaerobic stage was found to be 96.0 %. On the other hand, the color removal efficiency of the overall was found to be 100 %. The color removal efficiency of the aerobic reactor was only 4%. Thus, for complete color removal, a continuous anaerobic/aerobic system was found to be essential.

Qualitatively, dye decolorization products were identified by High Performance Chromatography (HPLC). The chromatograms of HPLC of the anaerobic/aerobic treatment process showed the formation of new products at the anaerobic stage and oxidation of the product at the aerobic stage. At the anaerobic treatment process a third new peak and another two peaks with a lower retention time and greater peak area were identified. This is due to the presence of one azo bond in RR 184 dye. According to (Pandey, 2006), azo dye reduction is taking place primarily by anaerobic reduction of the azo bond of the dye followed by aerobic oxidation of biodegradation products. The peak areas of the three metabolites decrease to a greater extent. This suggests that continuous anaerobic/aerobic treatment of dye containing effluents is a promising technology for dye decolorization as well as oxidation of decolorization products.

Based on the structure of most of the reactive azo dyes, the prediction is that under anaerobic condition, the products of dye reduction would result in the formation of aromatic compounds (Carvalho, 2007). And the chromatogram of the Thin Layer, showed the presence of two fragmented dyes. This is because the RR 184 under study was a monoazo dye and cleavage of the azo linkage would result in two structurally distinct aromatic amines.

According to the Literature (Sani and Banerjee, 1999), biodegradation of dyes by bacteria could be due to adsorption or biodegradation. After biodegradation, half of the decolorized media was autoclaved and inoculated into another media containing RR 184. The remaining non-autoclaved decolorized media was inoculated into another media containing RR 184. Then the biodegradation was monitored daily both visually and in a UV-Visible Spectrophotometer. In effect it is the non-autoclaved decolorized media showed biodegradation. This is because biodegradation of dyes proceeds primarily by degradation than physical adsorption.

The continuous anaerobic/aerobic reactor system was evaluated for Chemical Oxygen Demand (COD), total nitrogen and ammonia evolution. In the system, COD removal efficiency was 66.6 % at the anaerobic stage and the overall removal efficiency was 94.26 %. This shows that most of the chemicals and the dye are used by the consortia. Similarly, the removal efficiency of total nitrogen increases from 50.2 at the anaerobic stage to 77.9 in the overall. This is because the nitrogen sources in the original dye media were used as a nitrogen source for growth by the consortia. On the other hand, ammonia evolution increases after aerobic treatment than after anaerobic treatment in the continuous reactor system.

During decolorization of RR 184 by consortia in a continuous anaerobic/aerobic system, the pH of the synthetic dye containing wastewater, when it was treated anaerobically and increases slightly (Table. 5). According to (Sponza, 2005) the lowering of pH at the anaerobic stage is due to the formation of acids.

As figure 8 shows, the three isolates (S1, B3, and N5) were able to grow using the decolorized media as a sole nitrogen source and with the supplement of glucose as a carbon source. This shows that decolorization products can be further degraded into simpler molecules

6. CONCLUSIONS AND RECOMMENDATIONS

6.1. Conclusion

Treatment of synthetic wastewater containing azo dye, RR 184 by consortia of alkalophilic microorganisms was conducted under a continuous anaerobic/aerobic system. Biodegradation efficiency of the system and analysis of the degradation products was studied.

During the treatment, most of the color was removed at the anaerobic stage (96 %) than at the aerobic stage (4 %). Ultimately, a 100 % color removal efficiency was attained in the over anaerobic/aerobic system. In addition to color removal efficiency, Chemical Oxygen Demand (COD) and Total Nitrogen (TN) removal efficiency was studied. In the study a 66.6 % and 50.2 % COD and TN removal efficiency were obtained after anaerobic treatment respectively. In the overall treatment system, 94.26 % and 77.9 % COD and TN removal efficiency were obtained respectively.

After biodegradation of RR 184 in a continuous anaerobic/aerobic system, the intermediate products were studied by High Performance Liquid Chromatography (HPLC) and Thin Layer Chromatography (TLC). From HPLC analysis, it has been found that, the intermediate products which were appeared in the anaerobic treatment were treated at the aerobic reactor. Hence, a continuous anaerobic/aerobic system was found to be efficient in removing most of the biodegradation products. TLC analysis has shown that the consortia were able to degrade the dye under study in to two fragments.

The efficiency of a single isolate for color removal isolated from a continuous anaerobic/aerobic system containing consortia of alkalophilic microorganisms was evaluated in a batch test. In the study, isolate S1 has shown a 100 % color removal after 48 h of incubation. Similarly, the efficiency of three isolates from the continuous reactor were evaluated whether they can utilize the decolorized media as a sole nitrogen source or not. During the study, all the three isolates have shown growth.

6.2. Recommendations

1. Since the result of this study relies on a synthetic wastewater containing a single dye, further study on treatment real textile wastewater containing a variety of dyes should be done
2. Although decolorization was achieved in a combined anaerobic sequential system, the fate and mechanism of degradation of the decolorization product, aromatic amines, is not studied. Thus, future attention should be given.
3. The amount of aromatic amines produced in a continuous anaerobic system was not studied. Thus, future focus must be given to quantify the total aromatic amines produced.
4. The relation between dye structure and dye degradation also requires future attention.

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DECLARATION

I, the undersigned, hereby declare that this thesis is my original work; it has not been presented in other University, College or Institutions seeking for similar degree or other purposes. All sources of material used for the thesis have been duly acknowledged.

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