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Biodegradation of Textile Dye Reactive Black GDN by Free Cells Isolated from Soil and Textile Effluents

Palkesh D. Gandhi[1] and Sumaiya A. Shaikh[2*]

[1]Shree P.M Patel institute of PG studies and Research in Science, Affiliated by Sardar Patel University, Anand, India
[2]Department of Microbiology, C.B.Patel Computer College and J.N.M.Patel Science College, Bharthana (Vesu), Surat -395017, India

**Corresponding author*

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A B S T R A C T

Azo dyes, which are characterized by azo bonds, are a predominant class of colorants used in tattooing, cosmetics and consumer products. As per the requirement for dyestuff, dyed clothing in the effluent is less susceptible to acids, bases, and oxygen. Thus, conventional chemical and physical methods are not efficient in degrading the dyes. Some microorganisms have the capability to utilize the dyes as an energy source. These dyes are metabolized by bacteria to colourless aromatic amines or non-toxic compound bye enzymatic activity. Wastewater from textile industries poses a high environmental impact and their needs to be treated before discharged into the environment. The present study deals with the degradation of Reactive black GDN by different bacterial cultures isolated from a contaminated site. Amongst 5 cultures, the isolate 4 displayed 96% decolourisation of Reactive black GDN (100 mgl^{-1}) in 24h to 72 h. The colour removal efficiency of the isolate was further improved by optimizing various parameters. The decolourisation of the dye was 1.9 times higher under static as compared to shaking condition. The pH 7.0 and 37^{o}C temperature were found to be optimum for the decolourisation of the dye. The isolate was able to decolorize the dye in the range of 50-500 mg l^{-1}.

Introduction

Usually synthetic dyes are more stable against biodegradation because of having complex aromatic molecular structures (Aksu, 2005) and textile, cosmetic, pharmaceutical, paper and food industries use synthetic dyes widely (Pandey *et al.*, 2007). About 10,000 different dyes and pigments are used in textile industries and over 70-105 tons are produced worldwide per annum (Daneshvar *et al.*, 2007). The production and utilization of dyestuff is increasing because of the rapid increase of

industrialization and man's urge for colour (Mohan *et al.,* 2002). Because of their huge assortment of dye shades, high wet fastness profiles, ease of application, brilliant dyes and minimal energy consumption, reactive dyes are widely used in the textile industries (Shah *et al.,* 2013). There are three common groups of reactive dyes: Azo, pthalocyanine and anthraquinone (Axelsson *et al.,* 2006), most of which are harmful, carcinogenic and mutagenic (Acuner and Dilek, 2004; Rauf and Stiborova *et al.,* 2013). Irregular discharge of highly toxic and coloured effluent containing reactive dyes causes damage to the aquatic environment. Because of the presence of heavy metals, chlorides, aromatics, reactive dyes might be toxic to some aquatic life and may significantly affect photosynthetic activity in aquatic phototrophs because of reduced light penetration (Celia and Suruthi, 2016). Reactive azo dyes have high tinctorial value and less than 1 ppm of the dye produces obvious coloration (Gupta *et al.,* 2003). For the removal of dyes, colour and harmful compounds from wastewater, various physical and chemical methods such as adsorption, coagulation–flocculation, oxidation and electrochemical methods can be used (Lin and Peng, 1994).But these methods have many drawbacks like high-sludge production, high-energy costs, and formation of by-products (Sarioglu *et al.,* 2007). Conversely, being low cost and environmentally benign, bioprocessing can overcome these demerits (Kurade *et al.,* 2017).

India has emerged as one of the largest garment-manufacturing country in the world. The garment sector has become the largest sector of the country's foreign exchange earnings and employs about 50% of its industrial work force (Farhana *et al.,* 2015).The textile industries use large amount of reactive dyes in their production processes and discharge waste water into sewers and drains without any treatment (Chindah *et al.,* 2004). The physicochemical parameters of the effluents in India are much higher than the standard value recommended by Department of Environment (Shuchismita and Ashraful, 2015). The presence of reactive dyes in surface and subsurface water is making them not only aesthetically objectionable but also harmful for animals and causing many human health hazards resulting in diseases, viz. perforation of nasal septum, mucous membrane, dermatitis, and severe irritation of respiratory tract and toxicological effects as well as allergenic potential (Rovira and Domingo, 2019). Untreated textile effluents are spreading in the river, lake and other water body and impart a chemical concentration to the climate; its integrity renders the environmental quality fairly deplorable affecting plant growth and aquatic biodiversity. Because of this, people living around textile industries are now being threatened due to the environmental degradation (Sultana *et al.,* 2009). Therefore, a sustainable bioprocess is badly required to remedy the harmfulness imparted by the reactive dyes in the untreated textile effluents.

In the recent years, a number of studies have focused on using some wide variety of bacteria, fungi, yeast and algae (Mishra and Malik, 2014; Veena *et al.,* 2019) for degrading and absorbing dyes from wastewater. A wide variety of bacteria, fungi, yeast and algae are able to decolorize and degrade a wide range of dyes (Ayed *et al.,* 2010). Under optimum conditions, bacteria can rapidly degrade and even completely mineralize many reactive dyes (Chen *et al.,* 2003). The intermediate metabolites such as aromatic amines, and toxic or non-toxic compounds generated

during the decolourisation process, can be degraded by the hydroxylase and oxygenase produced by bacteria (Wanyonyi *et al.,* 2017).

In such manner, achievement of the textile reactive dye-degrading bacteria from the indigenous environment is very important. Bacteria present in the polluted textile effluents might have capabilities to degrade textile reactive dyes. Although several studies have been done showing absorption and degradation of dyes by microorganisms (Shen *et al.,* 2015) additional studies are needed to develop biotechnology to degrade and detoxify the reactive black GDN dyes in effluents and wastewaters generated from textile industries. In the present study reported herein, bacteria were isolated and identified from polluted textile effluents and the surrounding soils. These isolates decolorized reactive dyes used in the textile industries.

Different physicochemical parameters were optimized for decolourisation of reactive Black GDN, the reactive dye commonly used in textile industries, and bacterial biodegradation was shown as the mechanism of decolourisation of reactive Black GDN.

Materials and Methods

Dyestuff, media and chemicals

Stock solution of the reactive black GDNN was prepared by dissolving 1 g of dye into 1000 mL of sterile distilled water, filtered and stored in brown bottle at room temperature. From the stock, working solution was prepared to give a final concentration of 100 mg $L^{-1}$and used for isolation, enrichment and screening of the potent dye decolorizing bacteria. All reagents media and ingredients were of analytical grade with desired purity and purchased from HiMedia Laboratories, India; Merck and Germany.

Isolation of organism

The bacterial isolates were isolated by serial dilution method using streak plate technique on Nutrient broth (gL^{-1}Peptone-5, Meat extract-1, Yeast extract-2, NaCl-5, pH-7) A stock solution of the dye (1000mg L^{-1}) was prepared in distilled water and used for all studies. The flasks were incubated at 37°C under shaking conditions (130rpm) and steady condition. After 48h of incubation, 1.0 ml. of the culture broth was appropriately diluted and plated on Nutrient Agar (gL–1 Peptone-5, Meat extract-1, Yeast extract-2, NaCl-5, Agar-15, pH-7.0) containing 1000 mg L^{-1}Reactive black GDN.

The Morphologically distinct bacterial isolates showing clear zones around their colonies due to decolorization of dye were selected for further studies. The pure culture stocks of these isolates were stored at 4°C on Nutrient Agar slants containing 100 mg L^{-1} of Reactive black GDN. These isolates were screened for their ability to decolorize Reactive Black GDN in liquid culture.

Growth and colony characteristics

Growth and colony characteristics for all the 8 bacterial isolates were carried out by inoculating 1ml($1.5x10^9$ approximate suspension/ml according to McFarland standard) culture into Nutrient broth medium and streak on nutrient agar plate.

Cultures were grown overnight in Nutrient broth medium and next day a 1ml young culture was transferred to nutrient agar plate with dye (1000mg/lit) and slants. They were incubated at 37^o C for 24 h. (Fig.3)

Gram reaction and cell morphology

Gram's staining of the 24 h young cultures of all the isolates was performed to study Gram reaction and the cell morphology (Bartholomew J w., Mittwer t., 1952).(Fig.2)

Biochemical tests

All biochemical tests (Oxidase, Catalase, Indole, M-R, V-P, Citrate, Urease, Starch, H_2S Production, TSI, Gelatine, Glucose, Sucrose) were prepared in respective, test tubes, flasks, and petri dishes. Reagents required for different biochemical tests were prepared and stored at 4^o C in refrigerator. Overnight grown cultures of all 8 isolates were inoculated 10 µl in media and incubated at 37^o C for 24 hrs.

Dye decolorizing isolates were preliminary identified on the basis of morphological, cultural and biochemical characteristics according to Bergey's Manual of Systematic Bacteriology (Staley *et al.*, 2001).(Fig.4)

Screening for decolourisation

The bacterial isolates were analysed for the degradation of reactive black GDNN dye in broth cultures. The flask containing nutrient broth medium [Peptone 10.000 gram, Beef extract 10.000grm, Sodium chloride 5.000grm, and 1000ml distilled water] and dye was inoculated using 1ml ($1.5x10^9$approximate suspension/ml according to McFarland standard)of isolated bacterial suspension. The culture flasks were incubated on an orbital shaker with 130rpm, at 37^0 C. and in steady condition in incubator at 37^0c. The flasks without inoculation were kept as control. OD values and growth of organism were measured spectrophotometrically at 530nm to estimate the decolourisation process. The rate of decolourisation was calculated using the following formula.(Fig.1)

% Decolorization

$$= \frac{\text{Intial absorbance value} - \text{final absorbance value}}{\text{Intial absorbance value}} \times 100$$

Various factors were optimized to achieve highest degradation by the selected bacterial isolate.

Effect of pH

The bacterial culture was inoculated in 100 ml nutrient broth medium with Reactive black GDN dye at various pH. ranging from 4,6,8. The flasks with different pH were kept in incubator for 24 hrs, 48hrs, and 72hrs at 37^0c. These flasks were drawn at every 24 hours intervals for dye decolourisation assay. Readings were taken after every 24 hours for 3 successive days.(Graph.5&6)

Effect of Incubation time

The incubation time varying from 24hrs to 96hrs at 37^0c were examined for the detection of optimum incubation time required for the degradation of dye by bacterial isolate. These flasks were drawn at 24 hours intervals for dye decolourisation assay. Readings were taken after every 24 hours to 96 hours.(Graph.7)

Results and Discussion

The sample were collected in sterilized container from Sachin GIDC, Surat and analysed Physico-chemical characterization. The colour, temperature and pH of the sample were recorded on the site and samples were transported to the laboratory by storage at 4^o C. Other physico-chemical characteristics like colour, pH and temperature were measured on the same day of collection of sample as per Table 1. The raw sewage was black in colour because of the types of dyes generally used. As the stages of treatment progressed, the

colour of effluents changed from black- red and finally light brown. The black colour of the incoming effluent is due to wide use of black dye in dyeing and printing industries, thus, it contributes more to the effluent's colour compared to other dyes. The light brown colour of the finally released effluent after treatment may be due to the dirty water condition. The pH of the untreated effluent was 9.8, which reduced during treatment to near neutral 7.8.

Isolation and screening of bacterial strains

The selective enrichment of liquid effluent, sludge, and soil sample collected from the Sachin GIDC, Surat and waste disposal sites, led to the isolation of 8 morphologically different bacterial isolates. Gram strain of all isolates indicated the presence of 2 Gram positive and 6 Gram negative organisms (Table 3). The pure cultures were preserved on N-agar medium at 4^{o} C. All 8 isolates were tested individually for their ability to decolorize Reactive black GDN separately at the concentration of 100 mg L^{-1} each (Table 2). All isolates decolorize the dyes with different capacity ranging from lowest 12% to highest 94% in case of Reactive Black GDN. Five potential isolates namely; ISO1, ISO3, ISO4, ISO5, and ISO6 showed good decolourisation efficiency in Reactive black GDN. The dye concentration in effluent from textile printing house is approximately in the range of 50 to 500 mg L^{-1}. This value is typical of those used in studies on treatment for azo dye containing effluent. However, change in operating processes may lead to still high concentration of dye in effluent. Keeping in mind the above fact, we used 100 mg L^{-1} dye concentrations to check microbe's ability to decolorize different dyes. Decolourization of Reactive Black GDN was around 97% by ISO4, ISO5 and ISO6 at optimum pH. ISO1, ISO3, and ISO7 decolorized this dye at, 32%, 40%, and 75%, respectively. The dye that has been mainly studied, Reactive Black GDN, was decolorized to more than 70% by all the isolates whereas this dye was decolorize up to 98% by ISO4 and ISO6, the most studied organism in this study. The lowest and highest decolourisation of different concentrations of dyes by selected organisms were in the range of 12% to 94% for Reactive Black GDN. The isolation of different microorganisms from the sample indicates the natural adaptation of microorganisms to survive in the presence of toxic dyes. The difference in their rate of decolourisation may be due to the loss of ecological interaction, which they might be sharing with each other under natural conditions.

Growth and morphological characteristics

Morphological characteristics was obtained for all the 8 bacterial isolates. Wide variation in morphological characteristics was found indicating diversified bacterial species in textile effluent. The Gram's staining indicated that out of 8 isolates, Gm +ve rods-1, Gm -ve short rods -6, and Gm +ve cocci - 1, and (Fig.2). The additional information from Gram staining was in the form of cell morphology and arrangement. The growth pattern of these isolates on nutrient agar plate was filiform, echinulate and arborescent with moderate or large growth abundance (Table 4). It was found that most of the organisms were of rod shaped including short and big rods (Fig.2). The potential dye decolourizers were found in, Gm +ve and Gm -ve group. When organisms were grown on N-agar plate, there was characteristic pigmentation of colonies like white, dirty white, grey, light yellow and light brown. One isolates ISO5 were found to produce dark pigmentation of yellow(Fig.3). Size of colonies varied from small to moderate to large having smooth or rough texture with even, uneven, wavy filamentous margins and circular, rhizoid and irregular forms.

Table.1 Characteristics of samples collected from different stages of Sachin GIDC, Surat

Sr.	Sample	Nature of Sample	Colour	pH
1	Effluent 1	Liquid	Dark black	9.8
2	Effluent 2	Liquid	Dark black	8.2
3	Soli 1	solid	Black	5.7
4	Soil 2	solid	brown	4.6

Table.2 Decolourization of Reactive black GDN by bacterial isolates ISO1 to ISO8 (Dye 100 mg l^{-1})

Bacterial isolates	Decolourization (%) Reactive Black GDN
ISO1	26%
ISO2	15%
ISO3	40%
ISO4	94%
ISO5	40%
ISO6	90%
ISO7	73%
ISO8	12%

Table.3 Microscopic observation of isolates collected from soil and effluent the Sachin GIDC, Surat

No. of Isolates	Gram's reaction	Colour of cells	Motility
ISO-1	Gram Negative	Pink	Non motile
ISO-2	Gram Negative	Pink	Motile
ISO-3	Gram Positive	Purple	Motile
ISO-4	Gram Negative	Pink	Motile
ISO-5	Gram Negative	Pink	Motile
ISO-6	Gram Negative	Pink	Motile
ISO-7	Gram Negative	Pink	Motile
ISO-8	Gram Negative	Pink	Motile

Table.4 Colony characteristics of isolates

Isolates	Size	Shape	Margin	Elevation	surface	Consistency	Opacity	Pigmentation
ISO - 1	L	Round	Entire	Convex	Smooth	B	OP	NP
ISO - 2	S	Round	Even	Low convex	Smooth	Gummy	OP	DW
ISO - 3	L	Irregular	Irregular	Flat	Rough	Dry	OP	NP
ISO - 4	L	Round	Entire	Convex	Smooth	B	TP	NP
ISO - 5	L	Round	Even	Flat	Smooth	B	TL	GB
ISO - 6	S	Round	Entire	Flat	Smooth	B	TL	LY
ISO - 7	S	Irregular	Uneven	Convex	Rough	Dry	OP	NP
ISO - 8	S	Irregular	Uneven	Convex	Rough	Dry	OP	NP

NOTES: S = Small, L = Large, B = Buterious, OP = Opaque, TP = Transparent, TL = Translucent, NP= No pigment, DW = Dirty White, GB = Greenish Blue, LY = Light Yellow.

Table.5 Biochemical Characterization of selected bacterial isolates from effluent and soil

Biochemical Tests	ISO - 1	ISO - 2	ISO - 3	ISO - 4	ISO - 5	ISO - 6	ISO - 7	ISO - 8
Oxidase	**N**	**P**	**N**	**P**	**P**	**N**	**P**	**P**
Catalase	**P**	**N**	**P**	**P**	**P**	**P**	**P**	**P**
Indole	**N**	**N**	**N**	**N**	**N**	**P**	**N**	**N**
M-R	**N**	**N**	**N**	**N**	**N**	**N**	**N**	**N**
V-P	**N**	**P**	**P**	**N**	**P**	**P**	**N**	**N**
Citrate	**P**	**P**	**P**	**P**	**P**	**N**	**P**	**P**
Urease	**P**	**N**	**N**	**N**	**P**	**P**	**N**	**N**
Starch	**N**	**P**	**P**	**N**	**N**	**N**	**N**	**N**
H_2S Production	**N**	**P**	**N**	**N**	**N**	**P**	**N**	**N**
TSI	**P**	**N**	**N**	**N**	**P**	**P**	**N**	**N**
Gelatine	**N**	**P**	**N**	**N**	**P**	**N**	**N**	**N**
Glucose	**P**	**P**	**P**	**P**	**P**	**P**	**P**	**P**
Sucrose	**P**	**P**	**P**	**P**	**P**	**P**	**P**	**P**

Notes : P= Positive test, N = Negative test, MR = Methyl red test, VP = Voges Proskauer, TSI = Triple sugar iron test.

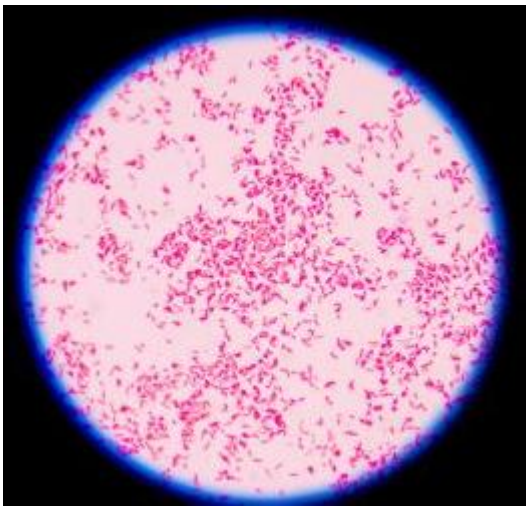
Fig.1 a) treatment with ISO5 and b) treatment with ISO4, Samples collected before and after treatment of textile effluent (Sachin GIDC, Surat, Gujarat.)

a)

b)

Fig.2 Microscopic Gram staining observation of isolates collected from soil and effluent the Sachin GIDC, Surat. a) Gram Negative and b) gram positive

a)



b)

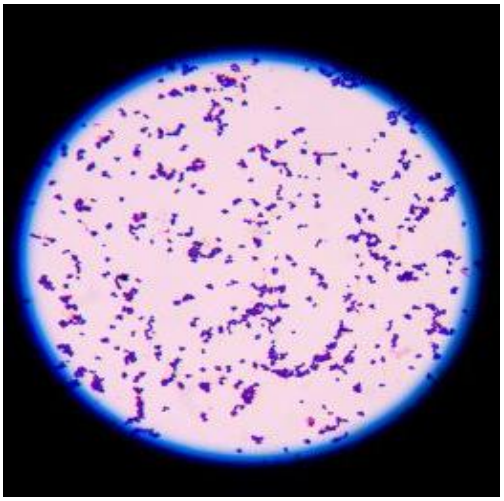


Fig.3 Isolates on Nutrient Agar Plate

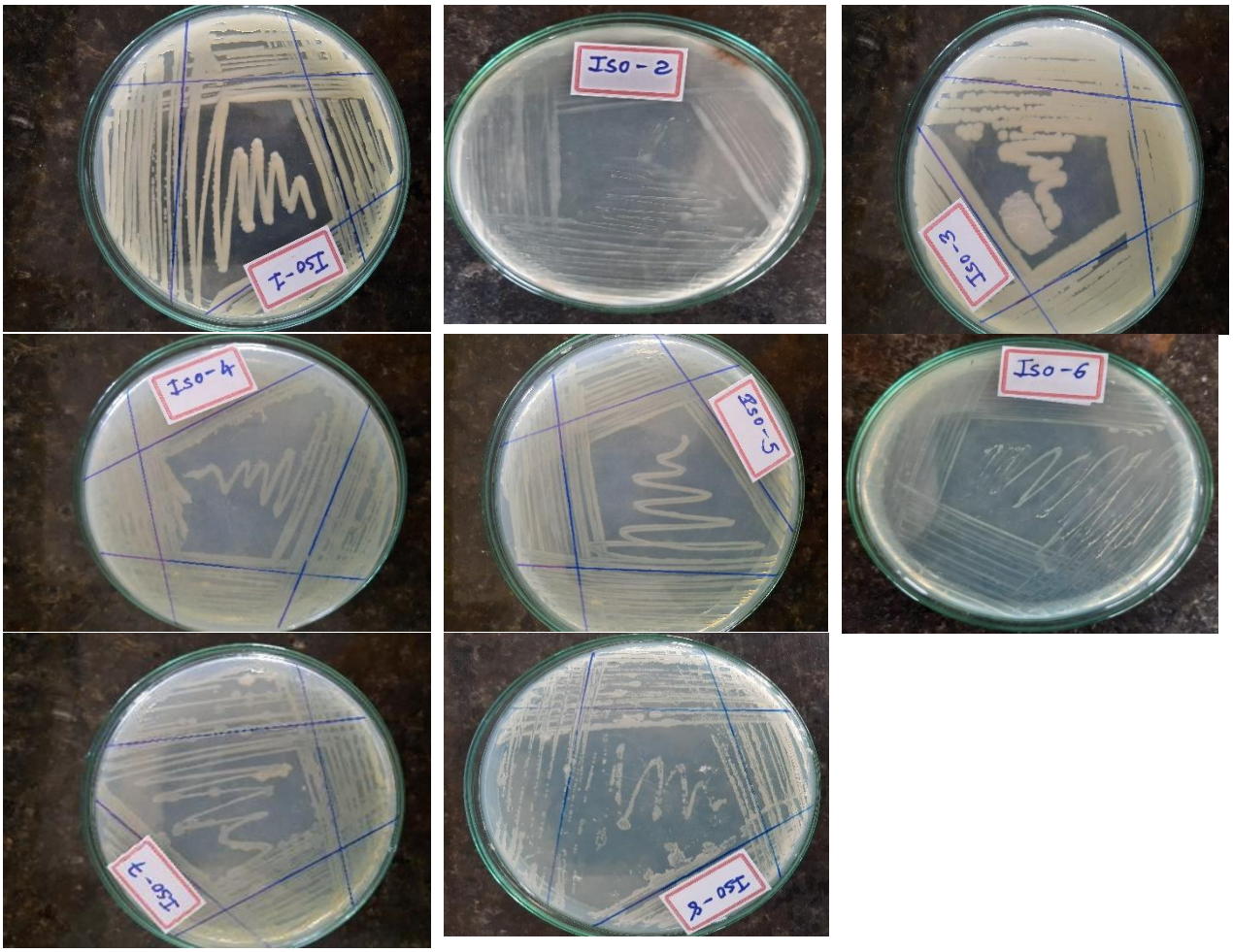


Fig.4 Results of various Biochemical test

a)+ve test on cimmon Citrate
b) control

a)+ve glucose fermenter test
b) control

a)+ve test on TSI Slant
b)control

Fig.5 Degradation of Reactive black GDN at pH 4 by various types of Isolates

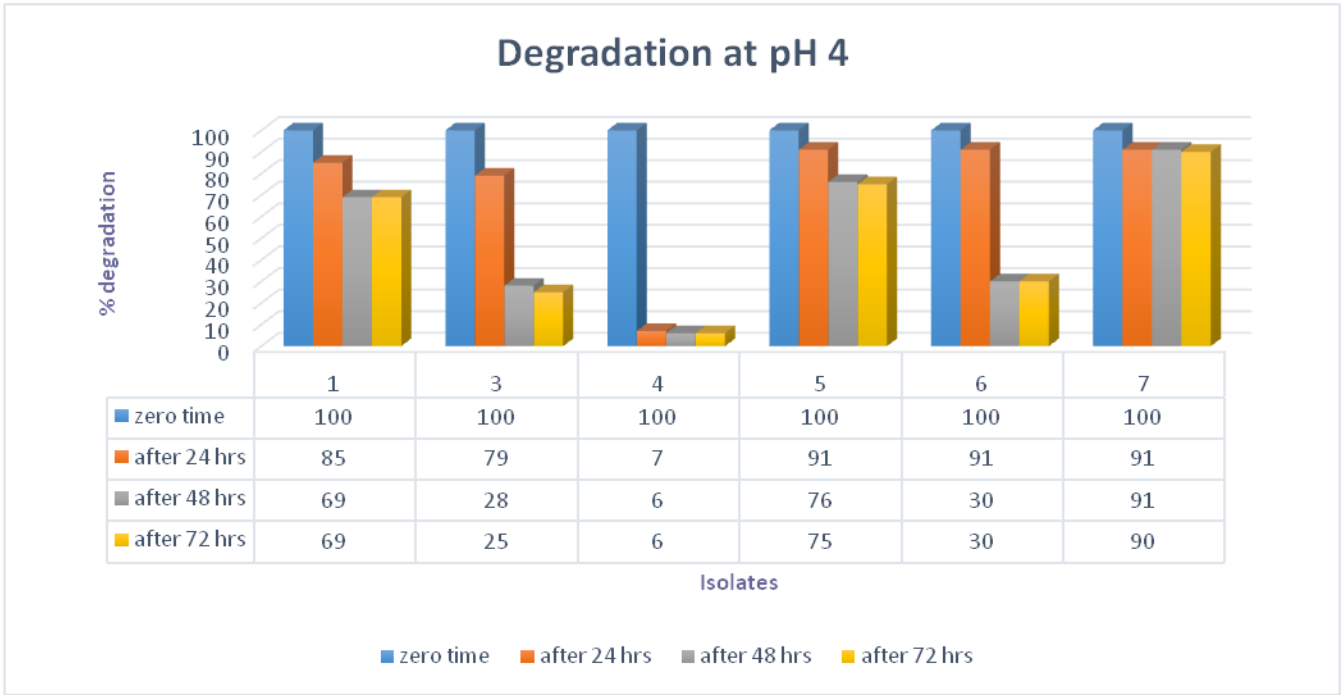


Fig.6 Degradation of Reactive black GDN at pH 8 by various types of Isolates

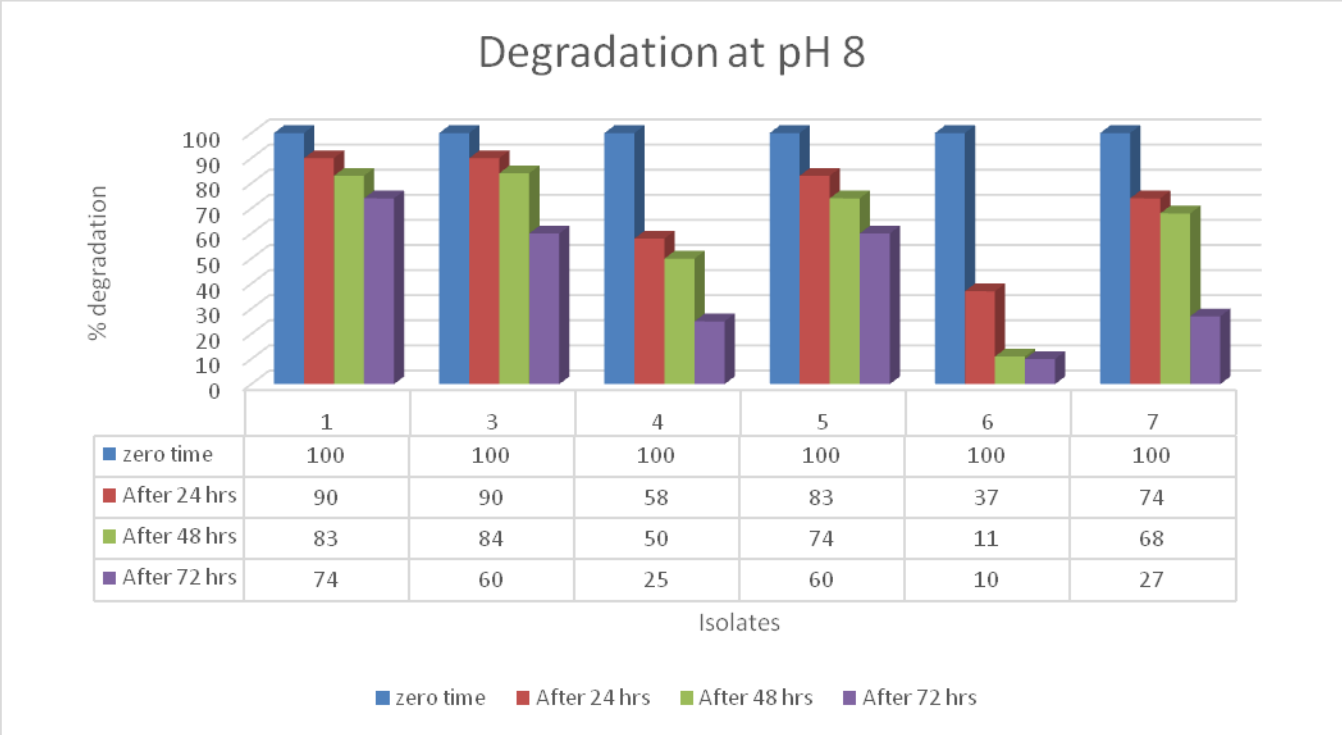
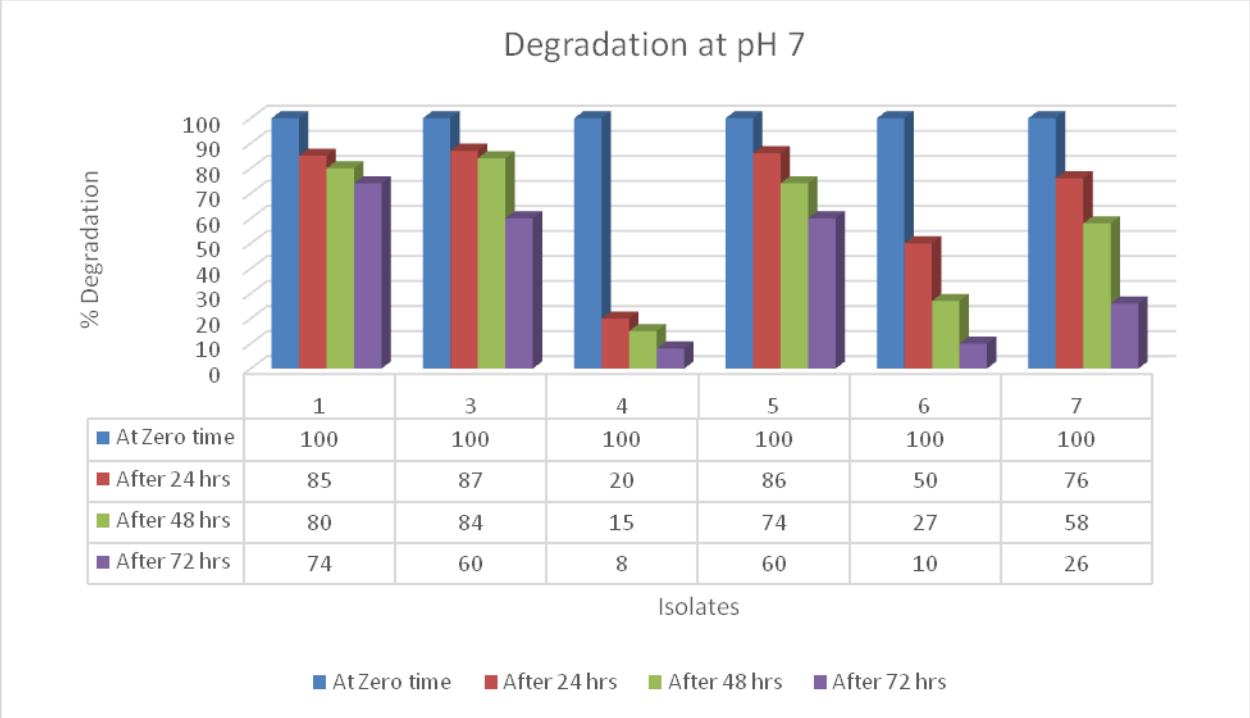


Fig.7 Degradation of Reactive black GDN varied with incubation time at pH 7 by various types of Isolates



Biochemical Tests

Bacterial isolates were considered for their characterization, based on Gram's reaction, cell morphology, colony characteristics, growth patterns in nutrient broth and biochemical tests. Table 3and Table 4 show results of various characters studied.ISO1, ISO1, ISO2, ISO3, ISO4, ISO5, ISO6, ISO7, and ISO8 produced acid present in TSI which is evidenced by conversion of slants from red to yellow. The result showed that many of them occurred commonly in such environment.

Effect of pH

The pH tolerance is an important consideration for industrial applications because processes using reactive azo dyes are usually performed under alkaline conditions. (Aksu and Tezer, 2005). The experiment was performed in 250ml Erlenmeyer flasks containing 100 ml nutrient broth medium with 100mg/l dye. It was observed that the percentage of dye decolourisation varied with change in pH of the medium (Graph.5, 6 and 7).

Although decolourisation rate peaked around pH 8 at 18 hrs, the organism decolorized more than 96% of the dye by incubation up to 24 hrs on wide range of pH (4-7-8). However, ISO1, ISO3, ISO6 and ISO7 organism showed very poor decolourisation at the pH 4.ISO4 shows 94% degradation at pH4 respectively (Graph.5). ISO7 and ISO8 shows No growth was observed at pH 4.

These observations indicated that the organism can treat basic dyeing waste water at normal operational pH and decrease the cost of acidification (Ayed *et al.,* 2009). Graph.5,6 and 7 depicted the effect of various pH on decolourisation process under steady condition. Under steady condition ISO6 showed reduced decolourisation of 87% and 48% at pH 8, respectively; while under steady the organism showed increased decolourisation of 100% and 93% at pH 8.

At lower pH values, the H+ ions compete effectively with dye cations, causing a decrease in colour removal efficiency.

Furthermore, at high pH, the surface of biomass gets negatively charged, which enhance the positively charged dye cations through electrostatic force of attraction.

Effect of incubation time

The experiment was performed in 250ml Erlenmeyer flasks containing 100 ml nutrient broth medium with 100mg/l dye. It was observed that the percentage of dye decolourisation varied with incubation time (Graph.7).ISO4 Shows 92% degradation in 24 hrs and ISO6 and ISO7 shows maximum degradation after 72 hrs at pH 7.

Two bacterial isolates,ISO4 shows maximum degradation at pH4 at 37^0cin 24hrs and ISO6 shows maximum degradation at pH8 at 37^0c in 48hrs under steady condition bacterial isolates having the best capability to decolorize reactive textile dyes were screened and their biochemical characteristics.

The decolourisation of Reactive Black GDN by the bacterial isolates is due to biodegradation and is dependent on various physico-chemical parameters.

Degradation and decolorizing activity against Reactive black GDN dye suggests that the bacterial isolates in this study have potential practical application in the biotransformation of various dye effluents. Currently, research is going on to characterize the enzymes, especially laccase, peroxidase and azoreductase, of these bacterial isolates.

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