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Isolation and Evaluation of Azo Dye Decolorizing Microorganisms from Ethiopian Alkaline Soda Lakes

By

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LIST OF ABBREVIATIONS

BOD	Biological Oxygen Demand
COD	Chemical Oxygen Demand
DO	Dissolved Oxygen
MSM	Mineral Salts Medium
RR 184	Reactive Red 184
TDS	Total Dissolved Solids
TSS	Total Susspended Solids

ABSTRACT

Textile dye decolorizing microorganisms were isolated from Ethiopian alkaliphilic Soda lakes. A total of 121 morphological distnict colonies were isolated from mineral salts medium-agar plate. When these isolates were subjected to confirmed decolorizer and non-decolorizer using mineral salts media containing 10 mg/l of Reactive Red 184, 95 isolates were found to be decolorizer while the rest were non-decolorizer. By selecting 25 isolates decolorizing the dye rapidly, further screening were carried out using 25 mg/l of Reactive Red 184. Among the 25 isolates, 8 isolates which decolorized the dye $\geq$ 80% in the first 24 h were selected and subjected to further screening using various concentration(50-150 mg/l) of Reactive Red 184. A55 which decolorized $\geq$ 82 % of the tested dye concentration in one day was selected as an efficient isolate for further studies. The effect of nutrient composition (carbon and nitrogen), culturing condition (anaerobic, anoxic, shaker, and aerobic), dye concentration and dye type on decolorization of Reactive red 184 was evaluated using isolate A55. Maximum extent of decolorization at the end of the fourth day was observed when carbon (98-100%) and organic nitrogen (100%) sources were used in the medium. The presence of sodium nitrate inhibited decolorization activity ranging from 1-29 % through four consecutive days of incubation. Decolorization was efficient (100%) in anaerobic and anoxic cultures. However, A55 showed good growth in aerobic and agitated culture, color removal was strongly inhibited. The decolorizing activity was found to decrease with increasing dye concentration from 50 - 250 mg/l in the first day, but at the end of the fourth day almost equal percentage of decolorization was observed. The isolate was found to decolorize a total of seven dyes ranging from 51-100 % at the fourth day of incubation.

Keywords: alkaliphilic, decolorization, decolorizing microorganism, Reactive Red 184 textile dye

1. INTRODUCTION

A lot of chemicals including dyes are manufactured and used in day-to-day life activity because of rapid industrialization and urbanization, (Sathiya, 2007). Textile industries, particularly those involved in dyeing and finishing processes are the major dyestuff consumers and causes considerable water pollution. However, textile wastewaters are characterized by various fluctuating parameters such as Chemical Oxygen Demand (COD), Biochemical Oxygen Demand (BOD), pH, temperature and color, the later which is resulted from dyes are the most undesirable contaminant (Dos Santos, 2005).

Dyes are either natural or synthetic aromatic compound, structurally consisting chromophore and auxochrome. As synthetic dyes designed to be chemically and photolytically stable, they are highly persistent in natural environments (Ramalho, 2005). Due to persistent aromatic structure and recalcitrant nature of various dyes, the disposal of colored wastewater creates not only aesthetically displeasing, but also impede penetration of sun light and causes depletion of dissolved oxygen into natural water bodies which seriously affects the biota. Moreover, there are considerable evidences that certain anaerobic metabolites of dyes are being toxic, carcinogenic and mutagenic agents of some microorganisms, aquatic life and human being (Tan, 2001;Weisburger, 2002). All these points converge to indicate the need of treating textile wastes before discharge to water bodies.

A wide range of methods used to treat textile effluent including biological, physical and chemical. However, the phsio-chemical methods are technically feasible for treatment of synthetic dyes in textile effluent, they have inherent drawbacks as high operative cost, formation of hazardous byproducts and intensive energy requirements (Kim & Shoda, 1999; Ramya *et al.*, 2007). As a viable alternative, biological treatment method have received increasing interest owning to their effectiveness, ability to produce less sludge and ecofriendly nature (Chen *et al*., 2003; Jang *et al.*, 2004). Biological processes have the

potential to convert or degrade the pollutant into water, carbon dioxide and various salts of inorganic nature (Stolz, 2001; Daneshvar *et al.*, 2006). Such a conversion fate of environmental pollutants are largely determined by metabolic activities of microorganisms (Ramlho, 2005).

Microorganisms have a wide range of catabolic enzymes that act on a wide range of natural and synthetic substances. Aerobic and anaerobic microorganisms have been involved in biodegradation of dyes (Tan, 2001; Dos Santos, 2005; Zille, 2005; Bhatt *et al.*, 2005). These authors mentioned that, isolation of such microorganism that are capable of decolorizing dye efficiently have great significance in both developed and developing countries. Ethiopia is a developing country having more than fourteen major state-owned and private textile and garment factories that discharge their untreated colored and toxic effluent into the nearby rivers, lakes and streams (Dierig, 1999). So, developing a system of cost effective and efficient treatment method is important in the country.

1.1. Objectives of the Study

General Objectives

The overall objective of this study was to isolate and evaluate azo dye decolorizing microorganisms from Ethiopian alkaliphilic Soda Lakes.

Specific Objectives

1. To isolate microorganisms from Reactive Red 184 (RR 184) enriched culture.
2. To screen potential microorganisms involved in decolorizing RR 184.
3. To evaluate the effect of various culture conditions on decolorization activity of the best isolate.
4. To evaluate decolorization of various textile dyes using the most efficient isolate.

2. LITERATURE REVIEW

2.1. Characteristic of Textile Wastewater

Industrialization is one of the sector contribute to the development of a nation's economy. By far, textile industry is the most important and largest industrial sector which contributes to income generation (Olukanni *et al*., 2006). It is found in most countries and their numbers have increased (Miao, 2005). However, these increments exert a strong problem on the surrounding environment regarding to effluent discharge and water consumption. Considering discharge of wastewater generated, textile industry rated as the most polluting among all industrial sectors (Delee *et al*., 1998; Vandevivere *et al*., 1998; Robinson *et al*., 2001). It is estimated that textile industry uses an average of 100-500 liter of water to produce one kilogram of fabric (Arafat, 2007).

A typical textile processing unit generates different types of wastewater, which differs in magnitude and quality. The wastewater contain several types of chemicals and dyes. The discharge of these wastes in to water body changes the COD, BOD, Total Suspended Solid (TSS), Total Dissolved Solid (TDS), pH, temperature , and color of the water (Delee *et al*., 1998; Robinson *et al*., 2001; Al-kdasi *et al*., 2004; Olukanni *et al*., 2006). Among many contaminants present in textile wastewater, color is considered the most undesirable. It is caused by dyes which are resulted from textile dyeing process (Daneshvar *et al*., 2006). Dyeing is one of the key activities in a typical textile processing set-up (Dos Santos, 2005). A brief description of the nature of wastewaters generated from common textile processes such as sizing, desizing, scouring, bleaching, mercerizing, dyeing and finishing (Figure 1) are presented as follows:

Sizing: is the first preparation step in which sizing agents such as starch or starch-derivatives, polyacrylates and polyvinyl alcohol (PVA) and carboxmethyl cellulose are added to provide strength to the fibers and minimizing breakage (Dos Santos, 2005; Arafat, 2007).

Desizing: involves the removal of sizing agent from the cotton fabric using enzymes, acid or alkali. Thus, desizing effluent generally has a high organic load and is characterized by high COD and BOD resulting from starch used during sizing process (Arafat, 2007). This stage is also characterized by high TSS and TDS values (Dos Santos, 2005).

Scouring*:* Subsequent to desizing, any remaining natural impurities (i.e. organic components other than cellulose) are scoured from the fabric by a process of prolonged boiling in alkaline solutions. Wastewater from this process usually contain sodium hydroxide and different kinds of anionic detergents (Carliell, 1993; CPP, 2002). It is characterized by a high COD and pH values, and a strong yellow-brown color (Carliell, 1993; Dos Santos, 2005).

Bleaching*:* The purpose of bleaching is to decolorize natural pigments (flavonoids) and to confer a pure white appearance to the fibres. The most common industrial bleaching agent is hydrogen peroxide, which is usually applied at alkaline pH and temperatures close to boiling (Couto and Toca-Herrera, 2006). Therefore, the wastewater from bleaching process is low in BOD and COD, but high in TDS and pH (Dos Santos, 2005).

Mercerizing: is a continuous chemical process used to increase dyeing ability, luster and fibre appearance. In this step, a concentrated alkaline solution (sodium hydroxide) is applied for treating cellulosic fibers (CPP, 2002). So, effluent from this process is characterized by high pH values (Dos Santos, 2005).

Dyeing: is the process of adding color to the fibres for increasing product value. Depending on the dyeing process, a wide range of dye classes and chemicals like metals, salts, surfactants, organic processing assistants, sulphide and formaldehyde are added to improve dye adsorption onto the fibres (Dos Santos, 2005). Wastewater in this process is characterized by intense colored and high temperature, BOD, COD, and TDS values. Some of the dyes also contribute to the complexes of toxic metals, like chromium and copper (CPP, 2002).

Finishing: refers to any processes used to improve the quality of the fabric after dyeing (Carliell, 1993). Almost all the finishing processes consume extensive energy but their contribution towards wastewater generation is not significant (CPP, 2002).

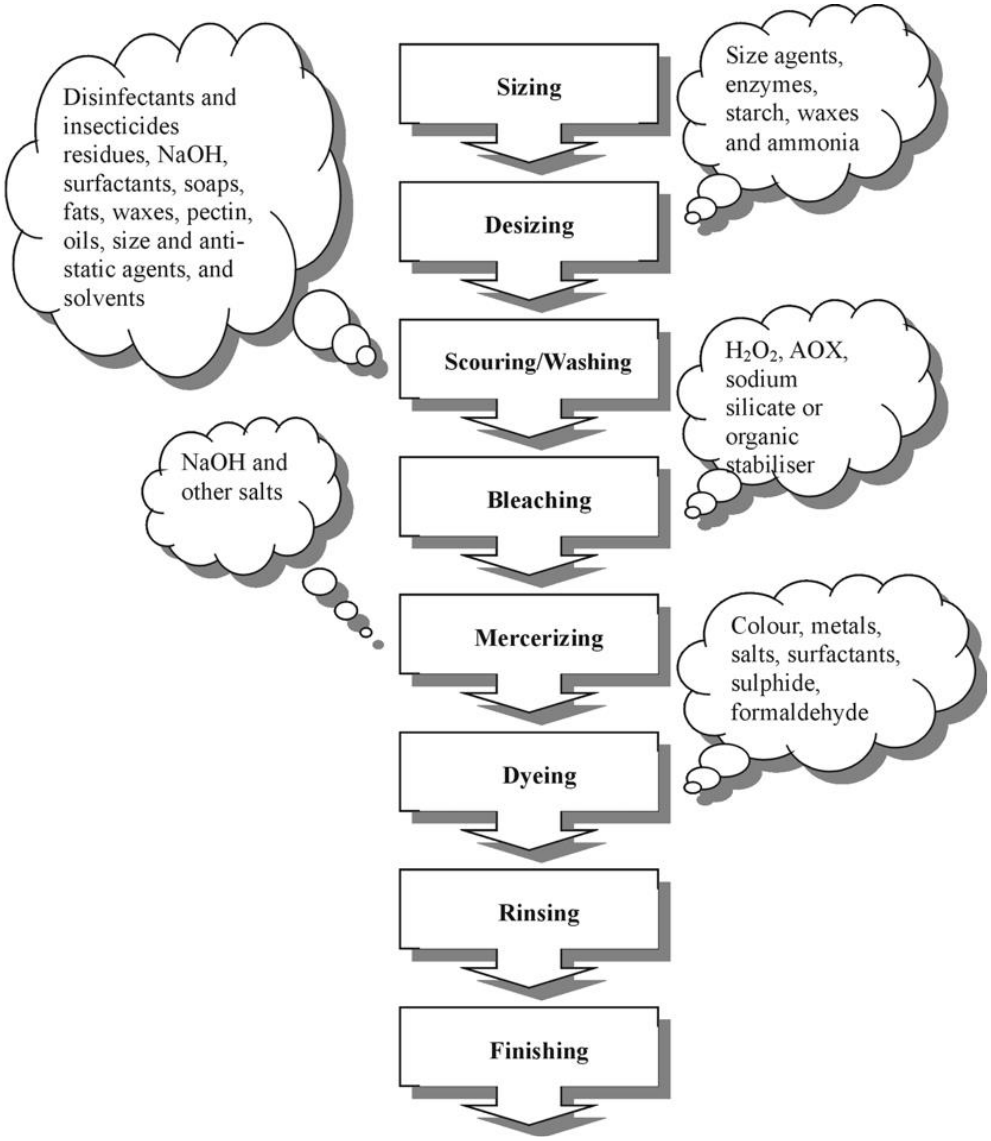


Figure 2.1. Schematic of operations involved in textile cotton industry and pollutants from each step.

2.2. Dye History and Classification

2.2.1. History of Dyes

Ever since the beginning of mankind, people have been using colorants for painting and dyeing their surroundings, their skins and their clothes. The first evidence of using colorant materials by man goes back to 15000-9000 BC, in the walls of the Altamira cave in Spain (Ramalho, 2005).

Formerly, colorants were obtained from animal and vegetable sources (Dos Santos, 2005; Ramalho, 2005). In 1500 BC, tyrian purple which was extracted from animals (molluscs) was used by Phoenicians. The pigment itself is not in the mollusc; however, when the precursor is extracted, it can be converted in to the dye by air or light (Zollinger, 1991; Clark *et al.*, 1993). The well known plant dye indigo, which was extracted from *Indigofera tinctoria* has been used since 3,000 BC (Clark *et al.*, 1993). Unlike to tyrian purple, indigo is still in use prepared other than plant source.

Up to the end of the nineteenth century, natural dyes obtained from plant parts are used to colorants for textile dyeing process. During dyeing process, they require several steps and their source diversity leads to application problem. For this reason, since the beginning of the 20 th century, synthetic dyestuffs had almost completely replaced natural dyes (Vander Zee, 2002; Ramalho, 2005).

The first synthetic dye ‘Mauve’ (aniline), a brilliant fuchsia color, was discovered by an Englishman, William Henry Perkin in 1856. His purpose was searching quinine for the cure for malaria, but the dye was accidentally synthesized (Zollinger, 1991). Since 1858, the German scientist P. Gries discovered the reaction mechanism of dyes (called diazotization); considerable numbers of new dyes were synthesized annually. At the moment, over 100, 000

dyes have been generated worldwide with an annual production of $7x10^5$ metric tones (Zollinger, 1991; Soares *et al.*, 2004). These dyes have a potential application in different industries, including the textile, leather, paper, pharmaceutical, plastic, paint, photograph, cosmetic, hair and food industries (Zollinger, 1991; Tan, 2001; Daneshvar *et al*., 2006). Among these industries, textile industry accounts for two-thirds of the total dyestuff market (Riu *et al*., 1998).

2.2.2. Dye Classification

Dyes are aromatic compound capable of coloring fabrics. Fundamentally, they are classified based on their chemical structures and method of application (Rastogi *et al.*, 1972).

Structurally, dyes consist of auxochromes and chromophores (Figure 2.2), which is together called chromogen (Rastogi *et al.*, 1972; Zollinger, 1991; Dos Santos, 2005). Auxochromes are electron-withdrawing or donating group, of which the most important ones are amine ($-NH_3$), carboxyl ($-COOH$), sulfonate ($-SO_3H$) and hydroxyl ($-OH$) (Dos Santos, 2005). On the other hand, chromophores have a delocalized electron system with conjugated double bonds having with drawing nature of electrons. The azo ($-N=N-$), carbonyl ($-C=O$), methine ($-CH=$), nitro ($-NO_2$) and quinoid groups are the most known chromophores (Vander Zee, 2002).

The first classifications of dyes based on chromophores are started in 1876 (Rastogi *et al.*, 1972). Based on chromophore, dyes are categorized as homocyclic (azo, nitroso, methine, anthraquione and quinoemine) and heterocyclic (indigoid, sulphur, and phthalocyanine) dyes.

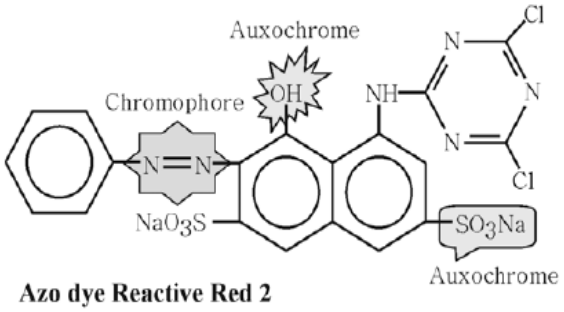


Figure 2.2. Examples of dye: auxochromes and chromophore for azo dye

A second classification of dyes are based on their mode of application to industries. The Color Index (C.I) distinguishes about 15 different application classes such as: acid, reactive, metal complex, direct, basic, mordant, disperse, pigment, vat, azoic and ingrain, sulfur, and solvent dyes (Ramalho, 2005). Various attractive forces have the potential of binding these categories of dyes to fibres. The types of bonds established between the dye and the fibre can be Van der Waals, hydrogen, ionic or covalent type (Table 2.1).

In structural and application category, classifying dyes as an azo group belong to one of the above classes. Azo dyes are a compound containing azo groups (─N═N─) which mainly forms a bond to benzene or naphthalene rings. They are the most widely used among synthetic dyes, representing almost 70% of the textile dyestuffs produced (Zollinger, 1991; Dos Santos, 2005). This enormous amount of azo dye using in textile and dyeing industries are due to their ease and cost effectiveness in synthesis, firmness and variety in color compared to that of natural dyes (Sathiya *et al.*, 2007; Seesuriyachan *et al.*, 2006).

Due to their chemical structure (chromophore), azo dyes absorb light in the visible range of wavelengths from 400-750 nm (Rastogi *et al.*, 1972; Vander Zee, 2002). Wavelengths smaller than that of violet (called Ultraviolet) and of wavelengths greater than that of red(called Infrared) is invisible (Table 2.2).

Table 2.1: Application categories of dyes (Ramalho, 2005).

Type of dye	Characteristics	Substrates
Acid	When in solution are negatively charged: bind to the cationic NH^{+3}- groups present in fibres	Nylon wool, polyamide, silk, modified acryl, paper, inks and leather
Reactive	Form covalent bonds with OH^-, NH^- or SH^- groups	Cotton, wool, silk and nylon
Metal complex	Strong complexes of one metal ion (usually chromium , copper, cobalt or nickel) and one or two dye molecules (acid or reactive)	Silk, wool and polyamide
Direct	Large molecules bound by Van der Waals forces to the fibre	Cellulose fibres, cotton, viscose, paper, leather and nylon
Basic	Cationic compounds that bind to the acid groups of the fibre	Synthetic fibres, paper and inks
Mordant	Require the addition of a chemical that combines with the dye and the fibre, like tannic acid , alum, chrome alum and other salts of aluminum, chromium, copper, iron, potassium and tin	Wool , leather, silk, paper, modified cellulose fibres and anodized aluminum
Disperse	Scarcely soluble dyes that penetrate the fibre through fibres swelling	Polyester, polyamide, acetate, acrylic and plastics
Pigment	Insoluble, non-ionic compounds or insoluble salts that retain their crystalline or particulate structure throughout their application	Paints, inks, plastics and textiles
Vat	Insoluble colored dyes which on reduction give soluble colorless forms (leuco form) with affinity for the fibre; on exposure to air are re-oxidized	Cellulose fibres, cotton, viscose and wool
Azoic and ingrain	Insoluble products of a reaction between a coupling components and a diazotized aromatic amine that occurs in the fibre	Cotton, viscose, cellulose acetate and polyester
Sulphur	Complex polymeric aromatics with heterocyclic s- containing	Cellulose fibre, cotton and viscose
Solvent	Non ionic dye that dissolve the substrate to which thy bind	Plastics, gasoline, vamish, lacquer, stains , inks, oils, waxes and fats
Fluorescence brighteners	Masks the yellowish tint of natural fibres	Soaps and detergents, all fibres, oils, paints and plastics
Food	Non- toxic and not used as textile dyes	Food
Natural	Obtained mainly from plants	Food, cotton, wool, silk, polyester, polyamide and polyacrylonitrile

Table 2.2: Regions of the electromagnetic spectrum and relationship between wavelengths and color (Rastogi *et al.,* 1972)

Electromagnetic region	Wavelength (nm)	Color perception
Ultraviolet	< 350	Nd
Visible light	350-400	Nd
	400-435	Violet
	435-480	Blue
	480-490	Greenish –blue
	490-500	Bluish-green
	500-560	Green
	560-580	Yellowish-green
	580-595	Yellow
	595-605	Orange
	605-750	Red
	750-780	Nd
Infrared	>780	Nd

Nd = Not detected by the eye.

2.3. Environmental Impact of Dyes

Wastewaters generated from various industries create the major environmental detrimental effects leading to imbalance of the bio-system (Somasiri *et al*., 2006). As mentioned so far, textile industry, which is the largest water consumer in the world, produces the wastewater comprising of various recalcitrant agents such as dyes, sizing agents, and dyeing aids. Dyes used in dyeing process are the most environmental problematic recalcitrant agent (Ramalho, 2005; Sathiya *et al.,* 2007). As dyes are designed to be chemically and photolytically stable, they are highly persistent in natural environments (Vander Zee, 2002). For instance, the half-life of hydrolyzed Reactive Blue 19 is about 46 years at pH 7 and 25 ºC (Hao *et al.,* 2000).

During textile processing, inefficiencies in dyeing result in large amounts of the dyestuff being directly lost to the wastewater, which ultimately finds its way into the environment. Approximately 10-15 % of the dyes are released into the environment during manufacturing and usage and then causes visual pollutants (Robinson *et al*., 2001). Many dyes are visually detected in water at concentrations as low as 1 mg/l (Vander Zee, 2002). It is estimated that dye concentration in textile effluent ranging from 10-200 mg/ l is highly colored (O'Neill *et al.,* 1999).

The uncontrolled release of these colored compounds in the environment causes aesthetic displeasing, impedes light penetration, decrease dissolved oxygen, damage the quality of the receiving stream and become toxic to treatment process, to food chain organisms and aquatic life (Hao *et al*., 2000; Tan, 2001; Somasiri *et al.,* 2006). Further more, degradation products of certain dyes are investigated and proven to be toxic, carcinogenic and mutagenic (Tan, 2001;Weisburger, 2002). The studies were made using bacteria, algae, fish and mammals. As tests indicate that the most acutely toxic dyes for algae are cationic dyes while for fish basic dyes, especially those with a triphenylmethane structure (Vander Zee, 2002).

The acute toxicity of azo dyes, as defined by the EU (Europian Union) criteria for the classification of dangerous substances is low. Mortality tests with rats showed that only a few azo dyes showed LD50 (Lethal Dose that kills half of the tested population) values below 250 mg / kg body weight (Van der Zee, 2002). However, occupational sensitization to azo dyes has been seen in the textile industry since 1930 (Ramalho, 2005), especially disperse dyes with monoazo or anthraquinone structures have been found to cause allergic reactions, i.e. eczema or contact dermatitis (Wallace, 2001).

Following oral exposure, azo dyes are metabolized to aromatic amines by intestinal microflora or liver azoreductases. After azo dye reduction in the intestinal tract, the released soluble aromatic amines are absorbed by the intestine and excreted in the urine, but those

derived from aniline, toluene, benzidine and naphtalene have been shown carcinogenic properties, especially bladder cancer (Vander Zee, 2002; Ramalho, 2005). Vander Zee (2002) noted that, the carcinogenicity mechanism probably includes as formation of acyloxy amines through N-hydroxylation and N-acetylation of the aromatic amines followed by O-acylation. These acyloxy amines can be converted to nitremium and carbonium ions that bind to DNA and RNA, which induces mutations and tumour formation.

2.4. Dye Treatment Methods

As stated so far, the textile industry is the most extreme user of synthetic dyes need an ecologically efficient solution for its colored effluents **(**Mechichi *et al.*, 2006**)**. Color is the first contaminant to be recognized in wastewater and has to be removed before discharging into water bodies (Miao, 2005; Ramalho, 2005). The removal of color is often more important than the removal of the soluble colorless organic substances, which usually contribute the major fraction of the BOD (Banat *et al.*, 1996; Miao, 2005). Methods for the removal of organic substances from most effluents are fairly well established in conventional Waste Water Treatment Plants (WWTP) (Vandevivere *et al*., 1998; Robinson *et al.*, 2001). However, treatments of dye in wastewaters by established WWTP processes are more difficult because of their synthetic origin and complex aromatic molecular structures (Miao, 2005).

In order to decrease the impact of textile effluents on the environment, non-biological (physico-chemical) and biological methods has been developed (Dos Santos, 2005). Biological and chemical methods involve the destruction of the dye molecule, whilst physical methods usually transfer the pollutant to another phase (Vandevivere *et al*., 1998; Robinson *et al*., 2001; Ramalho, 2005). According to Vander Zee (2002), when an individual dye removal method is designed, factors such as dye type, wastewater composition, dose and

costs of required chemicals, operational costs (energy and material) and environmental fate and handling costs of generated waste products should be carefully considered.

2.4.1. Non-biological Dye Treatment Methods

The two major non-biological color removal techniques are chemical and physical methods (Dos Santos, 2005). Chemical methods include Fenton's reagent, ozonation, Photochemical, Sodium hypochlorite (NaOCl) and Electrolysis (Dos Santos, 2005). Some of these methods involve the use of an oxidizing agent such as hydrogen peroxide for Fenton's reaction and ozone for ozonation (Ramalho, 2005). When these oxidizing agents are used in decolorization purpose, $HO^{\bullet}$ radicals are formed and increase the rate of chemical composition of the dye.

During Fenton's reaction, hydrogen peroxide is added in an acid solution (pH 2-3) containing Fe^{+2} ions [$Fe^{+2} + H_2O_2 \rightarrow Fe^{3+} + HO^{\bullet} + HO$]. In comparison with ozonation, this method is relatively cheap and also presents high COD removal and decolorization efficiencies (Vander Zee, 2002). In the ozonation process, hydroxyl radicals are formed when O_3 decomposes in water [$H_2O + O_3 \rightarrow HO^{\bullet} + 2\ O_2$]. However, ozone is a very good oxidizing agent due to its high instability (reduction potential 2.07 V) than to chlorine (1.36 V) and H_2O_2 (1.78 V), using it in the process has short half-life (20 minute) which requires continuous ozonation and making this method expensive to apply (Robinson *et al.*, 2001).

As a physical method, removal of dyes from wastewater be achieved by adsorption, coagulation, filtration, flotation and ion exchange (Table 2.3). Adsorption holds promise in the treatment of wastewater owning to simply design, easy to handle and provides sludge-free cleaning operations. However, peat, wood chips, fly ash and brown coal have been used as dye adsorbents; activated carbon has long been used in industry as a standard adsorbent for removing color (Robinson *et al*., 2001). Although, adsorption can efficiently decolorize textile effluents, its application has been limited by the high cost of adsorbents and the large volume of wastewater normally involved.

However, the phsio-chemical methods are technically feasible for treatment of synthetic dyes in textile effluent, they have inherent drawbacks as high operative cost, formation of hazardous byproducts and intensive energy requirements (Table 2.3) (Kim & Shoda, 1999; Ramya *et al.*, 2007). As a viable alternative, biological treatment method have received increasing interest owning to their effectiveness, ability to produce less sludge and ecofriendly nature (Chen *et al*., 2003; Jang *et al.*, 2004).

Table 2.3: Advantages and drawbacks of some non-biological decolorization processes applied to textile wastewaters (Robinson *et al*., 2001).

Physical/ Chemical Methods	Method description	Advantages	Disadvantages
Fenton reagents	Oxidation reaction using mainly H_2O_2-Fe (II)	Effective decolorization of both soluble and insoluble dyes	Sludge generation
Ozonation	Oxidation reaction using ozone gas	Application in gaseous state : No alternation of volume	Short half- life (20 min)
Photochemical	Oxidation reaction using mainly H_2O_2-UV	No sludge production	Formation of by-products
NaOCl	Oxidation reaction using CL^+ to attack the amino group	Initiation and acceleration of azo- bond cleavage	Release of aromatic amines
Electrochemical destruction	Oxidation reaction using electricity	Breakdown compounds are non-hazardous	High cost of electricity
Activated carbon	Dye removal by adsorption	Good removal of a wide variety of dyes	Very expensive
Membrane filtration	Physical separation	Removal of all dye types	Concentrated sludge production
Ion exchange	Ion exchange resin	Regeneration: no adsorbent loss	Not effective for all dyes
Electrokinetic coagulation	Addition of ferrous sulphate and ferric chloride	Economically feasible	High sludge production

2.4.2. Biological Dye Treatment Methods

Biological processes have the potential to convert or degrade the pollutant into water, carbon dioxide and various salts of inorganic nature (Stolz , 2001; Daneshvar *et al.*, 2006). Such a conversion fate of environmental pollutants are largely determined by metabolic activities of microorganisms (Ramlho, 2005). It was reported that, microorganisms have a wide range of catabolic enzymes that act on a wide range of natural and synthetic substances (i.e., dyes). As a result, a number of studies have focused on biological treatment of dyes using various microorganism such as bacteria and fungi (Abd El-Rahim *et al.*, 2003; Chen *et al.*, 2003; Sharma *et al.*, 2004; Bhatt *et al.*, 2005; Ramya *et al*, 2007).

2.4.2.1. Bacteria

Investigations to bacterial dye biotransformation have so far mainly been focused to the most abundant chemical class, azo dyes (Vander Zee, 2002). The ability of bacteria to metabolize azo dyes has been investigated by a number of research groups (Abd El-Rahim *et al.*, 2003; Chen *et al.*, 2003; Sharma *et al.*, 2004; Bhatt *et al.*, 2005). The bacterial metabolism of azo dyes is initiated in most cases by a reductive cleavage of the azo bond, which results in the formation of aromatic amines (Yoo, 2000). Aerobic and anaerobic bacteria involved in biodegradation of dyes (Tan, 2001; Dos Santos, 2005; Zille, 2005).

Although for a long time it was thought that azo dyes remained recalcitrant under aerobic conditions, some specific aerobic bacterial cultures are able to reduce the azo linkage via an enzymatic reaction (Tan, 2001). When the bacteria acclimatized long-term aerobic condition in the presence of simple azo compound, they synthesize an azoreductase specifically corresponding to the azo compound added (Stolz, 2001). In such a way (an extended period of acclimatization) researchers isolated bacteria that have the potential to aerobically biodegrade azo dyes including, *Pseudomonas* species strains KF46 and K22 (Zimmermann *et*

al., 1982; Zimmermann *et al*., 1984), *Xenophilus azovorans* KF46F (Blumel *et al.*, 2002**),** *Pagmentiphaga kullae* K24 (Blumel and Stolz, 2003), *Alcaligenes* sp. and *Aeromonas* sp. (Sharma *et al.*, 2004), *Bacillus firmus* (Arora *et al.*, 2007).

Under aerobic conditions low color removal efficiencies (10-30%) (Dos Santos, 2005) are achieved because oxygen is a more effective electron acceptor than azo dyes (Stolz, 2001). In a screening of redox potential values for different azo dyes, it was found that E_0 values are generally between –0.430 and –0.180 V (Dos Santos, 2005) (Figure 2.3).

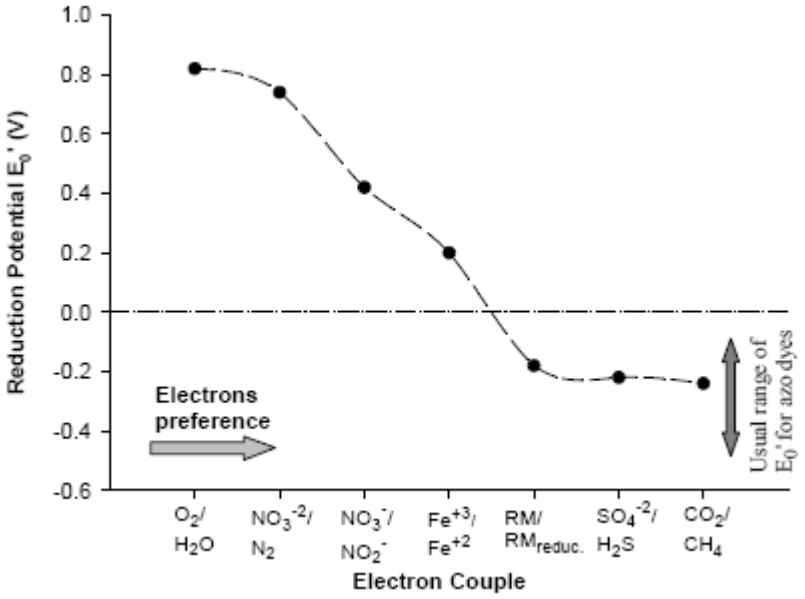


Figure 2.3. Electron flow preference as a function of the different electron couples (Dos Santos, 2005).

Unlike to aerobic biodegradation, azo dyes are readily decolorized in anaerobic conditions and forms two aromatic amines which do not absorb light in the visible spectrum. As a result, dye reduction represents a reductive decolorization (Haug *et al.*, 1991; Donlon *et al.*, 1997; Robinson *et al.*, 2001; Stolz, 2001; Vander Zee, 2002; Zille, 2005).

The exact mechanism of the anaerobic azo dye reduction is not clearly understood yet (Yoo, 2000). However, the term azo dye reduction may involve different mechanisms or locations

like enzymatic (Rafii *et al.*, 1990), non-ezymatic (Gingell and Walker, 1971), mediated (Kudlich *et al.*, 1997; Vander Zee, 2002), intracellular (Mechsner and Wuhrmann 1982) and extracellular (Carliell *et al.*, 1995).

According to the existing enzymatic anaerobic decolozation hypotheses, azo dyes are metabolized by unspecific, soluble, cytoplacmic enzyme called azoreducate (Rafii *et al.*, 1990; Yoo, 2000; Vander Zee, 2002). A precondition for anaerobic reduction of azo dyes is the presence and availability of organic substrate which serve as an electron donor for the azo dye reduction (Nigam *et al.*, 1996; Tan, 2001). Organic substrates like glucose, hydrolyzed starch, yeast extract, acetate, butyrate and propionate are used as electron donors for azo dye reduction (Tan, 2001; Vander Zee, 2002). Under anaerobic condition, azo dyes accept four electrons from these organic substrates and changed in to two aromatic amines in which the dye is originally synthesized [Figure 2.4 (a)] coupled to an ATP (Adenosine Triphosphate) gain. However, in the absence of azo dye, fermentative bacteria convert organic substrates in to alcohol and acetate using the formed acetyl-CoA as terminal electron acceptors [Figure 2. 4 (b)] (Yoo, 2000).

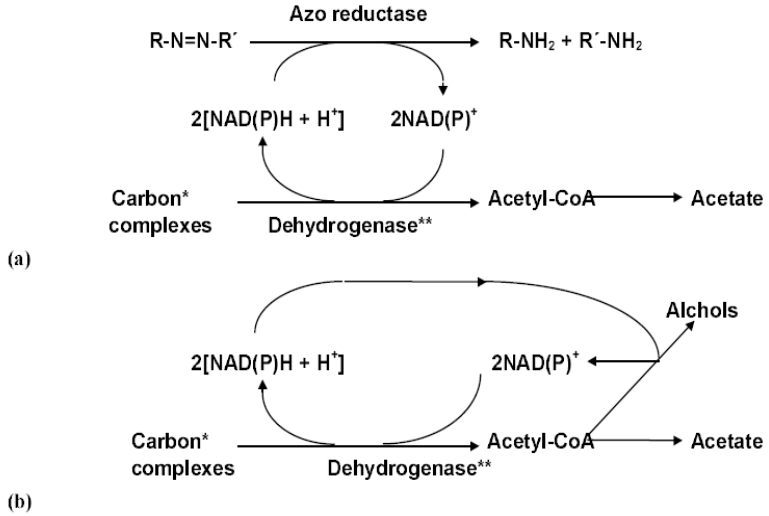


Figure 2. 4. (a) Hypothesis for anaerobic decolorization of azo dyes (R-N=N-R) by azo reductase and (b) general fermentation pathway without azo dyes:

R-NH2 and R´-NH2 are metabolites as the reduction products of the dyes. *=Glucose; **= Enzyme transmitting electrons (or e^- + H^+) from the carbon complexes.

As stated so far, anaerobic bacteria metabolize azo dyes in to the respective amines in which they are synthesized. The further mineralization of the formed amines are achieved using aerobic bacteria. This is why there are several studies that propose a combined anaerobic-aerobic system for the removal of dyes from wastewaters with bacterial consortium/sludge (Lourenco *et al.,* 2001; Tan, 2001; Libra *et al.,* 2004; Rahel Muche, 2007). Utilization of consortia offers considerable advantages over the use of pure cultures in the degradation of synthetic dyes. The reason is that the individual strains may attack the dye molecule at different positions or may use the decomposition products produced by another strain for further decomposition (Tan, 2001). However, the composition of mixed cultures may change during the decomposition process interfering with the control of the system (Ramalho, 2005).

2.4.2.2. Fungi

Like bacteria, fungi are also involved in biotransformation of azo dyes to various products. Currently, decolorization of dye wastewater by fungal metabolic activities is the subject of many studies (Miao, 2005).

Fungi from the Basidiomycetes group, known as white- rot fungi are a heterogonous group of microorganisms but have in common the capacity to degrade lignin as well as a wide variety of recalcitrant pollutants including various types of dyes (Vander Zee, 2002). Since degrading of dyes by the white-rot fungi was first reported in 1983, white-rot fungi have been the most widely studied, dye decolorizing microorganisms (Vander Zee, 2002; Ramalho, 2005; Yang, 2005). The most widely studied dye decolorizing white-rot fungi are like *Phanerochaete chrysosporium (*Glenn and Gold, 1983), *Trametes versicolor (*Swamy, 1998), *Coriolus versicolor (*Kapdan *et al.,* 2000), and *Bjerkandera adust* (Field *et al.,* 1992).

Several other non-white-rot fungi such as *Aspergillus niger* (Abd El-Rahim *et al*, 2003), *Geotrichum candidum* (Kim and Shoda, 1999), *Pleurotus ostreatus* (Palmieri *et al*., 2005) and *Cunninghamella elegans* (Cha *et al.,* 2001) have been investigated for successfully decolorization and mineralization of various dyes.

According to their life state, fungal decolorization can be classified as biodegradation and biosorption (Lacina *et al.*, 2003). While biodegradation refers to living cell activity, biosorption stands for dead cells or biomass (Miao, 2005). According to Dos Santos (2005), the uptake or accumulation of dyes (called biosorption) is achieved by fungal cell wall or biomass. It was reported by Miao (2005), fungal biosorption contributes less than 50% of color removal.

The ability of fungal living cell biodegradation is related to the production of enzymes such as lignin peroxidase, manganese peroxidase and laccase (Dos Santos, 2005). Since the laccase is produced in larger amount, using it as decolorization treatments have potentially advantageous (Zille, 2005; Sathiya *et al*., 2007). Laccase is a copper-containing enzyme that has very broad substrate specificity with respect to electron donors, e.g. dyes (Abadulla *et al.*, 2000; Duran *et al.,* 2002). Laccase catalyze the removal of a hydrogen atom from the hydroxyl group of phenolic compounds, to form free phenoxy radicals which results azo bond cleavage and release of molecular nitrogen (Dos Santos, 2005; Zille, 2005) (Figure 2.5)

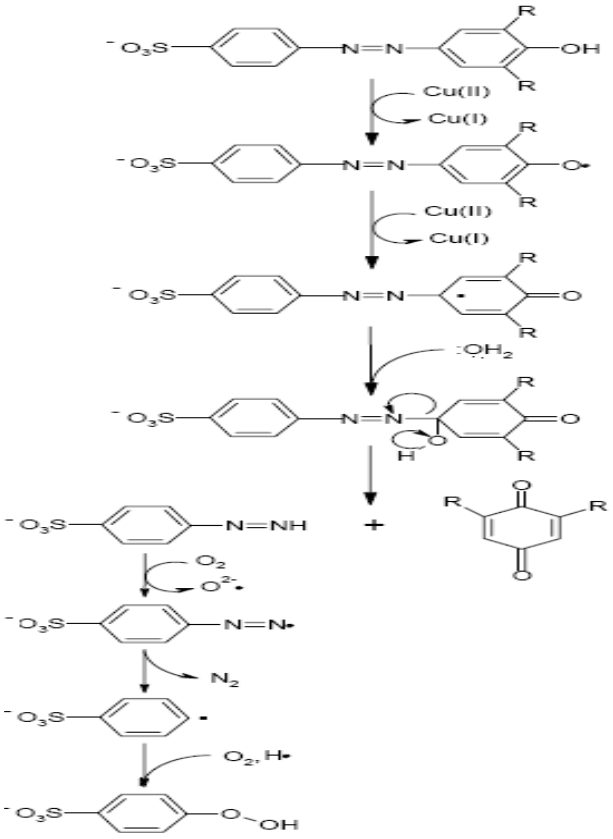


Figure 2.5. Oxidation of a phenolic azo dye by laccase (Dos Santos, 2005).

2.5. Factors Affecting Dye Decolorization

Due to highly variable nature of textile effluents, there are a number of factors that may affect the biodegradation rate of azo dyes.These factors are categorized as non-dye related parameters (temperature, pH, dissolved oxygen or nitrate concentrations and bacteria consortium) and dye related factors (type of dye, dye concentration and dye side-groups) all of which affects biological dye decolorization (Wallace, 2001; Miao, 2005).

Several researchers attempted to show the effect of non-dye related parameters on biological decolorization of dyes. Temperatures, which are too high or too low, can result in the exclusion of a particular group of microorganisms. Using activated sludge, Wuhrmann *et al*. (1980) investigated as, temperature has an increasing liner relationship with decolorization

rate up to 28 ºC. As seen in earlier sections of this review, the presence of oxygen generally inhibits the degradation of azo dye. Seesuriyachan *et al* . (2006) indicated that, in the absence of oxygen an azo compound will act as the sole oxidant, and its reduction rate will be governed by the rate of formation of the electron donor.

Co-subtrates such as organic carbon and organic/ inorganic nitrogen sources are considered to be essential medium supplements for the regeneration of NADH, which acts as the electron donor for the reduction of azo bonds (Arora *et al*., 2007). Moreover, the type of microorganism or consortium used for dye biodegradation will undoubtedly affect the reduction rate. Aerobic microbes do not have much ability to substantially decolorize azo dyes, but can oxidize the dye metabolites. The converse applies to anaerobic microbes (Wallace, 2001).

The azo dye structure can play a significant role in the dye biodegradation rate. Depending on the number and placement of the azo linkages, some dyes will biodegrade more rapidly than others. Experimental results have indicated that monoazo dyes are more effectively decolorized than diazo, triazo, and anthraquinone structures (Pisczek, 2005).

It has also been observed that the type, number and location of the substituents in the dye molecule affect the degradation rate (Ganesh, 1992; Pisczek, 2005). Dyes containing hydroxyl or amino groups were observed to degrade more rapidly than dyes containing methyl, methoxy, sulfo, or nitro groups.

A final and important factor for which biodecolorization efficiency depends on concentration of dyes present in the treatment plant. Attempts had been made to show effect of dye concentration using microbial consortium at laboratory and pilot scale (Sharma *et al.,* 2004; Abraham Mebrat, 2006; Arora *et al*., 2007). They conclude as decolorization progressively decreases as dye concentration increases. This may be due to the possibility of inhibition of metabolic activities of the strain at high concentration of the dye.

3. MATERIALS AND METHODS

3.1. Experimental Setup

A batch experiment of dye biodegradation study was conducted in bottles as shown in Figure 3.1.a. As it is schematically represented in Figure 3.1.b, it has sampling and gas removing ports on the left and right sides of the lid attached with a pieces of metal and glucose delivery plastic tubes. Carbon dioxide from the bottle was removed out via sampling port inserted in 3N KOH solution. Samples were taken with sterile syringe under aseptic condition.

Figure 3.1.a. Photographic representation of the setup.

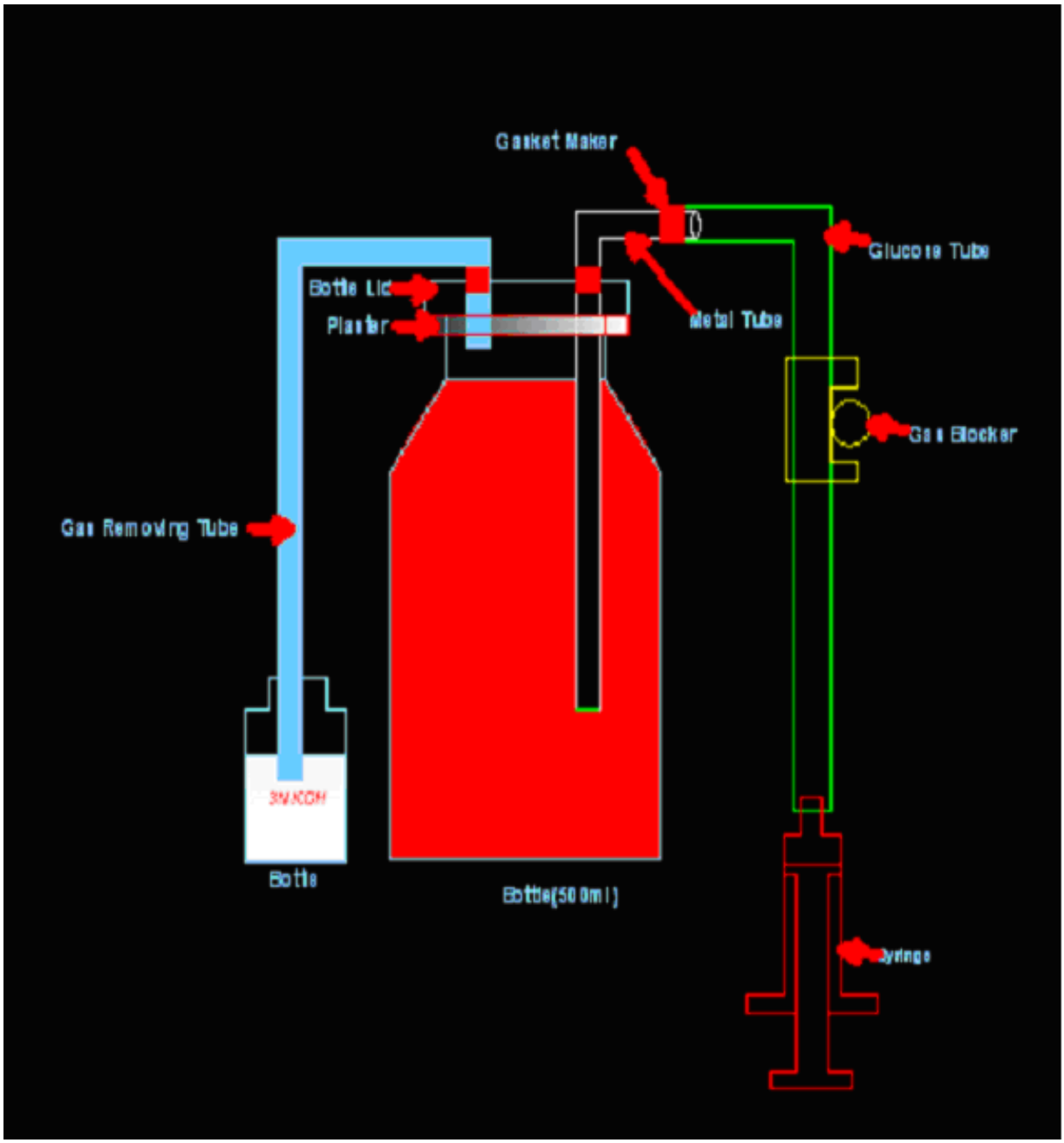


Figure 3.1 b. Schematic representation of the setup

3.2. Source of Microbial Culture

Samples were collected from Ethiopian soda lakes, namely Chitu, Abijata and Arenguadie. The rational behind using these soda lake inoculums is that, there are microorganisms adapted an alkaliphilic environment in the lakes and assuming these microorganisms adapt to the new environment and likely they can contribute in modifying dye containing environment (i.e., textile effluent) through their growth and function. Summary of the main physical and chemical characteristic of the three lakes are shown in Table 3.1.

The Crater Lake, Chitu in which the majority of the isolates were obtained for this study is the smallest of all known alkaline soda lakes found in the country. It is a closed basin with no obvious surface out flow with a moderate maximum depth of 21 m and covers diameter about 0.8 km^2 (Elizabeth Kebede, 1996). Location of Chitu and Abijata were located on Figure 3.2.

Table 3.1: Summary of the main physical and chemical characteristic of the three lakes (Garrett, 1992; Prosser *et al.*,1968)

Lakes	Depth, m	Temp, ^oC	pH	Ions						
				Na	K	Mg	Ca	HCO_3	Cl	SO_4
Abijata[a]	14.2	-	9.62	3730	133	3.1	5.1	6000	1710	37
Chitu[a]	21	27	10.2	14000	4109	0.7	2.5	5350	4800	800
Arenguadie[b]	18.5	22	10.3	67	8.1	<0.3	0.30	51.4	22	0.35

Measurement of ions: [a] mg/l

[b] milliequivalents per liter.

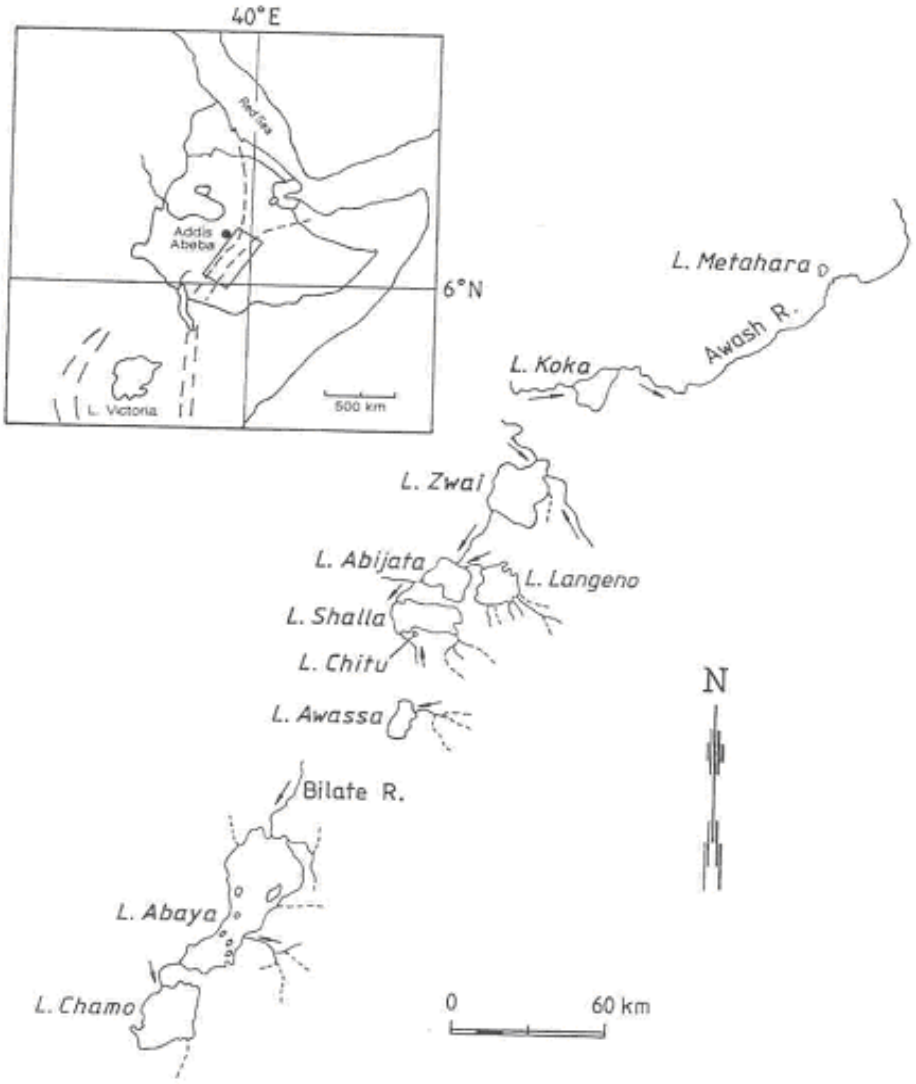


Figure 3.2. Location of the study area (Elizabeth Kebede, 1996)

3.3. Media Composition

Mineral Salts Medium (MSM) used by Arora *et al*. (2007) were followed with some modifications (ferric ammonium citrate by ferric citrate and neutral pH by alkaline pH). The composition includes (g/l): Na_2HPO_4 (3.6), KH_2PO_4 (1.0), $(NH_4)_2SO_4$ (1.0), $MgSO_4$ (1.0), $CaCl_2$ (0.10), $FeC_6H_5O_7$ (0.01) and 10 ml of trace element solution per liter. The trace element solution has the following compositions (mg/l): H_3BO_3 (30.0), $ZnSO_4.7H_2O$ (10.0), $Na_2MoO_4.2H_2O$ (3.0), $MnCl_2.4H_2O$ (3.0), $NiCl_2.6H_2O$ (2.0), $CoCl_2.6H_2O$ (1.0), and $CuCl_2.2H_2O$ (1.0). Stock solutions of glucose (50 % w/v) and yeast extract (10% w/v) were sterilized separately and added to the media to maintain final concentrations of 0.5 % (w/v) , 0.01 % (w/v) , respectively. During MSM-agar plate preparation, 2% (w/v) agar was added to the media. An alkaline pH of the media was maintained by using separately sterilized Na_2CO_3 (25 % w/v).

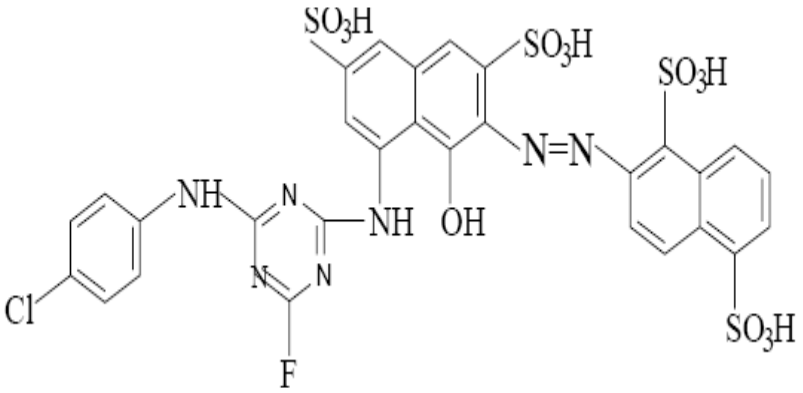
3.4. Dyes

RR 184 (Cibacron Red FB), Reactive Red 141 (Procion Red HE7B), Reactive Yellow 84 (Procion Yellow HE4R), Reactive Yellow 700 (Evercion Yellow ESL), Reactive Blue 198 (Evercion Blue HEGN), Vat 43 (Hydron Blue 3R stabisol) and Evercion Red ESL dyes which is mostly used in the textile industries were collected from Akaki and Adei Ababa textile factories. Description and molecular structures of some dyes are given in Table 3.2 and Figure 3.3. Among these dyes, RR 184 was chosen as a representative dye used for this study.

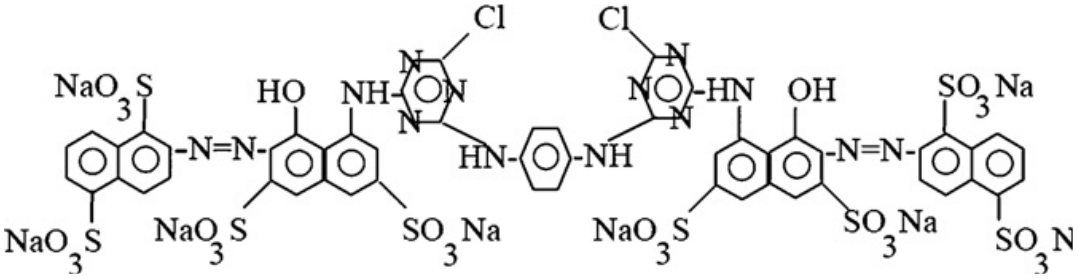
Table 3.2: Characteristic of dyes used for this study.

Color Index Name	Common Name	λ_{max} (nm)	Molecular formula
Reactive Red 184	Cibacron Red FB	540	A
Reactive Red 141	Procion Red HE7B	544	B
Reactive Yellow 84	Procion Yellow HE4R	411	C
Vat 43	Hydron Blue 3R Stabisol	600	D
Reactive yellow 700	Evercion Yellow ESL	415	Un
Reactive Blue 198	Evercion Blue HEGN	625	Un
Un	Evercion Red ESL	545	Un

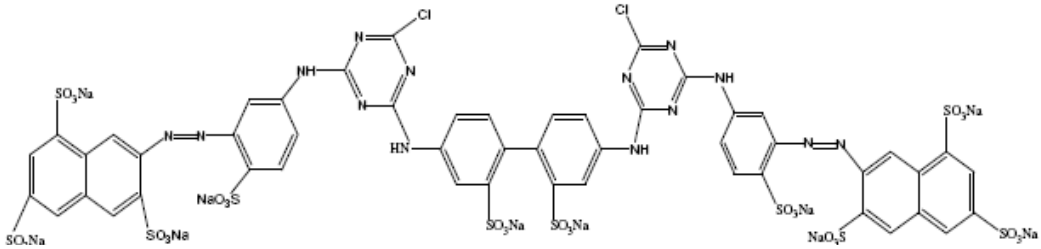
Un= Unknown



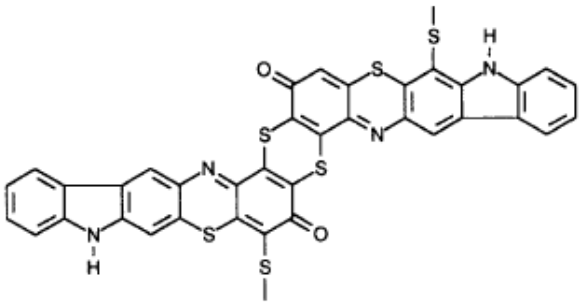
A] Reactive Red 184



B] Reactive Red141



C] Reactive Yellow 84



D] Vat 43

Figure 3.3. Molecular structure of some dyes used for this study

3.5. Analyses and Isolation Procedures

3.5.1. Wavelength of Maximum Absorbance (λ_{max}) and Calibration Curve

In order to determine wavelength of maximum absorbance, 100 mg/l of each dye was prepared and scanned in the ranges from 200 - 800 nm using Uv-visible spectrophotometer (Genesys 2pc, Shimazdu). Then, λ_{max} of each dyes were considered at one absorbance unit (Figure 3.4).

Calibration curve were prepared by using concentration ranged from 1 - 100 mg/l of RR 184. From concentration and measured absorbance data, calibration curve for RR 184 were constructed (Figure 3.5). Based on this calibration curve, final concentration of the dye residue were determined

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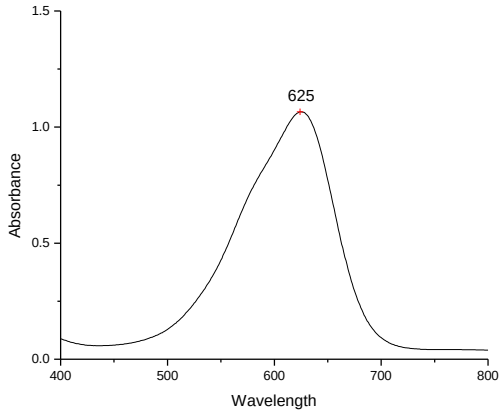
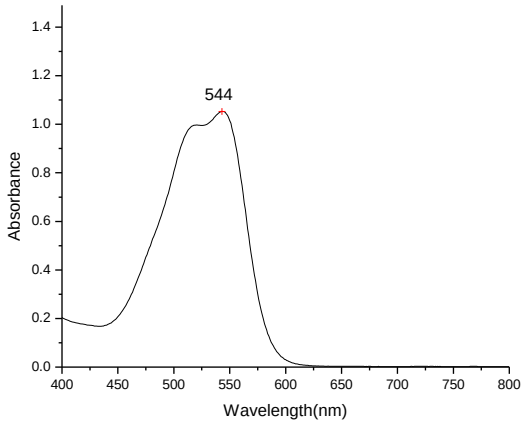
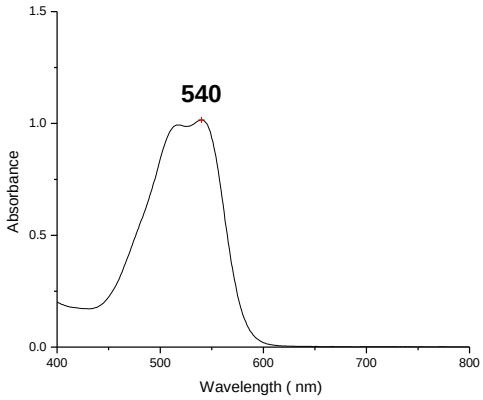


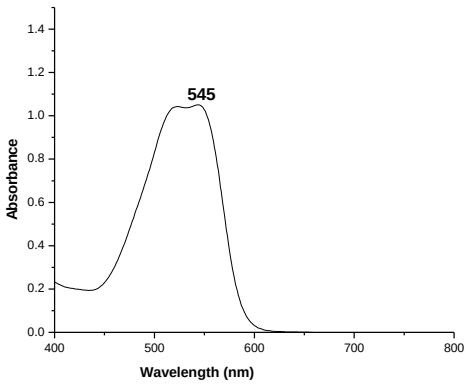
A) Reactive Blue 198



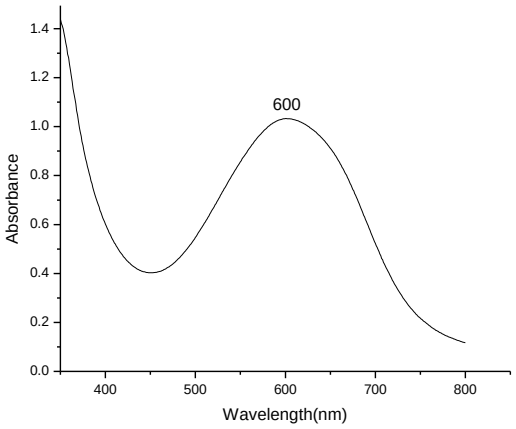
B) Reactive Red 141



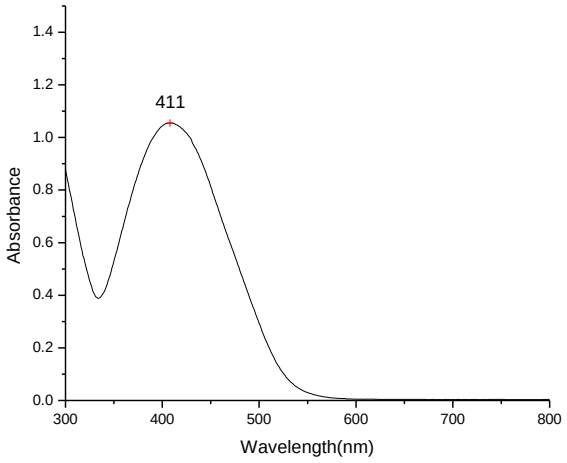
C) Reactive Red 184



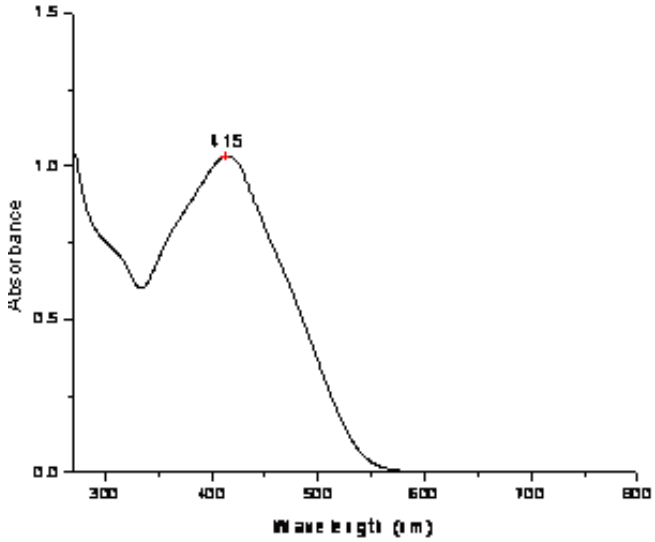
D) Evercion Red ESL



E) Vat 43



F) Reactive Yellow 84



G) Reactive yellow 700

Figure 3.4.Uv-visible spectrum of wavelength of maximum absorbance for different dyes

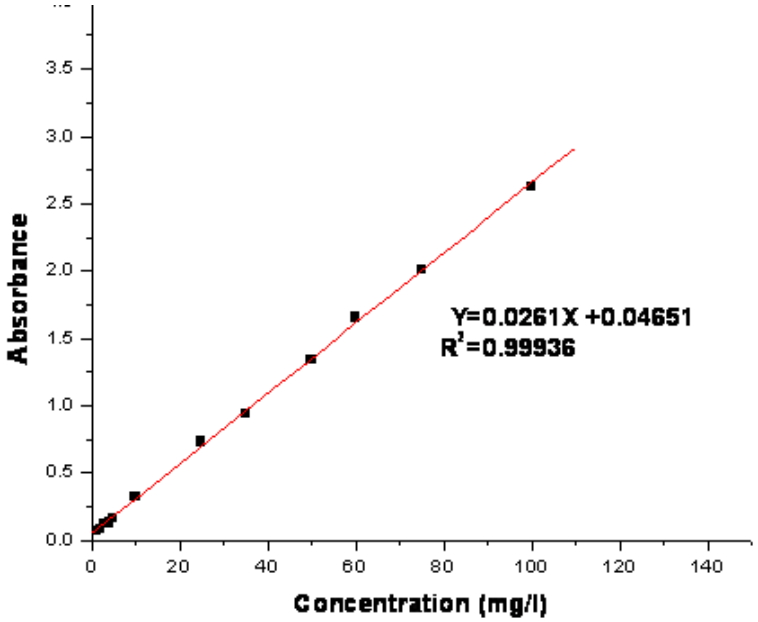


Figure 3.5. Calibration curve for RR 184 (λ max = 540 nm)

3.5.2. Decolorization Assay

The extent of decolorization was determined by measuring the absorbance of the samples at the start and at various time interval and the values were considered at each predetermined maximum absorbance wavelength. MSM without dye and inoculum was used as blank. For analysis, 10 ml of the liquid was taken out aseptically from the sample every 24 h and centrifuged at 6000 rpm (revolution per minute) for 30 min. Centrifuged cell free supernatant samples were scanned in the range of 200 - 800 nm using the aforementioned spectrophotometer.The percentage decolorization was calculated by using the following equation:

$$\% \text{ Decolorization} = \left[\frac{A_o - A_t}{A_o} \right] \times 100 \%$$

Where A_o = initial absorbance

A_t = absorbance after time t

3.5.3. Enrichment and Isolation of Dye Degrading Microorganisms

Alkaline samples collected aseptically from Lake Abijata, Arenguadie and Chitu were enriched on MSM containing 10 mg/l of dye. The sterilized MSM containing 10 mg/l RR 184 was inoculated with mud samples (10 % w/v) and incubated at ambient temperature under anoxic condition. Until a constant decolorization was achieved, 10 (% v/v) samples were further transferred to fresh MSM containing 10 mg/l of RR 184 within 5 - 8 interval of days. After each transfer, plating of serially-diluted enriched samples (10^{-1} - 10^{-7}) were carried on MSM-agar plates containing 10 mg/l of RR 184 and then incubated at ambient temperature. In order to create microaerophilic condition lighted candle in a jar was used (Figure 3.6). Finally, with out considering clear zone around the colony, one hundred twenty one (121) morphologically different isolates were selected and further purified. The colonies were given specific codes and stored on MSM-agar slants. Growths in liquid media were followed microscopically.

Figure 3. 6. Photograph which shows plate incubation under anoxic condition

3.5.4. Screening of Isolated Microorganisms for Dye Decolorization

Each of the isolates were tested for their color removal ability in liquid MSM containing 10 mg/l of RR 184. In order to achieve this confirmation, a loop full of cell culture from each slant were grown aerobically in 250 ml capacity of Erlenmeyer flask containing sterilized liquid MSM (100 ml) with out dye. The flasks were incubated on shaker at 120 rpm at ambient temperature for 4 - 5 days. Then, aerobically grown cells (10% v/v) were cultured in 500 ml capacity bottles containing liquid MSM and 10 mg/l of RR 184. The preparation were incubated at ambient temperature under anoxic condition. Decoloriation activities were monitored visually (Figure 4.4).

For further screening, twenty five isolates decolorizing 10 mg/l of RR 184 in liquid MSM rapidly were grown aerobically on the same liquid media with out dye. These aerobically grown cultures (10% v/v) were inoculated in 500 ml capacity bottles containing sterilized MSM supplemented with RR 184 (25 mg/l). The bottles were incubated at ambient temperature under anoxic condition. In such a way, screening out continued using 50, 100 and 150 mg/l of RR 184 until the best decolorizer were obtained.

3.5.5. Identification

Attempts were made to study morphological and biochemical characteristics of the selected microbial isolate.

3.5.6. Growth phase for Isolate A55 Growth

A loop full of cells from the slant was inoculated to 250 ml Erlenmeyer flask containing sterilized MSM (100 ml) with out dye and incubation were made on the shaker having 120 rpm at ambient temperature**.** From this 24 h grown culture, 1ml sample was transferred to the same flask containing fresh MSM and incubation made on the same condition as mentioned above. Then, Optical Density (OD) reading at 600 nm were carried out every 6 h intervals starting from 0 h to 144 h using Uv-visible spectroscopy.

3.5.7. Decolorization Activity of Isolate A55 under Different Conditions

3.5.7.1. Effect of Carbon Sources on Decolorization

Experiments were conducted using different carbon sources such as: glucose, lactose, sucrose, starch, sodium pyruvate, sodium citrate and media with out carbon sources. Media and stock solution sterilization were the same as stated in Section 3.3, except glucose which was substituted by these different carbon sources and ferric citrate excluded. The initial RR 184 concentration was fixed at around 150 mg/l and the preparation were incubated with 10

% v/v inoculum size. After every 24 h, the bottle contents were centrifuged at 6000 rpm for 30 min and decolorization were analyzed as mentioned in Section 3.5.2.

3.5.7.2. Effect of Nitrogen Sources on Decolorization

To evaluate the effect of nitrogen on decolorization activity, organic and inorganic nitrogen sources such as peptone, yeast extract, $NaNO_3$, $NaNO_2$, $(NH4)_2SO_4$ and media with out nitrogen were added to nitrogen free MSM. Stock solution (10 % w/v) of peptone and yeast extract were sterilized separately and added to inorganic nitrogen free sterilized MSM in a concentration of 0.01%(w/v). All inorganic nitrogen sources (1 g/l) were prepared and sterilized together with any nitrogen free MSM containing 150 mg/l of RR 184. The preparations were inoculated with 10 % v/v culture and incubated under anoxic condition at ambient temperature. After every 24 h decolorization were measured as described in section 3.5.2.

3.5.7.3. Effect of Different Culture Conditions on Decolorization

The effect of various culture conditions such as agitation, aeration, anoxic and anaerobic state on decolorization of RR 184 were examined. Agitation were operated on a rotary shaker running at 120 rpm. Anaerobic culture was bubbled with pure nitrogen at the beginning. Anoxic culture condition was made by filling the content of the bottle fully. Aerobic condition was maintained in a continuous aeration condition. All the experiments were operated at ambient temperature and alkaline pH under a constant initial dye concentration (RR 184) of 150 mg /l. The residue of RR 184, cell growth and DO (Dissolved Oxygen) were monitored as a function of time. DO and conductivity were measured using oxygen meter and conductivity meter, respectively. Biomass concentration was determined by dry cell weight after 24 h drying at 105 ^oC.

3.5.7.4. Effect of Dye Concentration on Decolorization

To determine the maximum RR 184 concentration tolerated by isolate A55, experiments with different initial dye concentrations (50, 100, 150, 200 and 250 mg/l) were performed in liquid MSM having the same composition as mentioned in Section 3.3 and measurement were made as described above.

3.5.7.5. Kinetics of Decolorization

In most experimental activity, decolorization functions are either first or second order type. In order to confirm kinetics of decolorization experiments were conducted using 10 % v/v inoculum of isolate A55 and 150 mg/l of RR 184 in MSM. Spectrophotometric measurements were made periodically by taking samples at different time interval.

3.5.8. Decolorization of Various Dyes by Isolate A55

In order to confirm whether the isolate decolorize dyes other than RR 184, six additional dyes possessing a concentration of 50 mg/l each were prepared separately and added to MSM. Then, each preparation were inoculated with aerobically grown culture of A55 (10 % v/v) and incubation were made as described above. Control with out inoculum was prepared for each. Samples were taken asepticall every 24 h for 96 h and analyzed as mentioned before.

4. RESULTS

4.1. Isolation and Characterization of Dye Decolorizing Microbial Isolates

Selective enrichment of mud samples collected from the three alkaliphilic soda lakes, led to isolation of 121 morphologically different isolates on MSM-agar plates containing 10 mg/l of RR 184. These colonies were individually tested for their color removal ability using liquid MSM containing 10 mg/l of RR 184. Among the total isolates, ninety five isolates showed decolorization activity in fourteen days of incubation. However, twenty six isolates were found to be non-decolorizers of the given dye even incubated for an extended period of time (Table 4.1). Decolorization was monitored visually (Figure 4.1).

Table 4.1: Summary of the isolates in the three lakes

Rate of decolorization (Day)	Lake			Total/day	Total
	Abijata	Arenguadi	Chitu		
1	3	NID	32	35	95**
2	8	1	22	31	
3	3	1	3	7	
4	NID	NID	5	5	
5	NID	NID	5	5	
6	NID	NID	NID	NID	
7	NID	NID	4	4	
8*	2	1	1	8	
> 14	3	1	22	26	26***
Total isolate /lake	19	4	98	121	121

* =Decolorization day range 9-14 day interval

**=Decolorizer

***=Non-decolorizer

NID =No Isolate Detected

Figure 4.1. Photographic representation of RR 184 (10 mg/l) decolorization by using different isolates.

Among the decolorizers, twenty five (25) isolates removing the color of RR 184 (10 mg/l) rapidly (within the first day) were selected for further screening using 25 mg/l of dye (Figure 4.2). The percentage decolorization of these isolates was monitored for 96 h within 24 h interval and the results were shown in Table 4.2.

Figure 4.2. Photographic representation of RR 184 (25 mg/l) decolorization using 25 isolates.

Table 4.2: Percentage decolorization of RR 184 (25 mg /l) by 25 isolates

Isolate Code	$OD_{600nm(Initial)}$	Decolorization (%)			
		24h	48h	72h	96h
A4L	1.25	34	44	51	55
A4S	2.13	51	51	62	100
A7*	1.74	91	91	99	100
A8	1.77	37	42	45	63
A17*	1.78	89	94	95	100
A38	1.15	35	36	52	94
A42	1.64	63	88	94	95
A44	0.97	27	28	37	66
A51	1.98	35	89	94	94
A52	2.36	48	79	93	95
A55*	2.13	83	86	96	100
A60*	1.92	80	93	100	100
A69*	1.9	89	93	100	100
A70*	1.9	94	99	100	100
Ab17*	0.86	88	91	100	100
B4	1.86	29	52	63	95
B6*	1.76	93	95	100	100
B8	1.65	46	76	59	92
B10	0.87	35	46	46	96

B11	1.42	31	36	37	39
B12	1.9	65	80	88	92
B13	1.32	39	70	76	84
B14	2.42	30	50	70	74
B17	1.5	29	30	40	55
B24	1.85	76	97	97	97

* Selected isolates

Eight isolates, designated as A7, A17, A55, A60, A69, A70, Ab17 and B6 which decolorizes the given dye $\geq$ 80 % and above within 24 h and completely decolorize at 96 h were subjected to further screening using various concentration of the RR 184 (Table 4.3).

Table 4.3: Percentage decolorization of RR 184 (50 - 150 mg /l) by the 8 isolates.

Isolate Code	$OD_{600nm(Initial)}$	Dye concentration	**Decolorization (%)**				pH	
			24h	48h	72h	96h	Initial	Final
	1.152	50	75	87	95	100	10.21	8.74
	0.828	100	72	87	91	98	10.1	8.77
A7	1.795	150	77	95	96	96	10.12	8.86
	0.603	50	70	96	96	100	10.23	8.72
	0.599	100	85	87	96	97	10.12	8.74
A17	0.790	150	1.6	2.1	4.7	5.2	10.12	9.65
	0.826	50	82	87	88	100	10.13	8.73
	0.775	100	88	89	97	98	10.16	8.88
A55*	1.322	150	89	96	97	97	10.08	8.69
	0.844	50	81	82	95	100	10.25	8.65
	0.862	100	72	79	82	97	10.18	8.65
A60	1.810	150	89	94	95	96	10.11	8.95
A69	0.793	50	61	70	95	100	10.09	8.79

	0.731	100	83	85	94	97	10.09	8.54
	1.690	150	89	95	95	96	10.12	8.67
	1.087	50	75	88	97	100	10.18	8.85
	1.264	100	78	86	98	98	10.13	8.79
A70	1.717	150	84	94	95	95	10.12	8.74
	0.962	50	58	70	70	100	10.24	8.32
	0.942	100	83	88	96	97	10.18	8.69
Ab17	0.820	150	2.5	2.7	94	95	10.16	9.18
	0.507	50	58	61	61	100	10.24	8.94
	0.862	100	78	91	92	99	10.15	8.54
B6	1.032	150	71	94	96	96	10.15	9.19

* Selected isolate

Among the above isolates, A55 which decolorized $\geq$ 82 % of the tested dye concentration in one day was selected for further studies. The characteristics of isolate A55 is summarized in Table 4.4.

Table 4.4: Morphological and biochemical characterization of isolate A55

Characteristics	Isolate A55
Morphology Test	
Shape	Rod
Color	White
Edge	circular
Elevation	Convex
Gram staining	Negative
Motility	+
Anaerobic growth	+
Aerobic growth	+
Biochemical Test	
Catalase	+
Oxidase	+

4.2. Time-course for Isolate A55 Growth

Growth of A55 was conducted at ambient temperature by monitoring the optical density at 600 nm (Figure 4.3). It was observed that the growth of A55 showed lag phase in the first day exponential phase starting from 24 h and ends before 84 h. Thus, the activation period was fixed by considering the late log phase and 10 % v/v inoculums were used for subsequent inoculations.

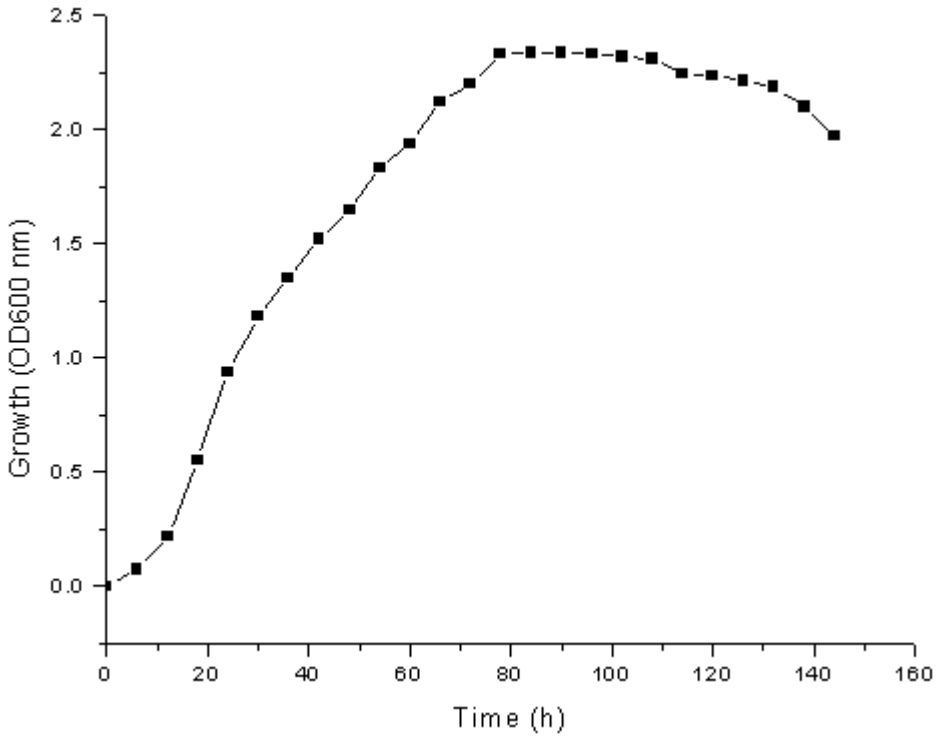


Figure 4.3. Growth profile of isolate A55

4.3. Decolorization Activity of Isolate A55 under Different Conditions

4.3.1. Effect of Carbon Sources on Decolorization

Using sodium citrate 90 % of decolorization was achieved during the first 24 h followed by sodium pyruvate and glucose 87 and 80 % decolorization, respectively. The least result during 24 h decolorization was 57% resulted from non-carbon supplemented culture (Figure 4.4). During 72 h decolorization assay, culture supplemented with starch took the highest decolorization percentage (100 %) compared to non-carbon supplemented source (84 %). Within 96 h, using carbon sources 96 - 100 % decolorization was achieved while 87% with out carbon source.

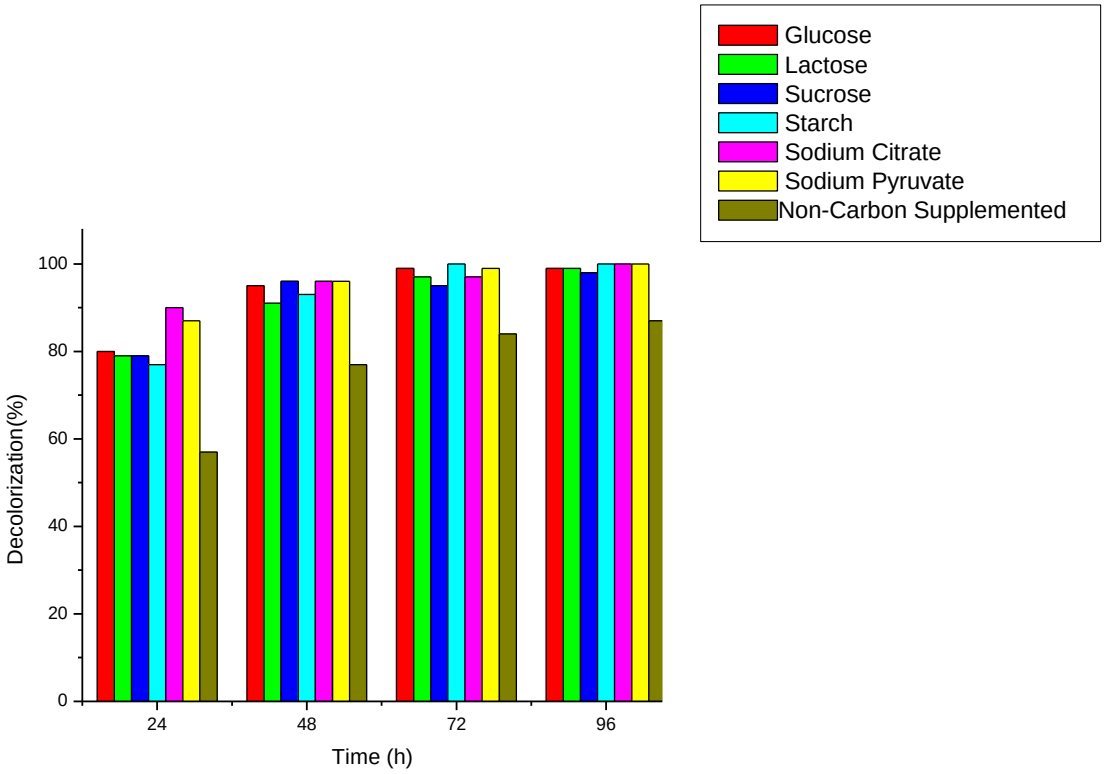


Figure 4.4. Effect of carbon source on decolorization

4.3.2. Effect of Nitrogen Source on Decolorization

Figure 4.5 shows the influence of various nitrogen (organic and inorganic) sources on the efficiency of decolorization of RR 184 by isolate A55. The highest decolorization was observed on cultures supplemented with organic nitrogen sources (i.e., 89 % for peptone and 91 % for yeast extract) within 24 h of cultivation. Within the first day of incubation cultures incubated in $NaNO_3$ and $NaNO_2$ showed lower percentage of decolorization than non-nitrogen supplemented culture. However, similar performances of color removal were observed in $(NH_4)_2SO_4$ and its combination with organic sources (10- 20%) incubated culture. Within 96 h, using organic nitrogen sources the highest percentage of decolorization

(100 %) was achieved while 64 - 80%, 29 - 53 %, and 39 % using combination of organic /inorganic, inorganic and with out nitrogen sources, respectively.

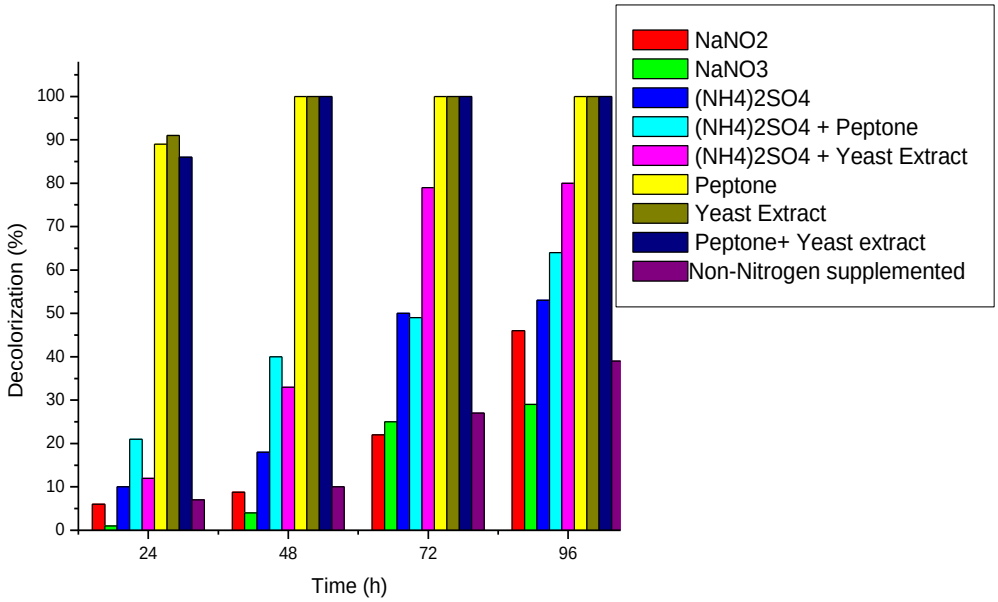


Figure 4.5.Effect of nitrogen source on decolorization.

4.3.3. Effect of Culture Condition on Decolorization

Table 4.5 showed anaerobic, anoxic, shaker and aerobic culture's dye residues, dissolved oxygen and biomass. In both shaker and aerobic cultures, the higher dissolved oxygen concentration increase biomass of the cell and decrease removal efficiency of A55. On the contrary, anaerobic and anoxic culture of A55 which have least concentration of dissolved oxygen contributed to highest color removal within four days of incubation. Figure 4.6 indicates the relation between dissolved oxygen, dry weight and dye absorbance of anoxic culture. The results showed higer concentration of oxygen in the culture was found to inhibit color removal in the first 24 h. However; progressive decline of dissolved oxygen in the culture was accompanied by an increase in biomass (dry weight) and complete disappearance of color.

Table 4.5: Effect of culture conditions on dissolved oxygen concentration, biomass and dye removal activity as a function of time.

Time (h)	Measured Parameters	Culture Conditions			
		Anaerobic	Anoxic	Shaker	Aerobic
0	DO [a]	0	2.31	2.76	3.82
	DW[b]	0.015	0.016	0.01	0.013
	DR[a]	150	150	150	150
24	DO [a]	0	1.64	2.39	2.55
	DW[b]	0.2	0.88	3.16	3.49
	DR[a]	62	55	104	113
48	DO [a]	0	0.26	0.56	2.53
	DW[b]	2.15	2.94	3.33	3.53
	DR[a]	4	1	81	110
72	DO [a]	0	0.11	0.42	2.5
	DW[b]	2.64	3.25	3.41	3.66
	DR[a]	1	1	89	100
96	DO [a]	0	0.02	0.35	1.6
	DW[b]	2.57	3.29	3.58	3.51
	DR[a]	0	0	74	99

DO= Dissolved Oxygen

DW= Dry Weight

DR= Dye Residue

Measurements: [a] = mg/l

[b=] g/l

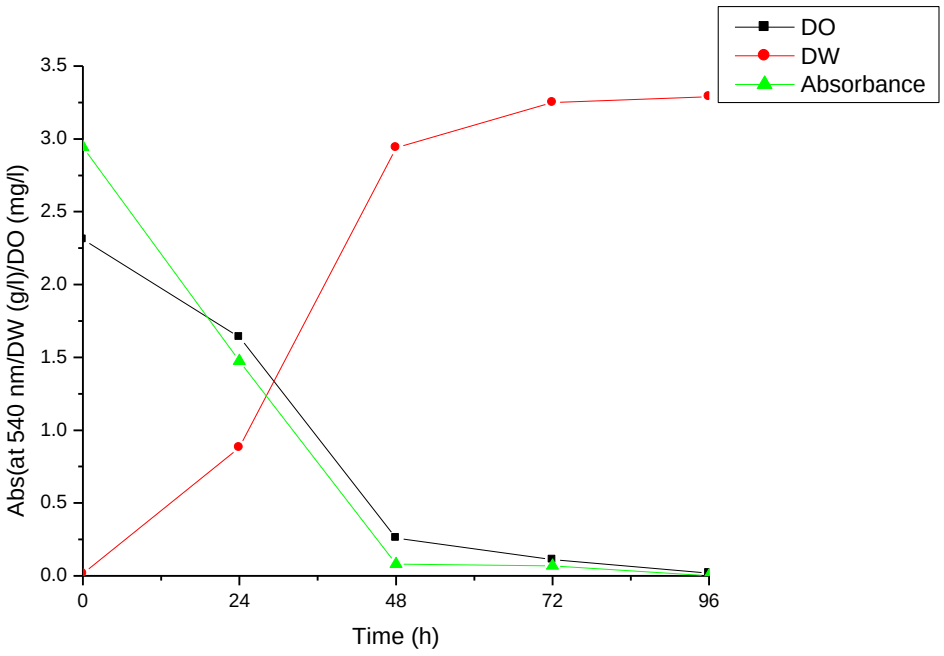


Figure 4.6. Effect of anoxic culture condition on color removal, dry weight and dissolved oxygen.

4.3.4. Effect of Dye Concentration on Decolorization

The decolorization efficiency was clearly seen visually (Figure 4.7) and decolorization percentage above 80 % for initial dye concentration less than 200 mg/l within 24 h cultivation, but it decreased with further increase in dye concentration. When the dye concentration was as high as 250 mg /l decolorization efficiency was 24 % (Figure 4.8). Within 96 h incubation isolate A55 showed equal percentage of decolorization. This means that an acceptable high color removal can be achieved by isolate A55 in an extensive range of dye concentrations.

Figure 4.7. A 24 h photographic representation of decolorization using different concentration of RR 184 and isolate A55

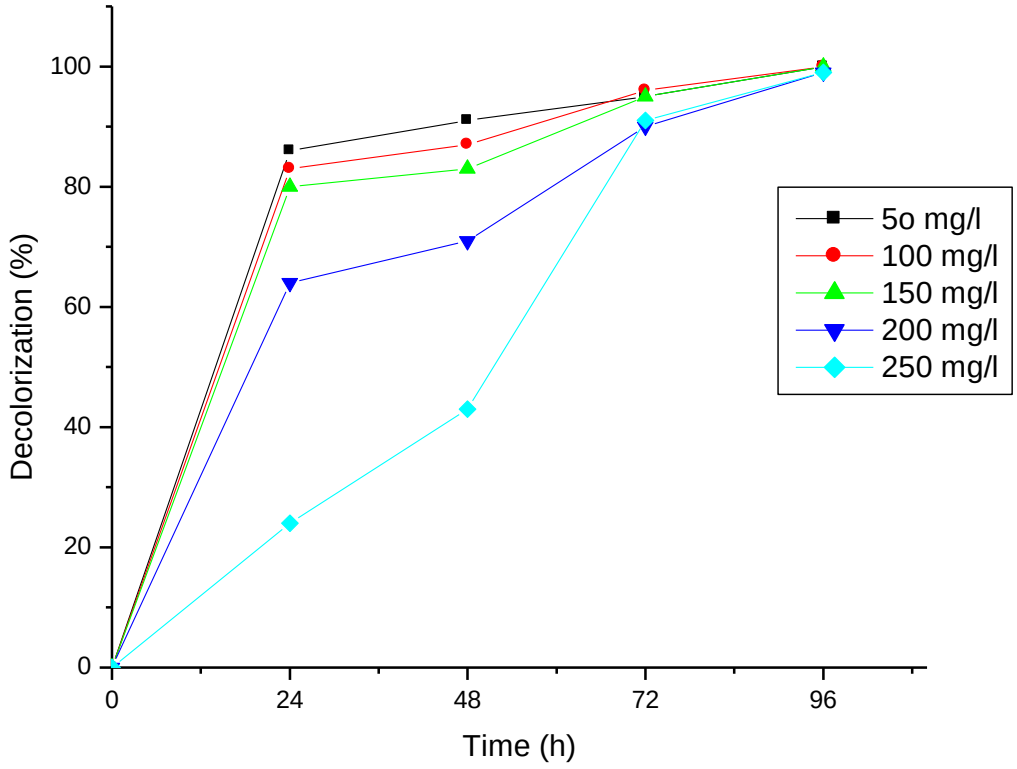


Figure 4.8. Effect of initial dye concentration

4.3.5. Kinetics of Decolorization

The experimental data obtained (Figure 4.9) was fitted with a mono-exponential function (first order kinetics) with a very good magnitude of correlation, R = 0.994. This result indicates to the existence of one component that is responsible for the overall decolorization process (i.e., biodegradation). Figure 4.10 shows a typical time dependent UV-visible spectrum of RR 184 solution during biodegradation. The absorbance peaks corresponding to dye, diminished with time indicates that the dye had been removed.

In order to develop a treatment plant, time is an important factor. Time related factors are calculated by the formula:

$\text{slope} = -1/\tau$, Where τ = The quantity used to characterize time dependent parameter.

$\tau = -1/\text{slope}$, from the data fit (ln A vs t) (Figure 4.12), Slope = (-0.0573 $\pm$ 0.00171) h^{-1}

τ = 17.5 h.

The above values indicate that, for complete degradation of a dye used for this study a time that is equal or greater to five times of τ should be considered (i,e, around 88 h) (Figure 4.10).

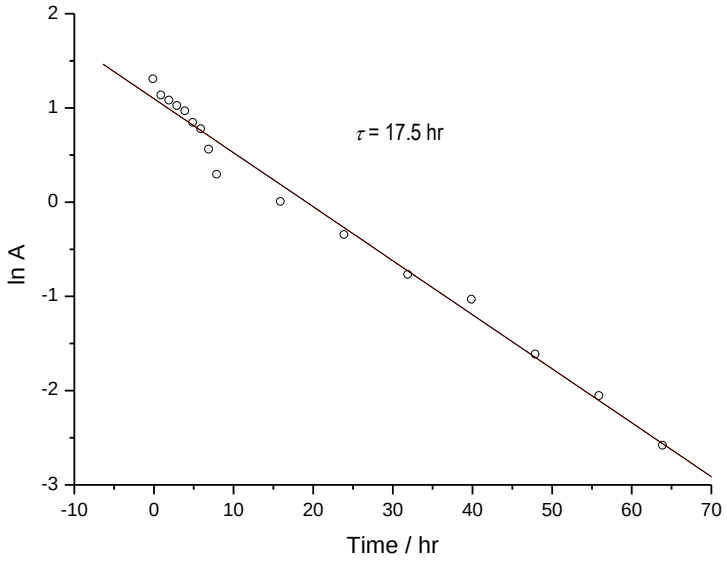


Figure 4.9. First order kinetic plot for RR 184.

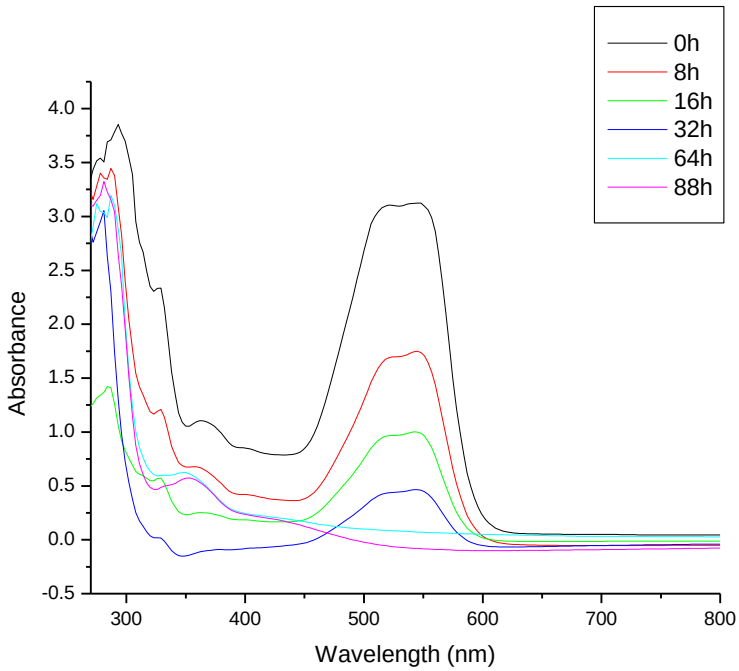


Figure 4.10. Uv-visible spectra of RR 184 (150 mg/l) biodegraded by isolate A55 at different time.

4.4. Decolorization of Various Dyes by Isolate A55

Isolate A55 showed that the potential to decolorize six additional textile dyes with a decolorization efficiency varying from 22- 98 % (Table 4.6). During the first day of incubation the isolate decolorized 86% for Reactive Yellow 84 and 22% for Reactive Blue 198. Within 96 h incubation period, the isolate decolorized all the tested dyes greater than 90% except Reactive Blue 198 (50 %). Results of pH value were found to vary from 10.11 - 10.32 at the start and 7.59 - 8.63 at the end. The drop of pH in all cases indicate that the supplemented organic substrates have been fermented by isolate A55.

Table 4.6: Decolorization of different textile dyes by isolate A55

			Decolorization (%)				pH	
Common Name	Color Index Name	λ_{max}	24h	48h	72h	96h	Initial	Final
Procion Yellow HE4R	Reactive Yellow 84	411	86	89	96	98	10.21	8.6
Procion Red HE7B	Reactive Red 141	544	24	34	86	98	10.23	8.31
Hydron Blue 3R stabisol	Vat 43	600	80	82	87	93	10.32	8.63
Evercion Blue HEGN	Reactive Blue 198	625	22	26	37	51	10.15	8.53
Evercion Yellow ESL	Reactive Yellow 700	415	32	92	93	95	10.22	8.62
Evercion Red ESL	NI	545	51	81	90	97	10.11	8.6
Cibacron Red FB	RR 184	540	86	91	95	100	10.29	7.59

NI=Not Identified

5. DISCUSSION

In the present study microorganisms capable of decolorizing textile dye were isolated from Ethiopian soda lakes (Abijata, Arengudi and Chitu) using azo dye, RR 184 enriched MSM. A total of 121 morphological distinct colonies were isolated from MSM-agar plate. Almost 81 % of the isolates were recovered from Lake Chitu followed by 16 % Lake Abijata and 3 % Arenguadi (Table 4.1).

When the total isolates were subjected to confirmed decolorizer and non-decolorizer using MSM containing 10 mg/l of RR 184, 95 isolates found to be decolorizer while the rest non-decolorizer. Almost all dye decolorizing microbial culture isolated within 8 days, where as the non-decolorizers were detected after 14 days of incubation.

Out of the 95 isolates, 25 of them which showed rapid decolorization were selected for further screening using 25 mg/l of RR 184. Their growth pattern ranges from 0.86- 2.42 which is the least and highest OD600nm reading displayed by isolate Ab17 and B6, respectively. The isolates decolorization rate varies from 27-100 % within 24-96 h (Table 4.2).

On the basis of percent decolorization, 8 isolates decolorizing the dye $\geq$ 80% (Table 4.2) in the first 24 h were selected and subjected to further screening using various concentration of RR 184. The isolates were A7, A17, A60, A69, A70, B6, Ab17 and A55, of which the later was selected based on its ability to decolorize 50- 150 mg/l of the dye in the range of 82-89 % within the first 24 h cultivation (Table 4.3). The isolate was found to be Gram-negative, rod shape, motile, catalase and oxidase positive that can grow aerobically and anaerobically.

As reported in many literatures, most researchers focus isolation of microorganisms that decolorize textile dyes using effluent-adapted organism. Sharma *et al.* (2004), Bhatt *et al.* (2005) and Ramya *et al*. (2007) isolated 5, 17 and 24 textile effluent-adapted microbial

cultures, respectively. In the present study, all the isolates were taken from alkaline soda lake sample which has no contact with textile effluents and yet degrading microorganisms were abundant. Soda lakes represent the most stable, naturally occurring alkaline environment characterized by high pH values and diverse range of microorganisms that have a potential of biotechnological application (Ulukanli and Diurak, *2002).* Because of the use of sodium hydroxide in the process of textile manufacture, textile industry effluents have alkaline pH. On the other hand, most biological treatment of textile effluents uses neutraliphilic microorganisms which require pH adjustment. Therefore, the use of alkaliphilic microorganisms which are adapted to high pH environment is expected to offer process advantage by avoiding or reducing the need for pH readjustment.

Textile effluent-adapted and non-adapted bacteria isolation was done by Olukan *et al.* (2006). He was successful to get eighteen effluent adapted and six non-effluent adapted isolates that showed 40.74-47.73% and 17.91-36.69 % of color removal, respectively. In the present study, the results obtained (Table 4.2) showed better than the results reported by Olukan *et al.* (2006).

Dye decolorization efficiency by isolate A55 was strongly influenced by the medium composition. Almost all carbon sources (glucose, lactose, sucrose, starch, sodium citrate and sodium pyruvate) were found to enhance decolorization more than 75 % within 24 h, compared to the non-carbon supplemented culture. The non-carbon supplemented culture also achieved 87 % decolorization within 96 h indicates that the yeast extract added as nitrogen source is likely to be deaminated and used as carbon source or the azo dye degradation products may have been used as a carbon source (Figure 4.4).

Better decolorization in the presence of different carbon sources than in the absence of any carbon source may indicate the requirement for the presence of sufficient electron donors. When organic carbon sources are metabolized they produce NADH which acts as electron donor for an azo dye reduction (Nigam *et al.*, 1996; Tan, 2001; Vander Zee, 2002). The other

probability of higher decolorization using organic carbon source could be nutritional contribution of the source which results fast growth of the organism. In the presence of actively growing cells that involved in respiration, oxygen is depleted and hence favourable condition for anaerobic reduction of dyes (Haug *et al.,* 1991; Donlon *et al.,* 1997; Vander Zee, 2002; Zille, 2005). In the present study, increased decolorization activity was observed when the DO level depleted in anoxic culture (Figure 4.6).

Generally, the results showed dependence of isolate A55 on carbon sources in enhancing decolorization efficiency. The ability of isolate A55 utilizing starch efficiently as a carbon source indicates its potential application in textile wastewaters that contain a large proportion of starch released during the desizing operation (Dos Santos, 2005; Arafat, 2007). Thus, under application conditions the organism may not need any other additional carbon source input to bring about dye decolorization.

On the contrary, the effect of nitrogen (especially inorganic) source on the decolorization rate of the dye by isolate A55 was none pronounced than the carbon sources. Figure 4.5 shows the organic nitrogen source (peptone and yeast extract) were found to enhance decolorization more than inorganic nitrogen sources such as $NaNO_3$, $NaNO_2$, and $(NH_4)_2SO_4$. Through out the entire cultivation period, the lowest rate of decolorization was recorded in cultures containing $NaNO_3$. A possible explanation for the low decolorization of A55 in the presence of $NaNO_3$ may be related to the nitrate preferencially consumed by the organism and serve as a final electron acceptor than the dye (Wallace, 2001; Dos Santos, 2005; Miao, 2005). Similarly Chen *et al*. (2003) and Ramya *et al*.(2007) reported the same results. The results clearly indicate that availability of organic nitrogen sources is extremely important to bring about complete decolorization.

In the present study the ability of isolate A55 to degrade RR 184 under anaerobic, anoxic, shaker (agitated) and aerobic culture condition was tested. Results showed that agitated and

aerated cultures have relatively higher biomass and dissolved oxygen but lower efficiency of color removal. In anoxic and anaerobic culture, a progressive increment in biomass followed by lower dissolved oxygen concentration was accompanied with improved decolorization efficiency (100%) (Table 4.5; Figure 4.6). During the first and second days of cultivation slightly more biomass was recorded in anoxic culture than for the anaerobic culture. Color removal in the first and second days of incubation was better in the anoxic culture than in anaerobic culture which may be related to higher biomass recorded. The results obtained in this study are in consistent with Chen *et al*. (2003), who found fast removal of an azo dye, Red RBN decolorization under anaerobic and anoxic culture of *Aeromonas hydrophilic.* Bhatt *et al*. (2005) also reported unagitated cultures of *Pseudomonas aeruginosa* exhibited 1.8 fold higher decolorization activity in comparison to agitated cultures. Generally, it was reported that microbial degradation of azo dyes is often an enzymatic reaction linked to anaerobiosis, because it is inhibited by oxygen that is in competition with the azo group as the electron receptor in the oxidation of the reduced electron carrier, i.e. NADH (Wuhrmann *et al*., 1980; Zimmerman *et al.,* 1982; Banat *et al*., 1996). However, some bacteria are able to decolorize azo compounds in the presence of oxygen (Wuhrmann *et al*., 1980).

In this study the effect of initial dye concentration on the rate of decolorisation of RR 184 by isolate A55 was studied in the presence of 50 - 250 mg/l dye in the medium. After 96 h of cultivation the organism decolorized almost all the dye present. Previous studies showed that dye concentration can influence the efficiency of microbial dye decolorization through a combination of factors including toxicity imposed by the dye at higher concentrations (Bhatt *et al*. 2005). However, results of the present study showed that, higher concentration of dye (250 mg/l) was not toxic to isolate A55. Given that dye concentration of textile industries wastewater is believed to be in the range of 10 - 200 mg/ l, this culture could be successfully employed for treatment of dye bearing industrial wastewaters.

In order to check if isolate A55 degrade other commonly used dyes, the culture was tested on MSM supplemented with 50 mg/l of six different additional dyes and RR 184 (Table 4.6).

The organism was found to decolorize all the dyes. However, the efficiency of decolorization varied from dye to dye in the range of 51 - 100 % at the end of the fourth day. During the first day of incubation, isolate A55 decolorize Reactive Red 184 and Reactive Yellow 84 with maximum decolorization efficiency (86 %) followed with the least decolorization efficiency by Reactive Red 141 and Reactive Blue 198 results 24 and 22 %, respectively. Similar observation was obtained by Chen *et al*. (2003). He was successful to get decolorization results ranging from 20-100% within 7^{th} day of incubation using DEC1 (*Aeromonas hydrophila)*. In the present study, it was possible to achieved 22-86 % of decolorization within the first dye of incubation which is better than results reported by Chen *et al.* (2003) 5- 81% within one day.

It was reported that decolorization variation depends on the structure and complexity of the dyes, particularly on the nature and position of substituent in the aromatic rings (Wallace, 2001; Miao, 2005). For instance, the half-life of hydrolyzed Reactive Blue 19 is about 46 years at pH 7 and 25 ºC (Hao *et al.,* 2000). In the present study, isolate A55 showed least color removal efficiency when Reactive Blue 198 were used. In general, the implication of this study indicate that to bring about complete dye degradation of all the dyes present other organism or mixed culture must be selected for textile wastewater treatment.

6. CONCLUSION AND RECOMMENDATION

6.1. Conclusion

Batch treatment of synthetic waste decolorization using azo dye, RR 184 and alkaliphilic soda lake inoculum was conducted under anoxic condition. An attempt was made to isolate different groups of microorganisms from this soda lake inoculum that can decolorize RR 184.

During the screening activity, isolate A55 was found to be effective decolorizer (more than 80%) within the first day of incubation using RR 184 dye concentrations of 50,100 and 150 mg/l. From the study, it has been found that the decolorization efficiency of isolate A55 was dependent on the presence of carbon and nitrogen sources. Organic nitrogen sources (yeast extract and peptone) were found better than inorganic sources.

Dye decolorization efficiency of isolate A55 was found to be higher in cultures incubated under anaerobic and anoxic condition which indicate involvement of oxygen sensitive metabolic (decolorization) process.

The ability of isolate A55 to decolorize seven different dyes with decolorization efficiency of more than 51 % and 100 % for the maximum dye concentration range tested indicates its potential application for treating textile effluents. Generally, the result of this work showed that, the potential of soda lake inoculum for bioremediation of dye–rich textile industry effluent.

6.2. Recommendation

1. The results of this study showed the potential application of alkaliphilic soda lake isolate decolorizing synthetic textile waste. Therefore, additional detail studies(pH, temperature and inoculum size) are needed to evaluate decolorization and other waste removal parameters (COD,BOD, total nitrogen) of an individual isolate using real textile wastewater., decolorization
2. Since the results of this study did not address identification of metabolic end product of the dye, future focus will be better to cover this gap and assessing the products to the level of mineralization.
3. Dye decolorization efficiency of isolate A55 was found to be higher in cultures incubated under anaerobic and anoxic condition which indicates involvement of oxygen sensitive reductase in the decolorization process. However, the study didn't address assaying enzyme activities in the culture and it requires further detailed investigation.
4. Evaluation of decolorization under continuous anaerobic-aerobic treatment system also requires future attention.
5. Identification of the isolate at molecular level is the limitation of this work. Therefore, future attention should focus to fulfill this gap.

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