Isolation of Reactive Red 3BX and Yellow 4GL dye degrading bacteria from the textile sludge

A DISSERTATION SUBMITTED TO THE DEPARTMENT OF MATHEMATICS AND NATURAL SCIENCES, BRAC UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIRMENT FOR THE DEGREE OF BACHELOR OF SCIENCE IN BIOTECHNOLOGY

Submitted by

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Declaration

I hereby declare that the thesis work titled "**Isolation of Reactive Red 3BX and Yellow 4GL dye degrading bacteria from the textile sludge**" has been written and submitted by me, Ahmed Abdullah Fahim (ID-11336004) Department of Mathematics and Natural Sciences under the supervision of Ms. Romana Siddique, Senior Lecturer Department of Mathematics and Natural Sciences without the use of other sources than those mentioned.

It is further asserted that this Bachelor's Thesis has never been submitted in the same or substantially similar version to any other examinations office. All explanations that have been adopted literally or analogously are marked as such.

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Abstract

This world is getting polluted in different ways. This world has many prime components including soil, water, and air. Every day to meet human needs thousands of mills, factories and are producing necessary commodities. And they are polluting the environment in different ways. Some factories are emitting poisonous gases, some are discharging effluents and liquid waste in the environment which are creating hazardous problems in the water system and in soil. And for this environment pollution one of the prime causes is discharging textile dyes into the environment without treatment. This is a common problem for many countries as well as for the Bangladesh. Textile dyes are chemical substances which can cause serious damages to the nearby eco life. The treatment of the textile dyes after using in the factories is either costly or time consuming. As a result mills and factories are not willing to do the treatment process every time, rather they discharge it without any treatment. In this project textile dye degrading bacteria were isolated from the textile sludge to degrade the textile dyes since this biological process is less expensive than the other existing process. Two bacterial strain ***Brevibacillus laterosporus and Staphylococcus nepalensis*** were isolated from the textile sludge which was tested with two textile dyes; they are Yellow 4GL and Reactive Red 3BX. And these both bacteria are very much effective to degrade these dyes. Especially ***Brevibacillus laterosporus*** *can* de-color the Reactive Red 3BX dye in a very short time; even the higher concentration of the dye can be degraded.

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LIST OF ABBREVIATIONS

Abbreviations	Descriptions
SM	Salts Media
NA	Nutrient Agar
YG2	Yellow 2G
NPK	Nitrogen, phosphorus, potassium
MIU	Motility, Indole, Urease test
MR	Methyl Red
VP	Voges-Proskauer
Y4G	Yellow 4G
rpm	rotations per minute
µL	microliter
mL	milliliter
g/L	grams per liter
v/v	volume by volume
RR	Reactive Red
nm	Nanometer
ETP	Effluent treatment plant

Chapter one: Introduction

1. Introduction:

From the very beginning of industrialization process some industries are still very much useful for mankind. Among these industries textile industry has a very significant role in human life and as well as on the total economy sectors. The textile industry is controlling the economy from the very early stage of industrialization. This has been possible since cloth is one of the basic needs of human. From the 18^{th} century garment sectors are totally depended on the textile factories as all the garment ingredients are made in textile factories. And for clothes color is one of the main components. People are used to wear colorful clothes from the very early stage. So different kind colors were invented to made clothes more attractive. By this dying industries raised up along with the textile industries. Now a day's dying section becomes must for every textile industry. For every color different kind of dyes are used in many textile factories. But these colors are different from those colors which are used in the foods. Because dyes are chemical compounds which are very hard to degrade. And most of them are very toxic substance. That is very harmful for the biological substance. Even these chemical substances are so much strong that to make the chemical degradation of them a lot of money, time and effort is needed to be given. Back then in 19^{th} centuries there was no strict rules for these dyes. That time textile factories used those harmful dyes on their products and after using them they just discharged them in the open environment without any treatment or purification of those dyes. Ultimately those dyes became the most harmful and hazardous components for the environment. That is one of the prime pollutants which are responsible to pollute the environment. But though they have such hazardous effects on the nature, to maintain the demand of clothes it becomes very elementary things to run a textile industry. So in many countries laws were developed to control the disposal of these dyes in environment without any treatment. Among those laws the prime law was every textile industry must have to have ETP plant. In these plants used dyes were gone under different kind of treatments to remove the toxic substances by using different kind of chemical methods. And even in Bangladesh this law is active. To set up a textile industry having an ETP plant is must. But it is a matter of sorrow that very few industries have the ETP plants. Maximum industries do not have any ETP plants. Those who have this they do not properly use it. The authorities just use it to save their skins from the law. As a result after having ETP plants

these industries are disposing dyes in the environment. And obviously they are polluting the nature. The main reason behind this attitude is to make a toxic azo dye into toxin less material, the chemical process is too much expensive. It is not an easy job to do. So that is many industries are not interested to do this process. As a result biological populations are getting destroyed.

1.1What are Azo dyes:

Azo dyes are a large class of very effective synthetic dyes used for coloring a variety of consumer goods such as foods, cosmetics, carpets, clothes, leather and textiles.

A small proportion of azo dyes contains, or can break down to form, a class of chemical substances referred to as aromatic amines.

Certain aromatic amines such as benzidine, 3,3'-dimethoxybenzidine and p-aminoazobenzene that may be derived from azo colourants are considered to be hazardous. Expert authorities such as the World Health Organization (WHO) International Agency for Research on Cancer (IARC) have classified some of these aromatic amines as known, or suspected, human carcinogens. A carcinogen is a substance that is capable of causing cancer. Exposure to a carcinogen does not mean cancer will result (1).

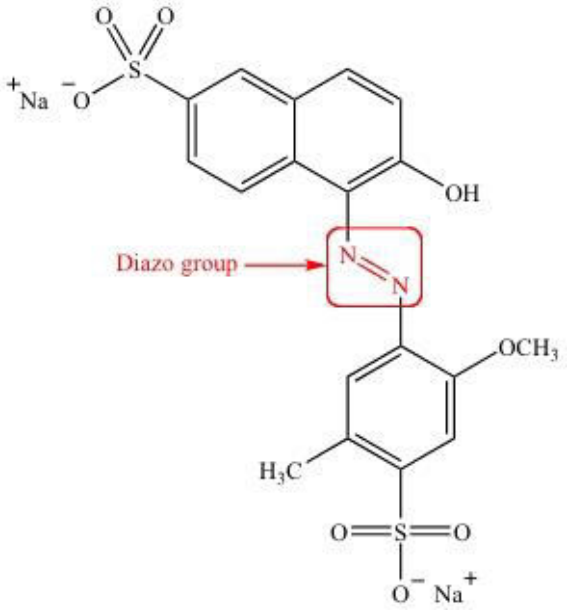


Figure1 : Bond in Azo dye (2)

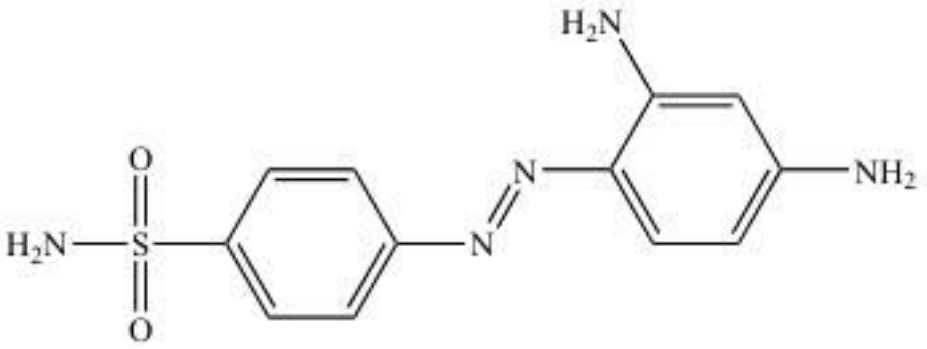


Figure 2 : Bond in Azo dye (3)

Those azo intensify population accounts for 60-70% about all dyes. Likewise it might be expected, they all hold numerous a azo group, -N=N-, which joins two sp2 hybridised carbon iotas. Often, these carbons need aid and only fragrant systems, yet this is not continuously the case. The majority azo dyes hold special case azo group, yet some hold two (disazo), three (trisazo) or more than 2.

In theory, azo dyes can supply a finish rainbow for colors. However, commercially they tend should supply a greater amount yellows, oranges and reds over whatever viable colors. Examine may be continuously continuing, though, with the goal that Right away there would a few feasible blue azo dyes on the market (2). The association between those color from claiming an azo color need been additional completely examined in the support for shade (4).

Some names of the azo dyes are given bellow,

1. Blue Br
2. Yellow 2G
3. Yellow 4G
4. Orange 2R
5. Reactive Red
6. Reactive violet e.t.c

1.2 Effects of textile dyes on environment:

The measurement of BOD and COD offers a good indication of the organic pollution of water. But these procedures alone are not sufficient to get information about the potential harmful effects of chemicals (APHA, 2005). The toxic effects of other unknown and undetermined substances in complex wastewaters can be estimated only through toxicity studies. Toxicity study refers to bio-analytical techniques applied to organisms at various levels to ascertain the harmful effects of chemicals on them (Blaise et. al., 2008, Slabbert& Venter, 1999). The assessment of toxicity is done by acute and enrichment toxicity tests (Schowanek et. al., 2001).

In addition to being toxic, dye effluents also contain chemicals that are carcinogenic, mutagenic or teratogenic to various organisms (Novotny et. al., 2006, Mathur&Bhatnagar, 2007). This is especially serious because many chemicals can cause damage to genetic material without being expressed immediately (Vogel, 1982). Azo and nitro compounds have been reported to be reduced in sediments of aquatic bodies giving rise to potentially carcinogenic amines (Chen, 2006). Many dyes are made from known carcinogens like benzidine and are also known to accumulate, thus posing a serious threat (Baughman &Perenich, 1988). Many dyes are also known to get reduced to toxic substances inside living organisms (Weber & Wolfe, 1987). The carcinogenicity of azo dyes, which constitute a significant proportion of textile dyes is well known (Weisberger, 2002, Umbuzeiro et. al., 2005). Some azo dyes have been linked to bladder cancer in humans; to splenic sacromas, hepatocarcinomas and nuclear anomalies in experimental animals and to cause chromosomal aberration in mammalian cells (Mendevedev, 1988, Percy et. al., 1989). There is evidence that malachite green not only has effect on immune & reproductive systems but also is a potential genotoxic and carcinogenic agent (Srivastav et. al., 2004). CI disperse blue has been shown to cause frame-shift mutation and base pair substitution in Salmonella (Umbuzeiro et. al., 2005). The genotoxic and cytotoxic effect of this dye on human cells have also been studied (Tsuboy et. al., 2007). The authors report the formation of micronuclei which are formed due to chromosomal breakage (clastogenicity) and aneuploidy. They also found DNA fragmentation due to single and double strand breaks (Tsuboy et. al., 2007) (Ratna&Padhi, 2012).

1.3 Water pollution by textile dyes:

Factories release a large number of gallons of this effluent as risky lethal waste, brimming with shading and natural chemicals from coloring and completing salts. Vicinity of sulfur, naph-thol, vat colors, nitrates, acidic corrosive, cleansers, chromium com-pounds and overwhelming metals like copper, arsenic, lead, lowlife chromium, mercury, nickel, and cobalt and certain assistant chemicals all things considered make the emanating very poisonous. Other hurtful chemicals present in the water may be formaldehyde based color altering operators, hydro carbon based conditioners and non-bio degradable coloring chemicals. The plant emanating is likewise regularly of a high temperature and pH, both of which are amazingly harming. The colloidal matter present alongside hues and slick filth builds the turbidity and gives the water a terrible appearance and foul smell. It keeps the entrance of daylight fundamental for the procedure of photosynthesis [28]. This meddles with the Oxygen exchange instrument at air water interface. Exhaustion of broke down Oxygen in water is the most genuine impact of material waste as disso-lved oxygen is extremely key for marine life. This likewise obstructs with self-sanitization procedure of water. Moreover when this effluent is permitted to stream in the fields it stops up the pores of the dirt bringing about loss of soil efficiency. The composition of soil gets solidified and entrance of roots is anticipated (K. Rita, 2011).

Figure 3: Pollution happened by Textile Dye (5)

1.4 Elimination of the poisonous effects of textile dyes from Effluent:

This is one of the prominent parts of textile factories. One of the main sources with severe pollution problems worldwide is the textile industry and its dye-containing wastewaters (i.e. 10,000 different textile dyes with an estimated annual production of 7.105 metric tonnes are commercially available worldwide; 30% of these dyes are used in excess of 1,000 tonnes per annum, and 90% of the textile products are used at the level of 100 tonnes per annum or less) (Baban et al., 2010; Robinson et al., 2001; Soloman et al., 2009). 10-25% of textile dyes are lost during the dyeing process, and 2-20% are directly discharged as aqueous effluents in different environmental components.

In particular, the discharge of dye-containing effluents into the water environment is undesirable, not only because of their color, but also because many of dyes released and their breakdown products are toxic, carcinogenic or mutagenic to life forms mainly because of carcinogens, such as benzidine, naphthalene and other aromatic compounds (Suteu et al., 2009; Zaharia et al., 2009). Without adequate treatment these dyes can remain in the environment for a long period of time. For instance, the half-life of hydrolysed Reactive Blue 19 is about 46 years at pH 7 and 25°C (Hao et al., 2000) (Zaharia Carmen and Suteu Daniela 2012).

There are different kinds of treatment processes to detoxify the textile dyes containing effluent. They are given bellow,

Physico-chemical treatments

1. Precipitation, coagulation flocculation,
2. Electrokinetic coagulation
3. Fenton process
4. Ozonation
5. Oxidation with NaOCl

Adsorption with solid adsorbents such as

1. Activated carbon
2. Peat
3. Coal ashes
4. Wood chips/ Wood sawdust
5. Silica gels
6. Irradiation
7. Photochemical process
8. Electrochemical oxidation
9. Ion exchange

Biological treatments

1. Aerobic process
2. Anaerobic process
3. Single cell (Fungal, Algal & Bacterial)

Emerging treatments

1. Membrane filtration
2. Photocatalysis
3. Sonication
4. Enzymatic treatment

(Anjaneyulu et al., 2005; Babu et al., 2007; Robinson et al., 2001) (Zaharia Carmen and Suteu Daniela 2012) .

1.5 About the project:

These involve microorganisms in dye degrading projects are mostly like fungi (Barr &Aust, 1994) and bacteria (Banet et. al., 1997). Microbial treatment is cost effective and is receiving wide attention (van der Zee &Villaverde, 2005). Biological treatment may involve aerobic and anaerobic degradation by microorganisms. Anaerobic treatment of dye waste water utilizes various anaerobic bacteria producing the enzyme azoreductase. A combination of anaerobic

treatment which results in reductive cleavage of azo linkages and aerobic treatment which degrades the products formed in the first step has been proposed (Frizters et. al., 2006). Zee (2005) has reviewed the work done utilizing the combined, sequential or integrated anaerobic-aerobic bioreactor treatment of dye wastewaters. Application of bacteria for dye removal has been extensively reviewed (Santos et. al., 2007). Some algae have also been used (Aksu, 2005). The role of fungi has been extensively researched and reviewed (Brar et. al., 2006, Kaushik& Malik, 2009). Fungus has proven to be a suitable organism for treatment of textile effluents and dye removal. The fungal mycelia have an advantage over single cell organisms as they can solubilise the insoluble substrates using extracellular enzymes. They have a large cell to surface ratio and hence greater physical and enzymatic contact with environment. They can tolerate high concentration of toxicants. Mucorales fungi have been used for treatment of model textile wastewaters and shown to reduce colour as well as toxicity (Olieveira et. al., 2007).

In this thesis project mainly dye degrading bacteria were isolated for the process which is called single cell treatment. Basically it is a post treatment for the effluent. This treatment has Good removal efficiency for low volumes and concentrations and Very effective for specific colour removal (Anjaneyulu et al., 2005; Babu et al., 2007; Robinson et al., 2001). Though it has some negative sides like, Culture maintenance is cost intensive and cannot cope up with large volumes of waste (Anjaneyulu et al., 2005; Babu et al., 2007; Robinson et al., 2001). But it has basically no side effects. And this process is environment friendly. Anyway in this thesis project two dyes were used, which are commercially available in the market. They are **Yellow 4GL** and **Reactive Red 3BX.**

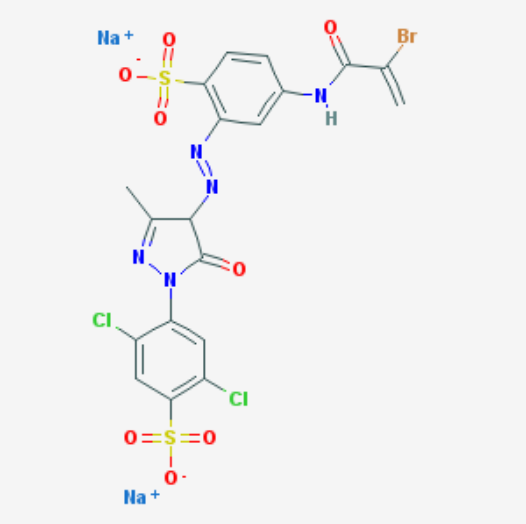


Figure 4: Yellow 4GL structure (6)

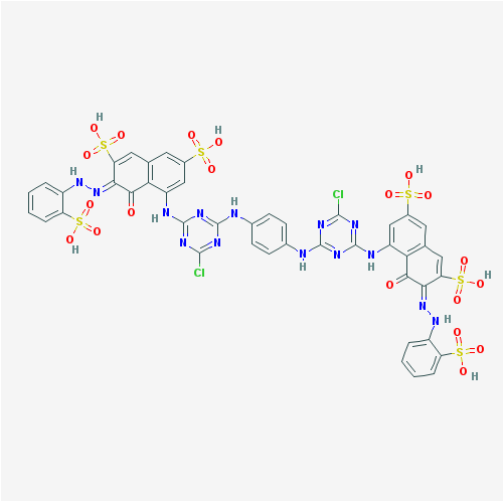


Figure 5: General Reactive Red dye's structure (7)

Chapter Two: Materials and Methods

2. Materials and Methods:

There are different types of materials being used. Some of them are chemicals and some them are apparatus. And also some machines were also used in this total process. Media compositions, apparatus and machines list are given into appendix section.

2.1 Place of research: This total research was conducted into the BRAC University biotechnology laboratory.

2.2 Soil sample collection: At the beginning some factories were visited to collect soil samples from their effluent plants. But the collection permission was cordially refused. So finally the soil sample was collected from a textile factory which is situated outside from Dhaka. And the name of that place is Bhalukha industrial area. The soil was found from the ETP treatment plant from that factory after cleaning it once. Approximately two to three kilogram of soil was collected into a polybag. The soil was wet muddy. It had a peculiar smell. After bringing it to the lab, it was kept in the refrigerator in low temperature. Low temperature was used to keep the microorganisms' viable (T. Teeresa, 1999).

Figure 6: Soil samples from ETP plant

2.3 Trial run to check the dye degrading bacteria's presence:

2.3.1 Preparation of SM broth:

The composition of SM broth is given bellow, since this media was used through the whole project.

For 1000ml of SM broth

Glucose – 10g, Peptone – 10g, Yeast Extract = 1g, Magnesium Sulphate – 1g, Potassium di Hydrogen Phosphate – 1.9g, Di potassium Hydrogen Phosphate – 0.6g

Then adjust the pH to 6.0 to 6.4.

2.3.2 Culturing dye degrading bacteria from soil:

To check the presence of bacteria, at first SM Broth was made. SM Broth is a salt media. But for the sake of experiment a little change was made into the composition of SM broth. The amount of glucose was decreased. So that only dye degrading bacteria can grow in that media. If there is no glucose in that media normal bacteria will not grow in that media. Only those bacteria which are capable to break the dye and can use the dye as their food source will grow. And in that media dye was added. The added dye was previously stored in the lab. The name of the dye was Yellow 2G. Three concentration of dye was given into the media. After preparing the SM broth it was split into 9 conical flasks and autoclaved into 121^0C for 15 minutes.

Figure 7: Dyes added media

After finishing the autoclaving dye was added. 10% solution of dye was made. 5g dye was dissolved into the 50ml distilled water. Then in 10% concentration flasks 1ml dye was directly added. Then in 5% concentration flasks 500 microliter dye and same amount of distilled water was added. And in 1% concentration flasks 100 microliter dye and 900 microliter distilled water was added. After that 5g soil was measured and mixed into the distilled water and 1ml of solution was given into each flask. After that flasks were kept into the shaker in 37^0C temperature. And those flasks were kept there for one week. And every day absorbances of those were taken. At the beginning the absorbance were taken into 580 Nanometer. And the last absorbance was taken by centrifuging those medias into 3000*g speed for five minutes. From each conical flask 4ml of media was taken by micro pipette into falcon tubes. After five days changes in the color was seen. The inoculated media color was changed from the control color. So here after centrifuge the supernatant absorbance was noted down.

Figure 8: Inoculated dyes in shaker

2.4 The absorbance table:

Normal Absorbance (without centrifuge)

Dye name: Yellow G2

Wavelength: 580 Nanometer

Table 2.4.1: Absorbance data for yellow G2 dye without centrifugation.

Concentration	Control	Flask 1	Flask 2
10%	0	-0.171	-0.028
5%	0	0.188	-0.004
1%	0	0.257	0.389

Supernatant's absorbance:

Centrifuge speed: 3000*g

Time: Five minutes

Wavelength: 580 Nanometer.

Dye name: Yellow G2

Table 2.4.2: Absorbance data for yellow G2 dye with centrifugation.

Concentration	Control	Flask 1	Flask 2
10%	0	0.028	0.104
5%	0	0.188	0.124
1%	0	0.146	0.252

Here for each concentration control was made auto zero to observe the changes in samples. Actually fluctuate of the sample's color from the control was seen. And the quartz cubet cells were used. Better the fluctuation, the better the de colorization.

2.5 Dilution and culture:

After taking seven days of absorbance from each sample conical flask 10ml of sample was taken into falcon tubes. And then it was centrifuged into 3000*g speed for five minutes. Then the supernatant was discarded and 10ml of autoclaved 0.85% NaCl solution was given. From that 10^{-5} times dilution was made. At first 0.85% NaCl solution was made into five test-tubes. Then 1ml pellet and saline solution was given into 1st test-tube. After that again 1ml from 1st test tube was taken and given into the 2nd test tube. By the same process 1ml given from 2nd to 3rd , then 1ml from 3rd to 4th and then 4th to 5th was done. By this way 10^{-5} dilution was made. After that 10^{-4} and 10^{-5} dilution was preserved. Then nutrient agar in Petridis was made. After autoclaving that for each two agar plate were made. One plate was for 10^{-4} dilution and another for 10^{-5} dilution. From each testube 200 microliter was taken with the help of micro pipette and poured on the agar. Then spread plate was made. After spreading those plates were kept into the incubator for 24 hours. After 24 hours different types of morphological colonies were seen in the plates. Some colonies were like concave in shape, some were like small bubble shape, and some were like creamy in color.

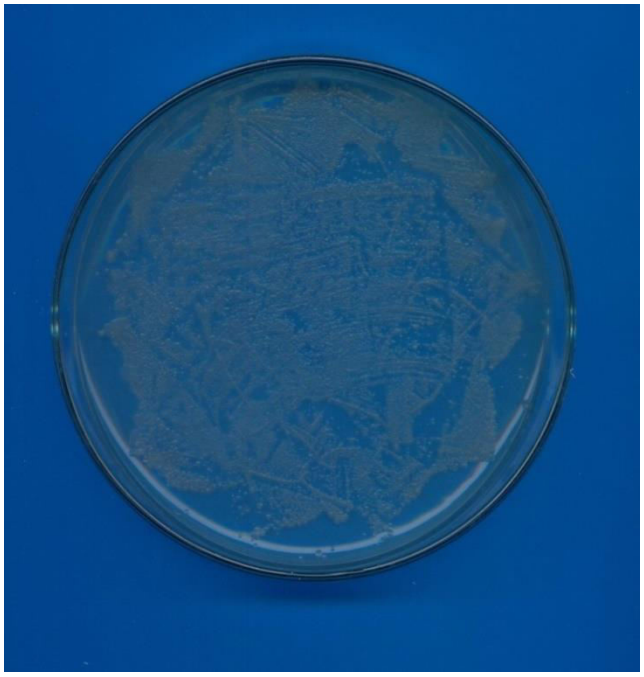


Figure 9: Different type of colonies after doing spread plateing from 10^{-4} dilution

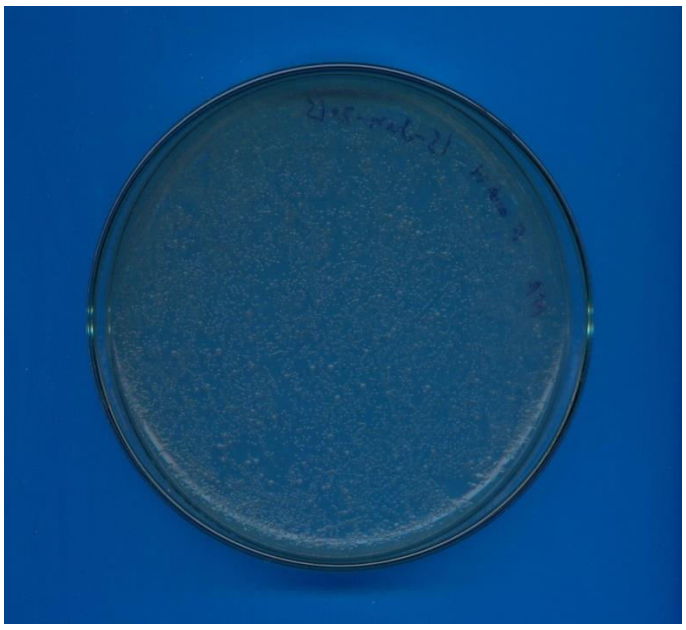


Figure 10: Different type of colonies after doing spread plateing 10^{-5} dilution

So from this trial it had been found that the soil sample contained dye degrading bacteria. After that trial run the original experiment was started. For the original run the two new dyes were

collected from oriental group, kawran Bazar. This time Yellow 4GL and Reactive Red 3BX were brought.

2.6 Original Project:

The initial work was almost same like the process which was written above. At first 18 conical flasks were taken. Then around 200ml SM broth was given into the conical flasks. After autoclaving was done in 121^0C for 15 minutes. Three concentrations of dyes were given into the flasks. 10% solution of yellow 4G and Reactive Red was made by adding 5g of dye into the 50ml distilled water. Then from that 10% stock dye solution three type of concentrations were given into Medias. They are like

Table 2.6: Different concentrations dye making amount.

10% dye concentration	1ml dye solution	0 ml distilled water
5% dye concentration	500 micro liter dye solution	500 micro liter distilled water
1% dye concentration	100 micro liter dye solution	900 micro liter distilled water

After that 5g soil was given into the 20ml distilled water and that solution was added into each conical flask of two dyes. And the amount of added soil solution was 1ml.

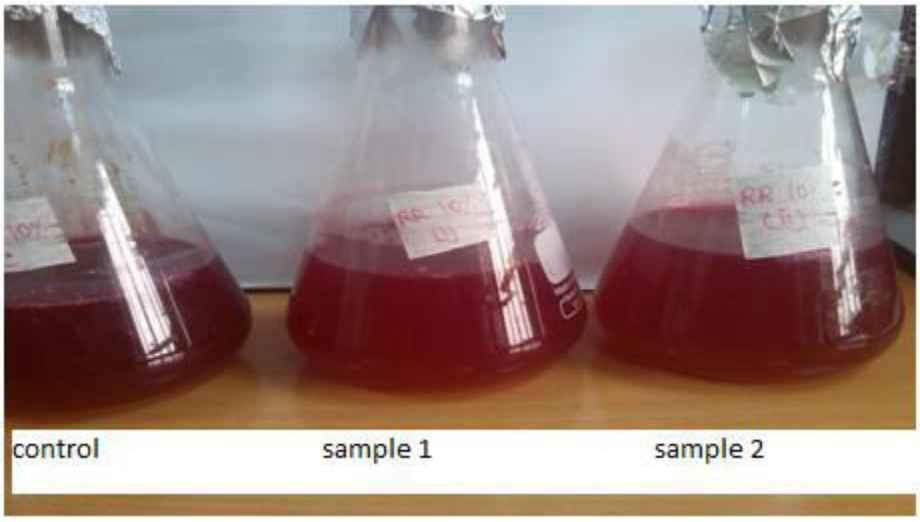


Figure 11: Red Dye was added into SM broth media

Figure 12: Yellow Dye was added into SM broth media

After adding inoculating in the media those were kept into the shaking incubator. And the temperature was set into 37^0C. This time they were kept for two weeks. And every one day intervals the absorbance was taken. And the absorbance was taken by doing centrifugation.

2.6.1 Table of Absorbance:

In this absorbance values the fluctuation from the control to dye was seen. In the control there was not any inoculation. Just media and color was added.

Yellow 4G = Y4G

Reactive Red = RR

For yellow 4G the wave length was set into 485 nm and for Reactive Red the set wavelength was 534 nm.

Day 0:

Table 2.6.1: Absorbance data of Yellow 4G and Reactive red dye

Y4G	control	Sample 1	Sample 2
10%	0	0.013	0.043
5%	0	0.015	0.040
1%	0	0.043	0.039
RR			
10%	0	0.022	0.043
5%	0	0.010	0.033
1%	0	0.009	0.10

Day 1:

Table 2.6.2: Absorbance data of Yellow 4G and Reactive red dye

Y4G	control	Sample 1	Sample 2
10%	0	-0.042	-0.044
5%	0	0	-0.003
1%	0	0.019	-0.001
RR			
10%	0	-0.021	-0.019
5%	0	0.008	-0.002
1%	0	0.030	0.033

Day 2:

Table 2.6.3: Absorbance data of Yellow 4G and Reactive red dye

Y4G	control	Sample 1	Sample 2

10%	0	0.076	-0.005
5%	0	-0.046	-0.037
1%	0	0.007	-0.001
RR			
10%	0	-0.231	-0.230
5%	0	-0.078	-0.073
1%	0	0.015	0.024

Day 3:

Table 2.6.4: Absorbance data of Yellow 4G and Reactive red dye

Y4G	control	Sample 1	Sample 2
10%	0	-0.048	-0.049
5%	0	-0.007	-0.014
1%	0	0.077	0.016
RR			
10%	0	-0.054	-0.064
5%	0	-0.145	-0.154
1%	0	0.002	-0.010

Day 4:

Table 2.6.5: Absorbance data of Yellow 4G and Reactive red dye.

Y4G	control	Sample 1	Sample 2
10%	0	-0.028	-0.005
5%	0	0.015	-0.023
1%	0	0.096	0.007
RR			
10%	0	-0.156	-0.146

5%	0	-0.108	-0.096
1%	0	0.007	-0.003

After taking four days absorbance data next three absorbance was taken into little modified method to show the fluctuation of the sample more accurately. In this method distilled water was used as the ideal transparent object. Controls' and samples' light absorbance was measured against the distilled water.

Day 5:

Table 2.6.6: Absorbance data of Yellow 4G and Reactive red dye

Y4G	Distilled water	Control	Sample 1	Sample 2
10%	0	0.936	0.970	0.969
5%	0	0.489	0.517	0.556
1%	0	0.275	0.361	0.292
RR				
10%	0	3.452	0.909	2.372
5%	0	1.974	1.117	0.556
1%	0	0.481	0.343	0.292

Day 6:

Table 2.6.7: Absorbance data of Yellow 4G and Reactive red dye

Y4G	Distilled water	Control	Sample 1	Sample 2
10%	0	0.901	1.003	0.988
5%	0	0.518	0.490	0.611
1%	0	0.266	0.316	0.243

RR				
10%	0	3.455	0.935	2.376
5%	0	2.024	1.129	1.276
1%	0	0.510	0.311	0.282

Day 7:

Table 2.6.8: Absorbance data of Yellow 4G and Reactive red dye

Y4G	Distilled water	Control	Sample 1	Sample 2
10%	0	0.095	0.951	0.820
5%	0	0.590	0.546	0.479
1%	0	0.284	0.277	0.268
RR				
10%	0	3.556	0.384	0.407
5%	0	3.079	0.264	0.330
1%	0	0.503	0.170	0.127

From this absorbance data it had been seen that the most effective data were found from the reactive red dye. Almost all the concentration of samples of reactive red dye was broken. And though the absorbance data of yellow 4G was not much effective like the reactive red but still some changes had noted down.

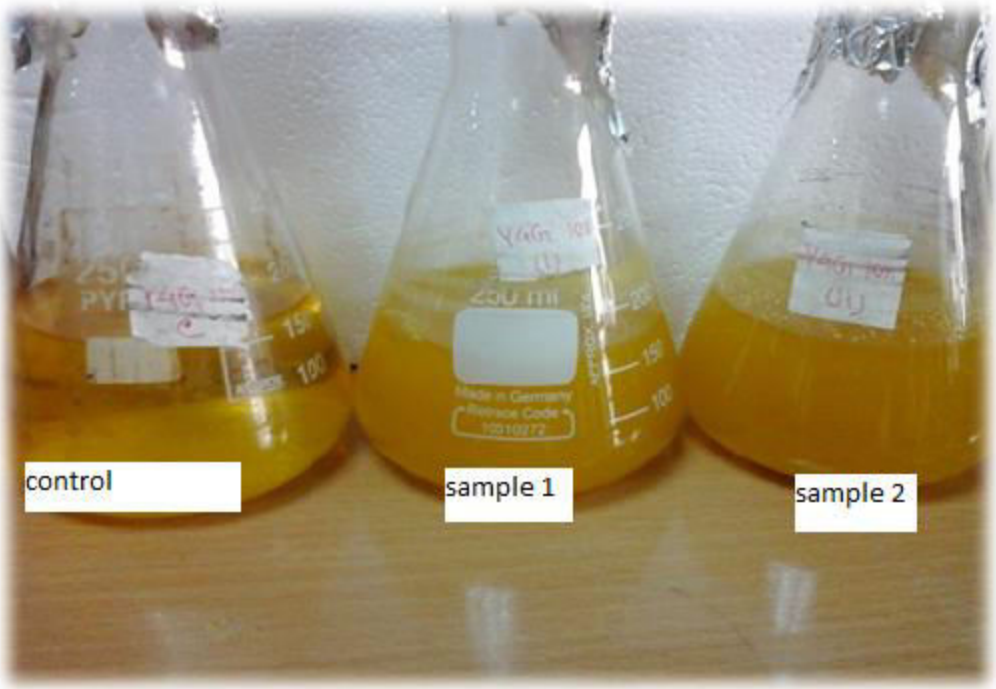


Figure 13: YELLOW-4G dye containing media after incubation

(Left conical flask = Control, middle conical flask sample 1, right conical flask = sample 2)

Figure 14: Reactive Red 10% samples. Here middle conical flask's dye containing media's color is degraded. (left conical flask = Control, middle conical flask sample 1, right conical flask = sample 2)

Figure 15: Reactive Red 5% samples, here middle conical flask's dye containing media's color is degraded after incubation.(left conical flask = Control, middle conical flask sample 1, right conical flask = sample 2)

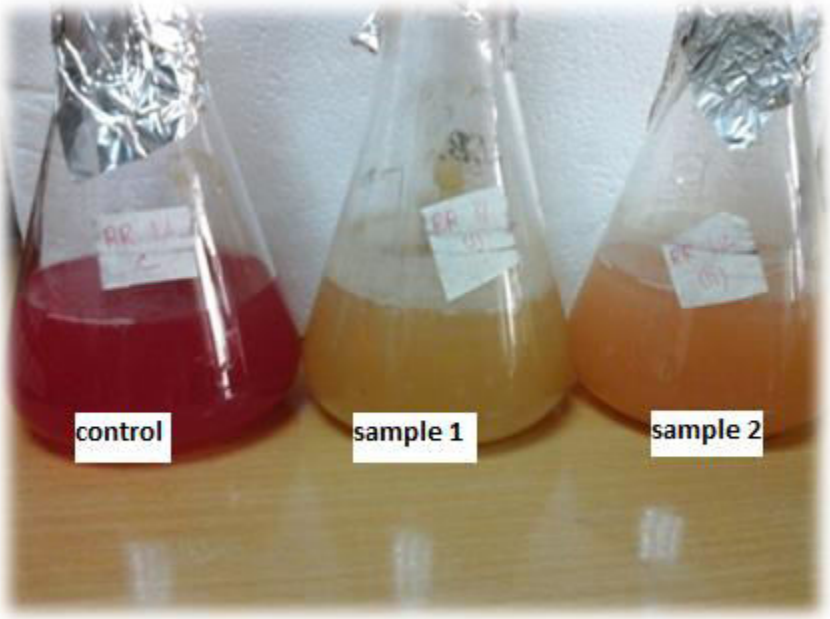


Figure 16: Reactive Red 1% samples, here both inoculated dye containing media are showing de-colorization from the control

(Left conical flask = Control, middle conical flask sample 1, right conical flask = sample 2)

2.6.2 Dilution:

After the absorbance part bacteria were needed to be isolated from that media which were responsible for dye degrading. At first to isolate bacteria the first step was to make dilution of the media containing dye and sample.

1. To make dilution 18 falcon tubes were taken.
2. Then 10ml media from each conical flask was transferred into each falcon tube.
3. Then they were centrifuged for 5minutes in 3000*g speed.
4. Then in the falcons media were separated into supernatants and pellets.
5. After that the supernatants were discarded and in the falcons 10ml of 0.85% NaCl autoclaved solution was given into all falcons.
6. After that for each falcons 5 test-tubes were taken filled with 10ml of autoclaved 0.85% NaCl solution.
7. Then 1ml mixture of pellet and NaCl solution was transferred into the 1^{st} test-tubes. That made 10^{-1} diluted solution.
8. Then from test-tube 1ml again 1ml of mixture was transferred into the 2^{nd} test-tube. That made 10^{-2} diluted solution.
9. By this way 1ml from 2^{nd} to 3^{rd} then 3^{rd} to 4^{th} then 4^{th} to 5^{th} was done.
10. And the final two diluted solution was 10^{-4} and 10^{-5} solution.
11. After that 10^{-4} and 10^{-5} solution were preserved into the 4'C temperature into the fridge.

2.6.3 Culture:

The reason behind making culture was to isolate bacteria from the culture plates. From those bacterial cultures different morphological colonies were selected. To get fine isolated colonies 10^{-4} and 10^{-5} diluted mixture was used. Because less diluted mixture could create matt on the nutrient agar. And in the Petridis nutrient agar media was used. The steps are given bellow,

1. At first Petridis were collected and sufficient amount of nutrients agar was taken and autoclaved.
2. After media was prepared total 36 Petridis plates were used to pour the media.
3. Then and total 36 test-tubes were present with the two dilutions.
4. For each dilution one plate was assigned.
5. From every test-tube 200microliter solution mixture was taken with micropipette and poured on the media.
6. After that spread plate was made on the media.
7. After it was done, all the inoculated Petridis were kept into the incubator for 24 hours in 37^0C temperature.
8. After 24 hours incubation period those Petridis were observed and different type of colonies were marked.

2.6.4 Isolation of the selected culture:

Selected colonies were marked by their different type of morphological outcomes. Those colonies which were common in different plates were taken for once. Twenty colonies were selected. And they were grown in single media. And the process is given bellow,

1. At first colonies were marked.
2. After that nutrient broth media was made in 20 test-tubes.
3. After that they were autoclaved in 121^0C.
4. After finishing autoclaving each marked colony was collected with the inoculums loop and deepens into the nutrient broth containing test-tube.
5. When the inoculating was done all the inoculated test-tubes were kept into the incubator.
6. And the time period was set 24 hours. And the temperature was 37^0C.
7. When 24 hours done inoculated nutrient broth containing test-tubes were taken outside.
8. After that nutrient agar media was made in 20 Petridis.
9. Then from each test-tube an inoculums loop was deepen in to the nutrient broth and streaking was done on the nutrient agar plate.

10. When all the inoculation was done, those inoculated nutrient agar media were kept into the incubator for 24 hours in 37^0C temperature.
11. After 24 hours incubation plates were taken out from the incubator and kept into the refrigerator in 4'C.

2.6.5 Finding of the dye degrading capability of isolated colonies:

Since 16 bacterial colonies were isolated into single plate, they were needed to be observed the property of dye degradation. Because it was sure that 16 type of bacteria can not have the ability to degrade dye. Highest three or four bacteria can do the dye degradation. To find out this property some steps were followed. And those steps are given bellow,

1. At first 20 test-tubes were taken and nutrient broth was made into them.
2. After that autoclaving was done.
3. Then each test-tube was inoculated with the each colony, which were preserved into the nutrient agar media.
4. After that they were kept into the incubator for the growth for 24 hours in 37^0C temperature.
5. When the growth had come, SM broth was made into 40 conical flasks.
6. Here 40 conical flasks were used because 20 colonies would be in two individual dyes. Which were Yellow 4G and Reactive Red.
7. Those conical flasks were autoclaved with media containing.
8. After autoclaving 1% solution of both of dyes were made.
9. To make 1% dye solution 1g of each dye were dissolved into 100ml distilled water.
10. After that 1ml of dye was given into all the conical flasks.
11. Reactive Red dye was given into 20 conical flasks and same for Yellow 4G.
12. After the dyes adding in every conical flask 1ml of each colony which was grown into the nutrient broth was given.
13. After that they were kept into the incubator in 37^0C for two weeks.

2.6.6 Decolorization:

During this step everyday all the conical flasks were checked. And the change in color was noted down for that individual colony. Within 24 hours one colony made the dye discolor. By this way after two weeks it was found that almost in 19 conical flasks, dye was broken down into different colors. And the de-colorization happened into 16 conical flasks of 20 Reactive Red added medias. And only in 4 conical flasks of Yellow 4G added media was broken. Though colonies could not affect much on the Yellow 4G, but it was found that 3 common colonies had the capability to degrade the both dye. And they were very efficient to break the red dye. They decolorize red dye within 24 to 48 hour. Whereas other took a long time compared to those. After two weeks of incubation absorbance were taken for 15 conical flasks containing re active red dye and 3 conical flasks' containing Yellow 4G dye. By the way here two controls of Re-active red dye and yellow 4G dye were kept. In control there was no bacterial inoculation. Just color was added. After that there opening were sealed with paraflim.

2.6.7 Gram Staining:

Six isolated colonies were isolated and grams staining was done. Because of grams staining always fresh bacterial culture is needed to be prepared. The process is given bellow,

1. At first bacterial culture was done into the nutrient agar.
2. After that glass slide was taken and a single colony was taken by using a loop.
3. Then a drop of distilled water was taken and that colony on the loop was spreaded.
4. When spreading was done on the glass slide heat fix was done.
5. Then cristal violet was poured and then washed.
6. After that grams iodin was given for 1minutes and washed away.
7. Then 100% ethanol was given and washed away.
8. After washing ethanol safranin was poured for 45 seconds then washed away
9. After all these steps glass slide was kept for drying.
10. When drying was done it was observed under the microscope.

As it was the initial level of grams staining so some pictures were taken , though this gram staining was done just to take notes about bacteria. Because the final grams staining pictures were taken after isolation of dye degrading bacteria.And they are added in the result part.

In this grams staining one bacterial colony was found as the small rod sahpe. And other are cocci in shape.

Table 2.6.7: Gram staining results for chose colonies.

Colony name	Shape	type
Colony C	Small rod	Gram positive
Colony D	Cocci	Gram positive
Colony I	Cocci	Gram positive
Colony K	Cocci	Gram positive
Colony L	Cocci	Gram positive
Colony M	Cocci	Gram positive

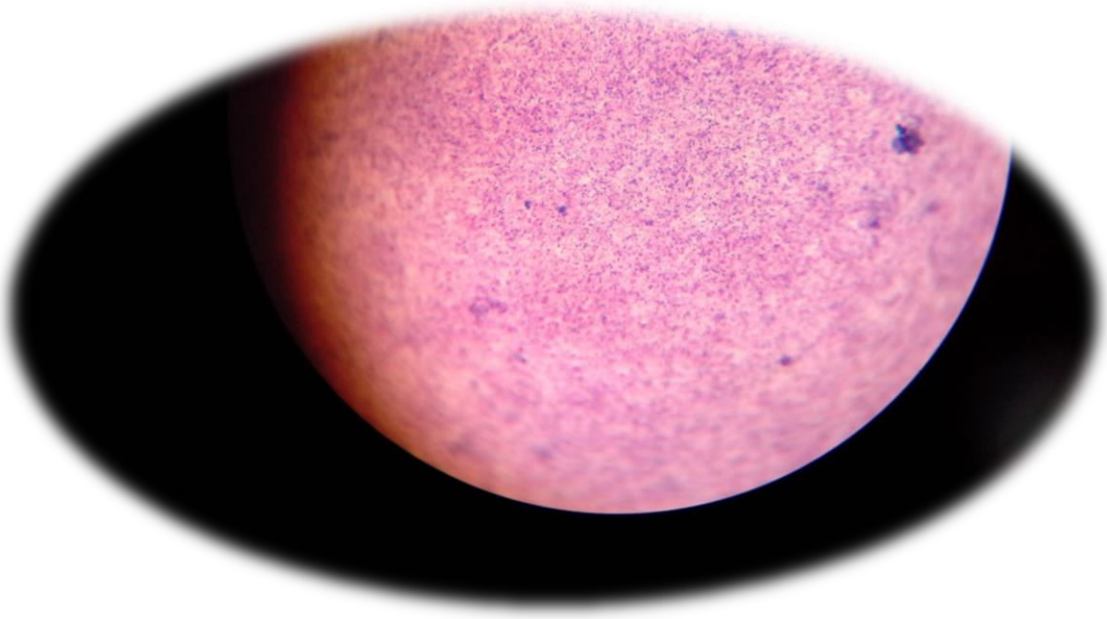


Figure 17: Grams staining photo of Colony C (small rod, gram ve+)

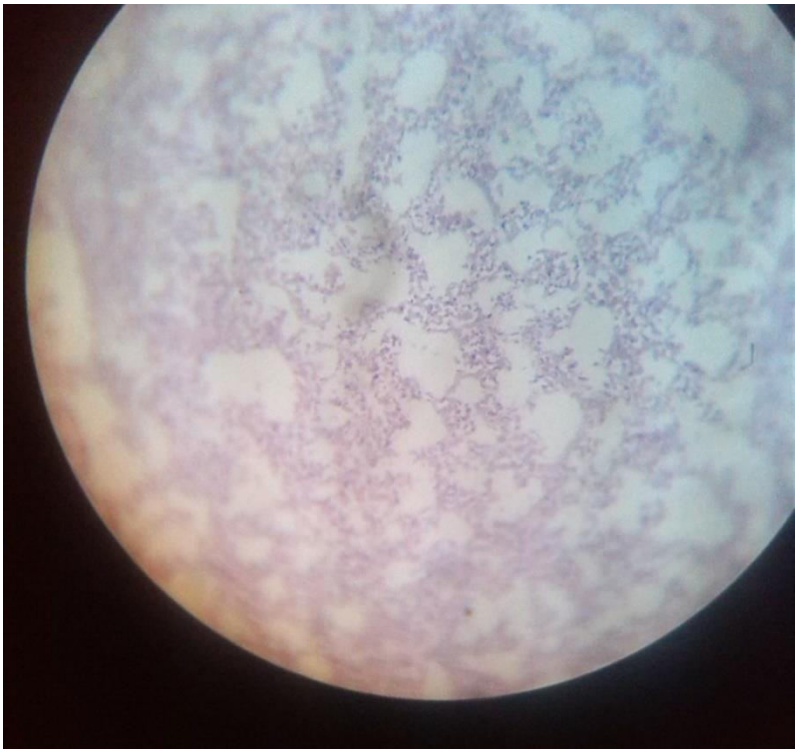


Figure 18: Grams staining photo of Colony I (gram ve+ cocci)

2.7.1 Bio chemical tests:

Bio chemical tests are very important part of any kind of project. Because they are the key parts of identify a bacteria. By doing these tests an unknown bacteria can be identified. For each type of bacteria there are different types of biochemical tests. For example to identify rod shape bacteria there are several biochemical tests. And for cocci shape identification, there are other tests. Names of the biochemical tests are given which were done in this project.

1. Catalase
2. Oxidase
3. Casein hydrolysis
4. Cimmon citrate
5. Nitrate reduction test

6. Starch hydrolysis test
7. MIU test (motility in dole urease)
8. Methyl red test
9. In dole test
10. Vogas-proskuras test.
11. TSI test = 1. Slant color 2. Butt color 3. Gas production 4. Hydrogen sulphide production
12. Aerobic growth test
13. Growth in 10% NaCl
14. Growth in 15% NaCl
15. Fructose test
16. Galactose test
17. Glucose test
18. Lactose test
19. Maltose test
20. Manitol test
21. Sucrose test
22. Treshalos test

2.7.2 Catalase test:

It is a easy biochemical test. And needed time is very short. Catalase is an enzyme which breaks the Hydrogen per oxide. If bubbles come then it is a positive result. If no bubbles then it is a negative result. Hydrogen peroxide is a potent oxidizing agent that can wreak havoc in a cell; because of this, any cell that uses oxygen must have a way to get rid of the per oxide. (8)

2.7.3 Oxidase test:

Basically, this is a test to see if an organism is an aerobe. It is a check for the presence of the electron transport chain that is the final phase of aerobic respiration. Normally, oxygen is the final electron acceptor for this system. In the oxidase test, an artificial final electron acceptor (N,N,N',N'-tetramethylphenylenediaminedihydrochloride) is used in the place of oxygen. This

acceptor is a chemical that changes color to a dark blue/purple when it takes the electron from the last element (cytochrome oxidase) in the electron transport chain. (9)

2.7.4 Citrate utilization test:

The citrate test screens a bacterial isolate for the ability to utilize citrate as its carbon and energy source (Difco**.** 1998 and MacFaddin, J. F. 2000). A positive diagnostic test rests on the generation of alkaline by-products of citrate metabolism. The subsequent increase in the pH of the medium is demonstrated by the color change of a pH indicator. The citrate test is often part of a battery of tests used to identify gram-negative pathogens and environmental isolates (Tang**,** Y. W. et el, 1998).). For instance, test kits such as the API-20E (bioMerieux) and Enterotube II (BD Diagnostics) include citrate utilization medium as one of the diagnostic tests. (10)

2.7.5 Starch hydrolysis:

Starch agar is a differential medium that tests the ability of an organism to produce the extracellular enzymes (exoenzymes) α-amylase and oligo-1,6-glucosidase that are secreted out of the bacteria and diffuse into the starch agar. These enzymes hydrolyze starch by breaking the glycosidic linkages between glucose subunits and allow the products of starch hydrolysis to enter the cell.

Starch agar is also used in differentiating members of various genera which have both amylase-positive and amylase-negative species, including *Streptococcus*, *Clostridium, Corynebacterium*, *Fusobacterium*, *Enterococcus, Pseudomonas,* and *Bacillus* (Leboffe MJ, Pierce BE**.** 2010 and. MacFaddinJF**.** 2000). (11)

2.7.6 Casein Hydrolysis Test

Casease is an exoenzyme that is produced by some bacteria in order to degrade casein. Casein is a large protein that is responsible for the white color of milk. This test is conducted on milk agar which is a complex media containing casien, peptone and beef extract. If an organism can produce casein, then there will be a zone of clearing around the bacterial growth (12). The hydrolysis reaction causes the milk agar, normally the opacity of real milk, to clear around the

growth area as the casein protein is converted into soluble and transparent end products — small chains of amino acids, dipeptides, and polypeptides (Jackie Reynolds, Fall 2011).

2.7.7 Nitrate reduction test:

This test determines the production of an enzyme called nitrate reductase, resulting in the reduction of nitrate (NO3): the test is also called the nitrate reduction test. With this enzyme,nitrate is reduced to nitrite (NO2). It then forms nitrous acid that reacts with the first reagent sulfanilic acid, and that reacts with the other reagent naphthylamine to form a red color. Reduction of nitrate is generally an anaerobic respiration in which an organism derives its Oxygen from nitrate. (Jackie Reynolds, Fall 2011). Nitrate reduction by bacteria is mediated by nitrate reductase and indicates that the organism can use NO_3^- as an electron acceptor (Balows, A., and B. I. Duerden (ed). 1998, Willey**,** J. M., L. M. Sherwood, and C. J. Woolverton (ed.). 2011, and Balows, A., and B. I. Duerden (ed)**.** 1998). Nitrite may be reduced to a variety of nitrogen products (Willey, J. M., L. M. Sherwood, and C. J. Woolverton (ed.). 2011..) including NO, N_2O, N_2, and NH_3, depending on the enzyme system of the organism and the atmosphere in which it is growing. Reduction of nitrate often indicates a shift to or facilitation of anaerobic metabolism, as some organisms can use nitrate as an electron acceptor during anaerobic respiration or anaerobic chemolithotrophy (Balows, A., and B. I. Duerden (ed). 1998). (13).

2.7.8 Motility Indole urease test:

Motility has long been recognized as an important taxonomic tool and biological characteristic of microorganisms (Jordan, E. O., M. E. Caldwell, and D. Reiter. 1934, Leifson, E.1951.,Leifson, E. 1960, and Stanier, R. Y., and C. B. van Neil. 1941.). Motility in bacteria can be provided by a variety of mechanisms, but the most common involve flagella (Jarrell, K. F., and M. J. McBride. 2008, and Jordan, E. O., M. E. Caldwell, and D. Reiter. 1934). The presence of flagella occurs primarily in bacilli but there are a few flagellated cocci, thus motility is a very important means of identification in the family *Enterobacteriaceae* (Jordan, E. O., M. E. Caldwell, and D. Reiter. 1934, Leifson, E. 1960). Motility test medium with triphenyltetrazolium chloride provides an easy method for determining motility. TTC in its oxidized form is colorless. As bacteria grow in the presence of TTC, the dye is absorbed into the bacterial cells where it is reduced to the

insoluble red-colored pigment formazan (MacFaddin, J. 1972). Growth is indicated by the presence of the red color, and as motility occurs, small to very large regions of color can be observed around the area of inoculation. (14)

2.7.9 Methyl red test and Voges- proskauer test:

The Methyl Red test includes including the pH marker methyl red to inoculated container of MR-VP stock. In the event that the creature utilizes the blended corrosive aging pathway and produces stable acidic deciding items, the acids will defeat the cradles in the medium and produce an acidic domain in the medium. At the point when methyl red is included, if acidic finished items are available, the methyl red will stay red. (15)

The VP test identifies organism that use the butylene glycol pathway and produce acetoin. At the point when the VP reagents are added to MR-VP soup that has been inoculated with a living being that uses the butylene glycol pathway, the acetoin finished item is oxidized in the vicinity of potassium hydroxide (KOH) to diacetyl. Creatine is likewise present in the reagent as an impetus. Diacetyl then responds to create a red shading. Hence, red is a positive result. In the event that, after the reagents have been included, copper shading is available, the outcome is negative. The MR and VP tests are especially valuable in the recognizable proof of the Enterobacteriaceae. (15)

2.7.10 Carbohydrate utilization test:

During the fermentation process, an organic substrate serves as the final electron acceptor (MacFaddin JF**.** 2000, and Stanier RY, Doudoroff M, Adelberg EA. 1963). The end-product of carbohydrate fermentation is an acid or acid with gas production (Forbes BA, Sahm DF, Weissfeld AS. 2007,& Mahon CR, Lehman DC, Manuselis G. 2011). Various end-products of carbohydrate fermentation can be produced. The end-product depends on the organisms involved in the fermentation reaction, the substrate being fermented, the enzymes involved, and environmental factors such as pH and temperature (Stanier RY, Doudoroff M, Adelberg EA.

1963). Common end-products of bacterial fermentation include lactic acid, formic acid, acetic acid, butyric acid, butyl alcohol, acetone, ethyl alcohol, carbon dioxide, and hydrogen (Forbes BA, Sahm DF, Weissfeld AS**.** 2007, MacFaddin JF. 2000, Mahon CR, Lehman DC, Manuselis G. 2011).

Fermentation reactions are detected by the color change of a pH indicator when acid products are formed. This is accomplished by adding a single carbohydrate to a basal medium containing a pH indicator. Because bacteria can also utilize peptones in the medium resulting in alkaline by-products, the pH changes only when excess acid is produced as a result of carbohydrate fermentation (Cowan ST, Steel KJ. 1965).

Phenol red is commonly used as a pH indicator in carbohydrate fermentation tests because most of the end-products of carbohydrate utilization are organic acids (MacFaddin JF. 2000.). However, other pH indicators such as bromocresol/bromcresol purple, bromothymol/bromthymol blue, and Andrade's can be used. (16).

2.7.11 Growth at 10% and 15% NaCl:

This test was used to observe the saline tolerance of isolated microorganisms. Here salt was mixed with the nutrient agar media.

Chapter 3: Result and Observation

3. Result and observation:

3.1 The values of absorbance are given bellow:

Dye name: Reactive Red

Wavelength: 534nm

Table 3.1.1: chosen colonies absorbance results for Reactive Red.

colony name	Absorbance (nm)
Reactive Red control	0.529
Colony A	0.166
Colony B	0.156
Colony C	0.380
Colony D	0.237
Colony E	0.194
Colony F	0.175
Colony G	0.299
Colony H	0.257
Colony I	0.399
Colony J	0.060
Colony K	0.391
Colony L	0.090
Colony M	0.143
Colony N	0.182
Colony O	0.1937

Dye name: Yellow 4G

Wavelength: 485nm

Table 3.1.2: chosen colonies absorbance for Yellow 4G dye.

Colony name	Absorbance
Yellow 4G control	0.065
Colony C	0.346
Colony D	0.360
Colony I	0.426

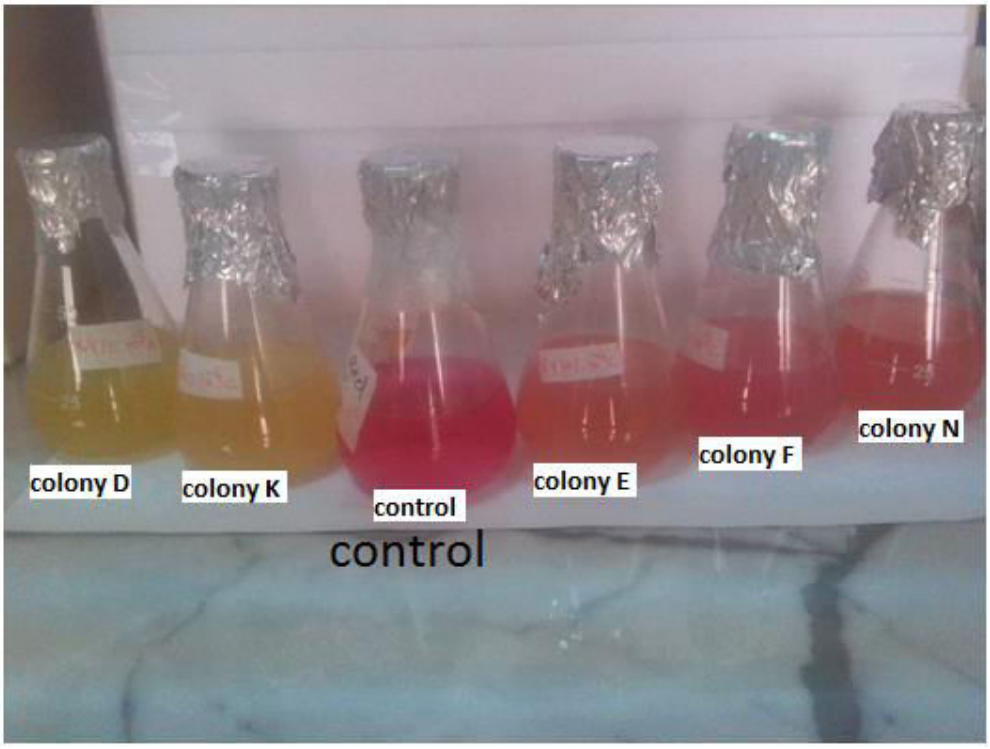


Figure 19: Decolorization of Reactive Red Dye

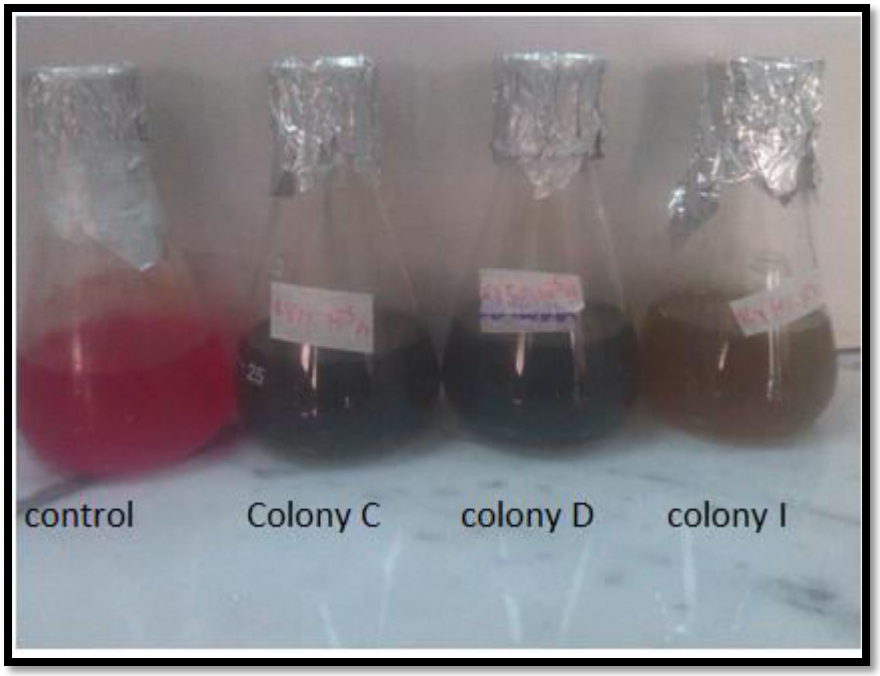


Figure 20: Decolorization of Reactive Red Dye by colony C, D and I.

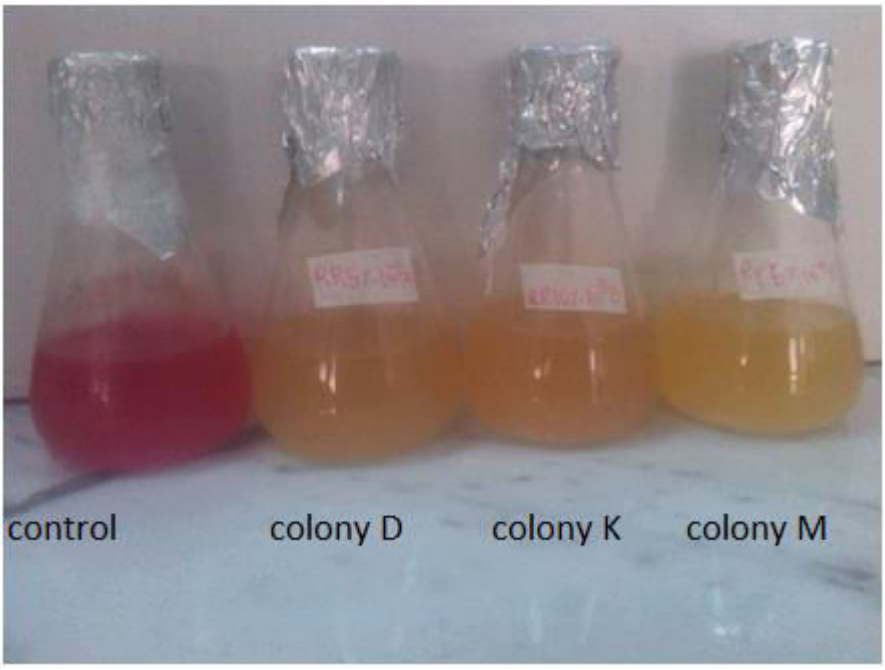


Figure 21: Decolorization of Reactive Red Dye by colony D, K and M

Figure 22: Decolorization of Yellow 4G

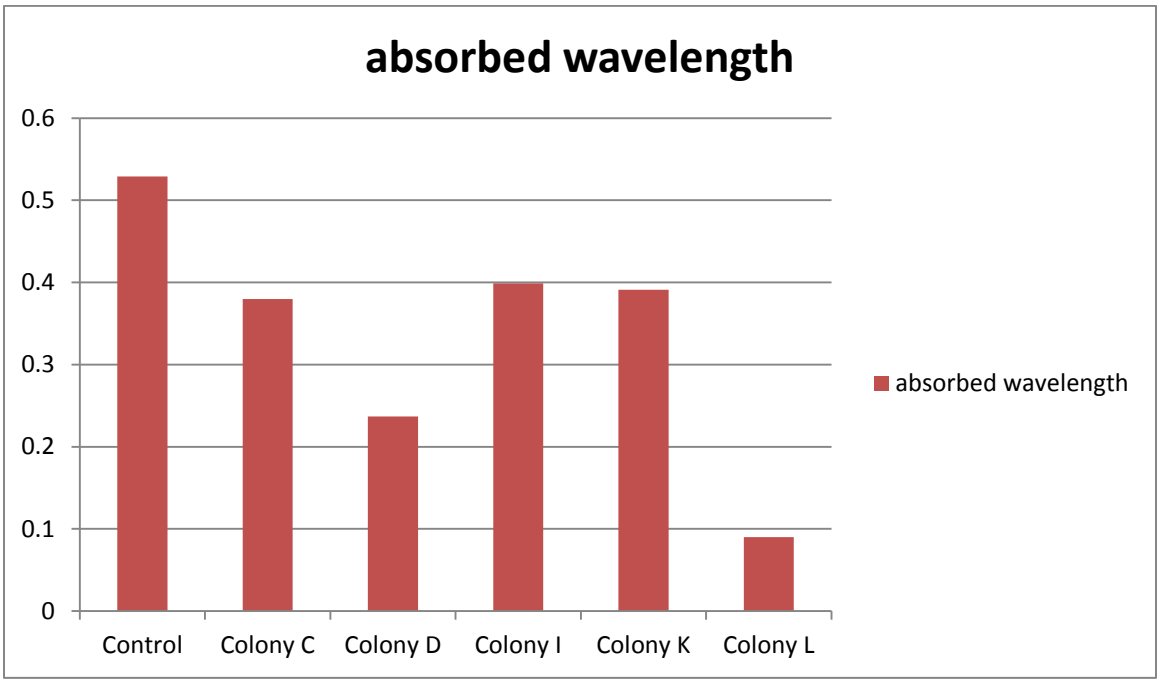
From total 18 colonies 6 colonies were taken. As they degrade the dye very efficiently and very fastly.

Name of the colonies which were choosen are given bellow:

1. Colony C
2. Colony D
3. Colony I
4. Colony K
5. Colony L
6. Colony M

Here Colony C, D, I had the capability to degrade the both of dyes.

Their Reactive Red dye degradation graph is given bellow by the chose colonies,



Graph 1: Chart representing the dye degradation property of colonies Y axis is representing absorbance data and X axis is representing colony names)

3.2 Result of biochemiocal tests for chosen Colonies:

Result of catalase test:

Table 3.2.1: Catalase test result

Colony name	Result
Colony C	Positive
Colony D	Positive
Colony I	Positive
Colony K	Positive
Colony L	Positive
Colony M	Positive

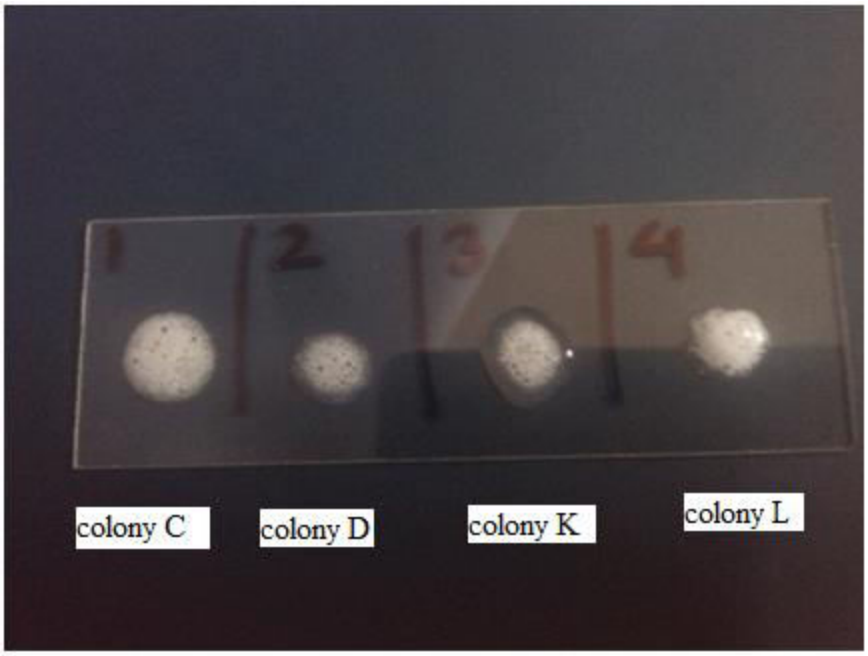


Figure 23: Catalase test's positive result

Oxidase test result:

Table 3.2.2: oxidase test results for chosen colonies.

Colony name	Result
Colony C	Negative
Colony D	Negative
Colony I	Negative
Colony K	Negative
Colony L	Negative
Colony M	Negative

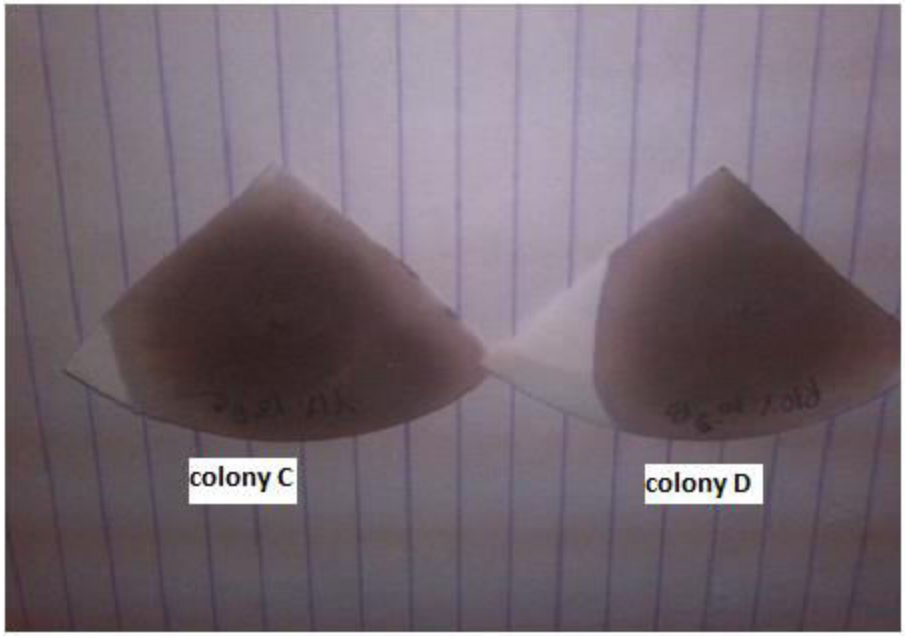


Figure 24: Oxidase test's negative result

Citrate utilization test results:

Table 3.2.3: Citrate utilization test results for chosen colonies.

Colony name	Result
Colony C	Negative
Colony D	Negative
Colony I	Positive
Colony K	Positive
Colony L	Positive
Colony M	Positive

Figure 25: Citrate test's positive result

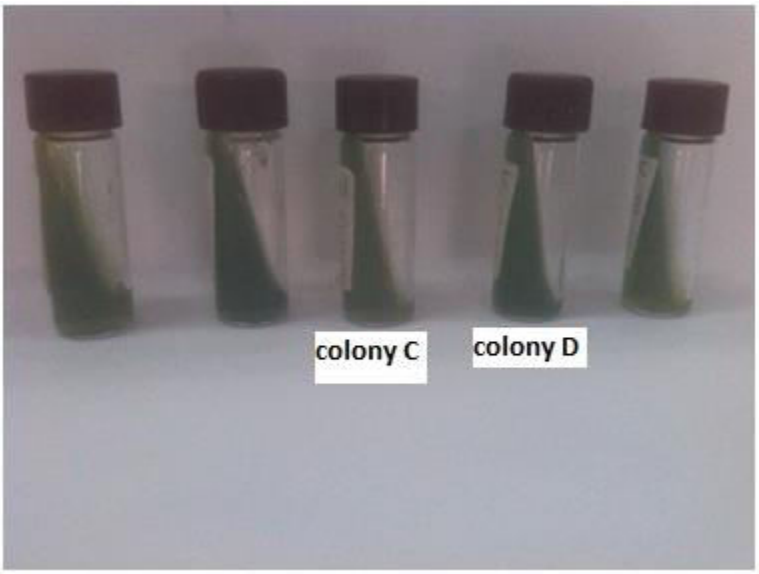


Figure 26: Citrate test's negative result

Starch hydrolysis test Result:

Table 3.2.4: Starch hydrolysis test Result for chosen colonies

Colony name	Result
Colony C	Negative
Colony D	Negative
Colony I	Negative
Colony K	Negative
Colony L	Negative
Colony M	Negative

Results in casein hydrolysis test:

Table 3.2.5: Results in casein hydrolysis test for chosen colonies.

Colony name	Result
Colony C	Positive
Colony D	Negative
Colony I	Negative
Colony K	Negative
Colony L	Negative
Colony M	Negative

Figure 27: Positive Result of casein hydrolysis test (colony C)

Results of nitrate reduction test:

Table 3.2.6: Results of nitrate reduction test chosen colonies.

Colony name	Results
Colony C	Positive
Colony D	Negative
Colony I	Negative
Colony K	Negative
Colony L	Positive
Colony M	Negative

Figure 28: Nitrate broth test(positiv result = Red color, negative result = Transparent color)

Results of motility indole urease test:

Table 3.2.7: Results of motility indole urease test for chosen colonies.

Colony name	Motility	indole	Urease
Colony C	Positive	Negative	Negative
Colony D	Negative	Negative	Negative
Colony I	Negative	Negative	Negative
Colony K	Negative	Negative	Negative
Colony L	Negative	Negative	Negative
Colony M	negative	Negative	Negative

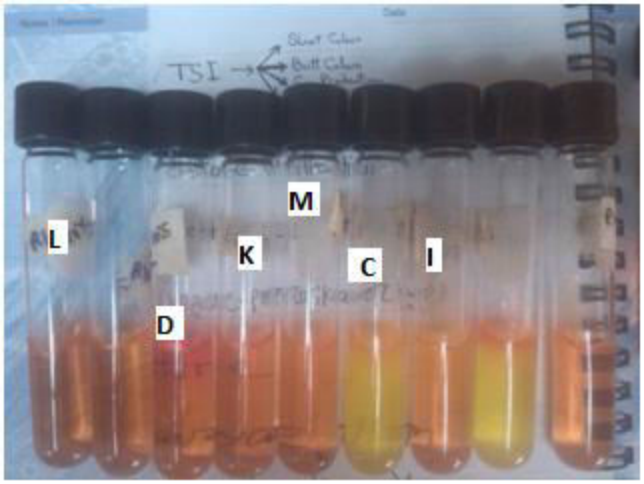


Figure 29: MIU Test(Yellow = positive result, Red = negative result)

Result of MR – VP test:

Table 3.2.8: Result of MR – VP test for chosen colonies.

Colony name	MR result	VP result
Colony C	Positive	Negative
Colony D	Negative	Negative
Colony I	Negative	Negative
Colony K	Negative	Negative
Colony L	Negative	Negative
Colony M	Negative	Negative

Carbohydrate utilization test:

Table 3.2.9: Carbohydrate utilization test for cosen colonies.

Colony name	fructose	galactose	gulcose	lactose	maltoe	manitol	sucrose	thrshalos
Colony K	positive	positive	negative	positive	positive	positive	negative	negative
Colony M	negative	positive	negative	positive	positive	negative	positive	positive
Colony L	positive	positive	positive	positive	positive	positive	positive	negative
Colony D	positive	positive	negative	positive	negative	positive	positive	positive

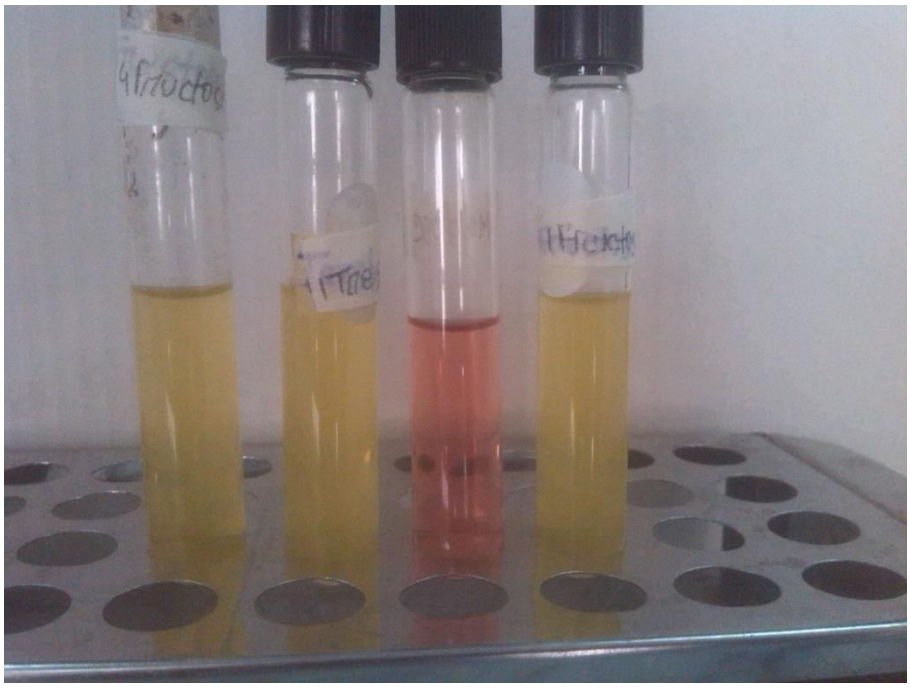


Figure 30: Carbohydrate utilization test (yellow = positive Red = negative)

Results of Growth at 10% and 15% NaCl test:

Table 3.2.10: Results of Growth at 10% and 15% NaCl test for chosen colonies.

Colony name	10% NaCl	15% NaCl
Colony C	Negative	Negative
Colony D	Negative	Negative
Colony I	Negative	Negative
Colony K	Negative	Negative
Colony L	Negative	Negative
Colony M	Negative	Negative

3.3 Small rod shape bacterium result (colony C):

Since total six bacteria were isolated from the soil, and five bacteria were taken for identification. So different type of biochemical tests were done to identify those microorganisms. In those six bacteria one was small rod in shape and other were cocci in shape. As the small rod shape microorganism was very efficient in dye degrading so it was the prime choice to identify. So the result of this bacterium was accumulated. As this bacterium was isolated from soil, so it was assumed that it must be a soil bacterium. And the possible choices were Bacillus, *Paenibacillus*, *Brevibacillus*, *Geobacillus*, *Lysinibacillus*, *Sporosarcinagenuses*. Results of this bacterium's biochemical test are given bellow.

Table 3.3.1: Small rod shape bacterium biochemical results (colony C)

Test name	Result
Gram staining	Positive
Catalase	Positive
Oxidase	Negative
Nitrate reduction	Positive
Citrate utilization	Negative

Indole	Positive
Voges-Proskauer test	Negative
Casein hydrolysis	Positive
Growth on 7% NaCl media	Negative
Motility	Positive
Growth on usual media	Positive
Starch hydrolysis	Negative
Acid production from Glucose	Positive
Acid production from Fructose	Positive
Acid production from Maltose	Positive
Acid production from Mannitol	Positive
Acid production from Trehalose	Positive

Now these results were added into the bacterial identification software ABIS software (17).

Figure31: Biochemical results were added (here different biochemical test's results were inputted)

After inputting the biochemical test results the shown possible bacteria's names are

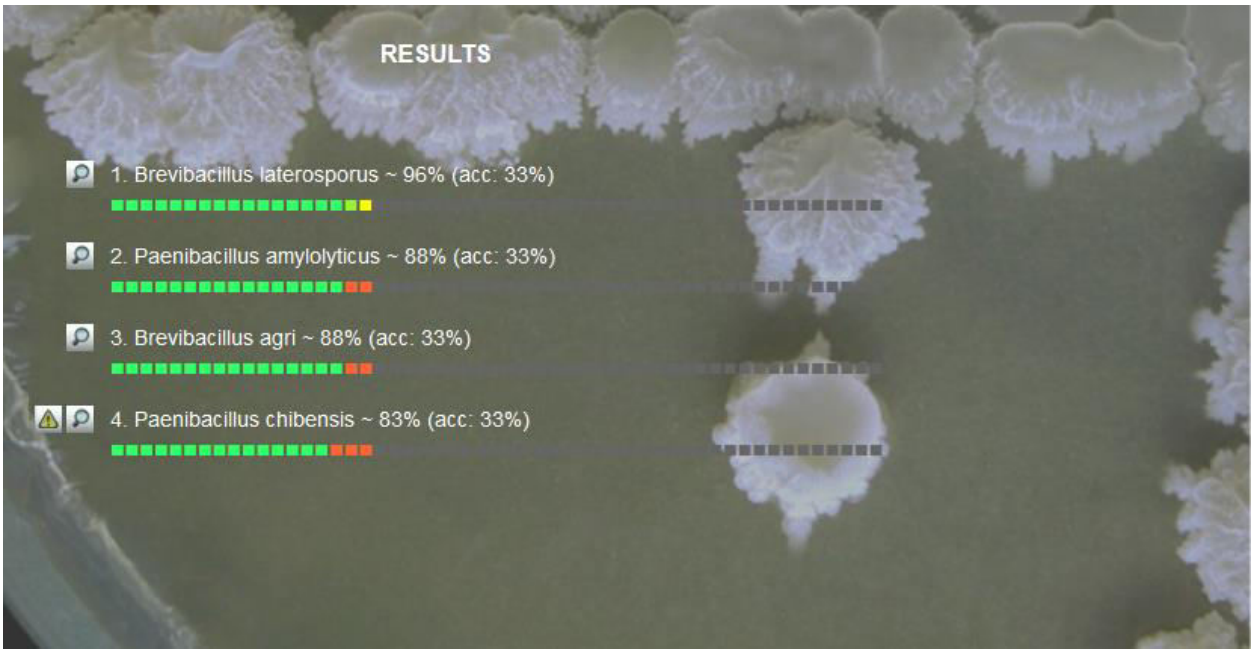


Figure 32: The possible results of Colony C

Name of the possible bacteria are

1. *Brevibacillus laterosporus* ~ 96% (acc: 33%)
2. *Paenibacillus amylolyticus* ~ 88% (acc: 33%)
3. *Brevibacillus agri* ~ 88% (acc: 33%)
4. *Paenibacillus chibensis* ~ 83% (acc: 33%)

But the possible bacterium is *Brevibacillus laterosporus*, because most of the biochemical results were matched with this. So it was assumed that the small rod bacterium is *Brevibacillus laterosporus*.

Though its biochemical results matched with other three organisms. But emphasis was given on this bacterium. Because all the biochemical tests which were done, matched perfectly with its

properties. And it is also found in soil (18). And the grams staining photo also matched with this isolated organism which is assumed as *Brevibacillus laterosporus*.

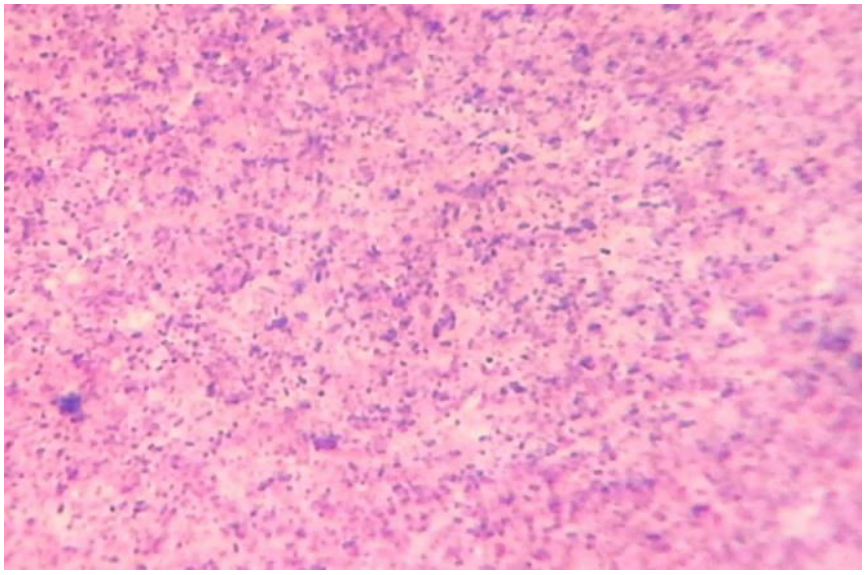


Figure 33: Grams staining photo of Colony C which is *Brevibacillus laterosporus* (19)

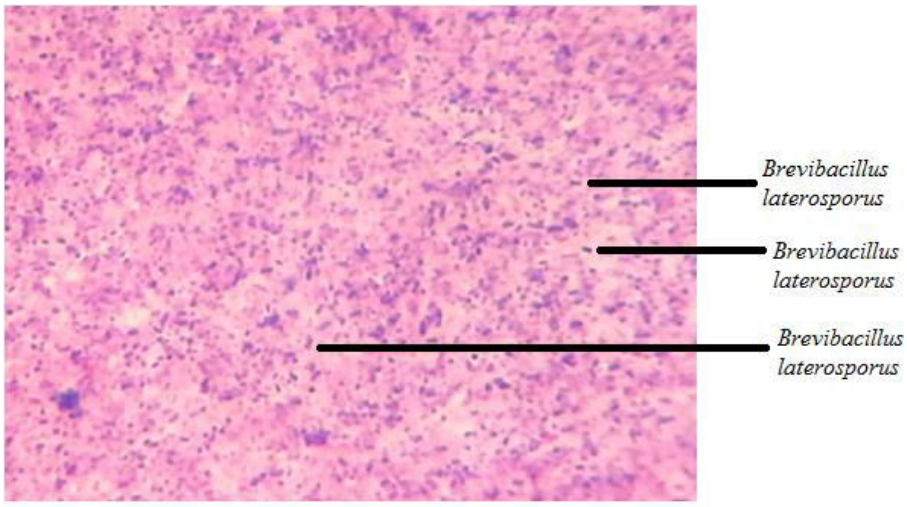


Figure 34: Grams staining photo of colony C, here small rod shaped bacteria were labeled.

And In this project *Brevibacillus laterosporus* degraded Reactive Red 3BX dye very efficiently in a short time which was like in 24 hours. And also it degraded the Yellow 4GL dye.

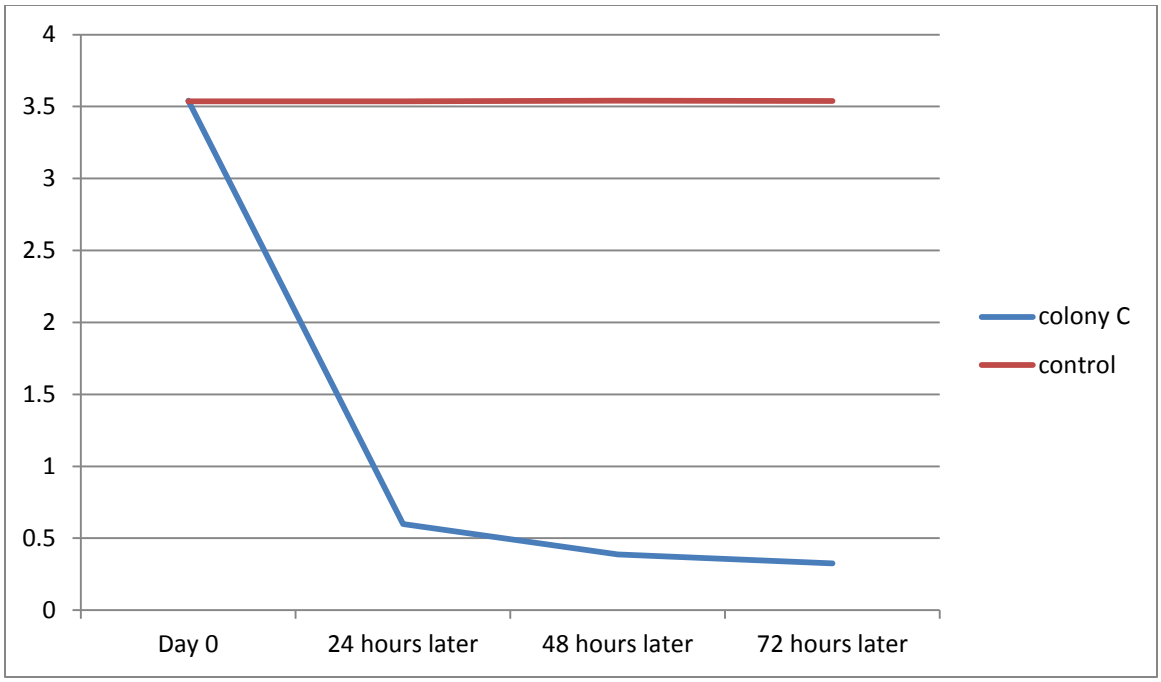
So again to test this *Brevibacillus laterosporus* isolate colonies' dye degrading capability, this bacterium was inoculated into dye. But this time the concentration of dye was increased. Again SM broth was made. Then 5% concentration of both dyes Yellow 4GL and Reactive Red 3BX were mixed into the media. And it was kept for one week to observe their dye degrading capacity.

And the colony C which was assumed as the *Brevibacillus laterosporus* showed better result in red dye. And also it showed slight decoloriztion in yellow dye. But in this short period just in one week Brevibacillus *laterosporus* showed better result.

The absorbance taken for *Brevibacillus laterosporus* in red is given bellow,

Table 3.3.2: *Brevibacillus laterosporus's* absorbance data

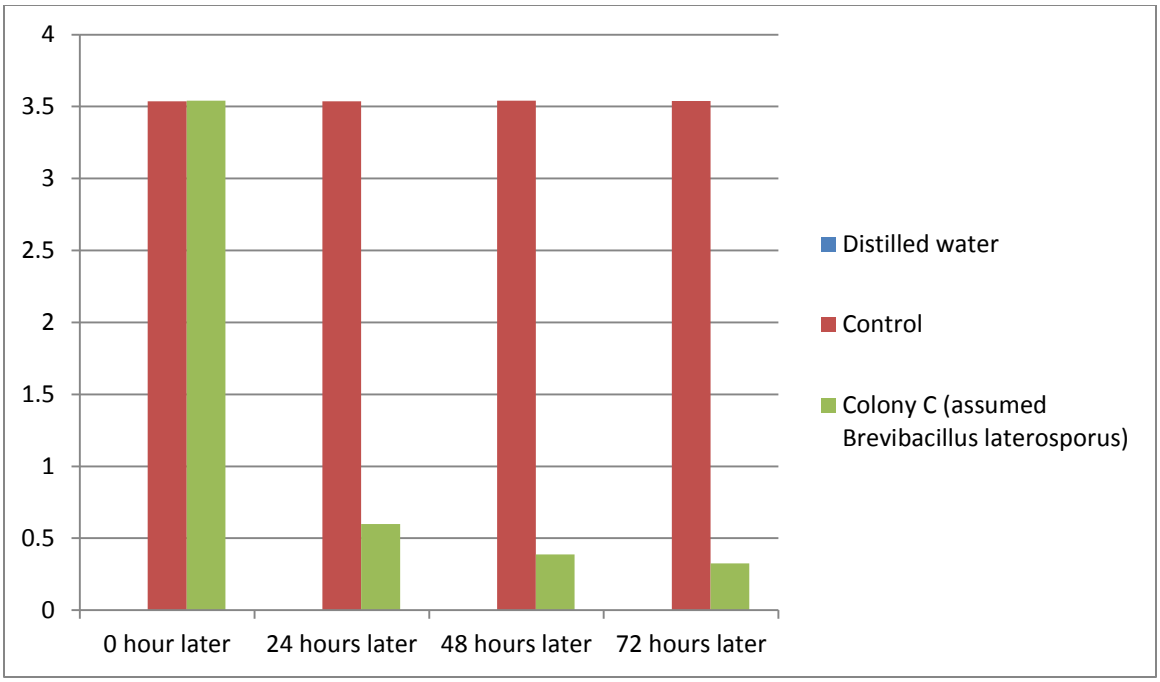
Reactive Red	Distilled water	Control (nm)	Colony C (assumed *Brevibacillus laterosporus*) (nm)
0 hour later	0	3.537	3.541
24 hours later	0	3.537	0.598
48 hours later	0	3.541	0.388
72 hours later	0	3.539	0.326



Graph 2: Decolorization by *Brevibacillus laterosporus* in chart(Y axis stands for Absorbance Range in nanometer)

And X axis is showing the time period.

In this graph the absorbance of control remained same but the absorbance of colony C which was ***Brevibacillus laterosporus*** decreased rapidly within 72 hours.



Graph 3: Decolorization of assumed *Brevibacillus laterosporus* (Y axis is representing absorbance data and X axis is representing time period)

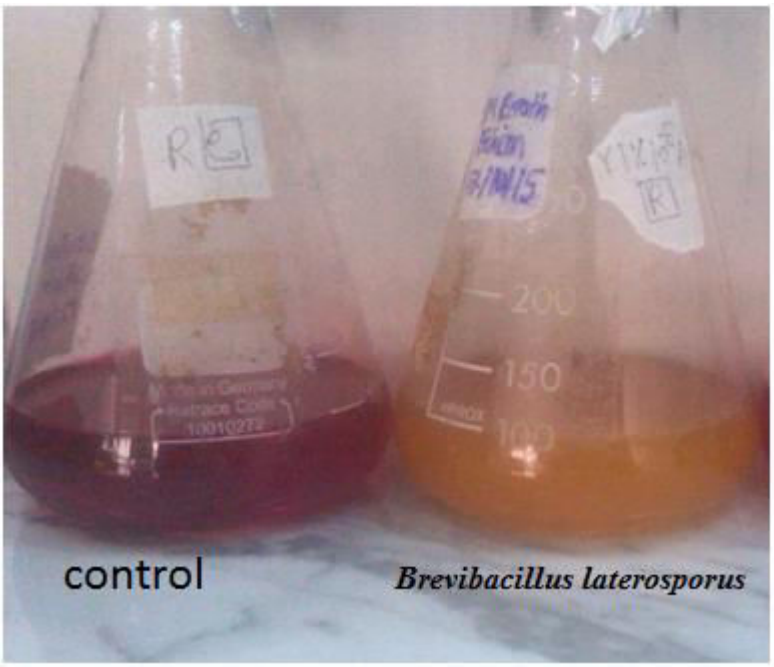


Figure36: The decolorization of colony C which was assumed as *Brevibacillus laterosporus*

.

Decolorization percentage of *Brevibacillus laterosporus*:

Initial OD = 3.541

Final OD = 0.326

Dye concentration = 5%

Decolorization% = {(Initial OD – Final OD) / Initial OD} * 100

= {(3.541 – 0.326) / 3.541} *100

= **90.8 %**

So the colony C, *Brevibacillus laterosporus* showed an excellent decolorization.

3.4 Cocci shape bacteria's result:

Then the cocci bacteria's biochemical tests were accumulated. As they were all gram positive bacteria, their possible genuses were *Staphylococcus* and *Macrococcus*.

Table 3.4.1: Cocci shape bacteria's biochemical results

Test names	Colony K	Colony M	Colony L	Colony D
Aerobic growth	positive	positive	positive	positive
Growth on 10% NaCl agar	Negative	Negative	Negative	Negative
Growth on 15% NaCl agar	Negative	Negative	Negative	Negative
Nitrate reduction	Negative	Negative		Negative
Urease	Negative	Negative	Negative	Negative
Catalase	positive	positive	positive	positive
Oxidase	Negative	Negative	Negative	Negative
Sugar production from				
Glucose	Negative	Negative	positive	Negative
Fructose	positive	Negative	positive	positive
Galactose	positive	positive	positive	positive
Maltose	positive	positive	positive	Negative
Mannitol	positive	Negative	positive	Positive
Lactose	positive	positive	positive	Positive
Sucrose	Negative	positive	positive	Positive
Thehalose	positive	positive	Negative	Positive

For Colony K, the found result was

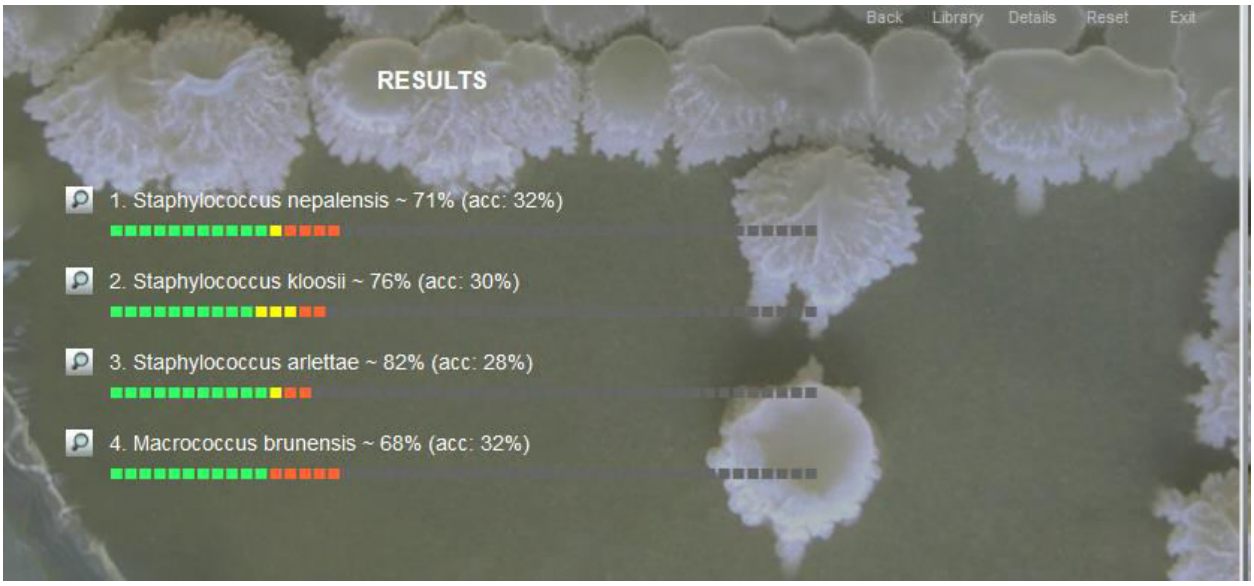


Figure 37: Results for colony K

1. *Staphylococcus nepalensis* ~ 71% (acc: 32%)
2. *Staphylococcus kloosii* ~ 76% (acc: 30%)
3. *Staphylococcus arlettae* ~ 82% (acc: 28%)
4. *Macrococcus brunensis* ~ 68% (acc: 32%)

But the result was not taken as its percentage was low.

For colony L:

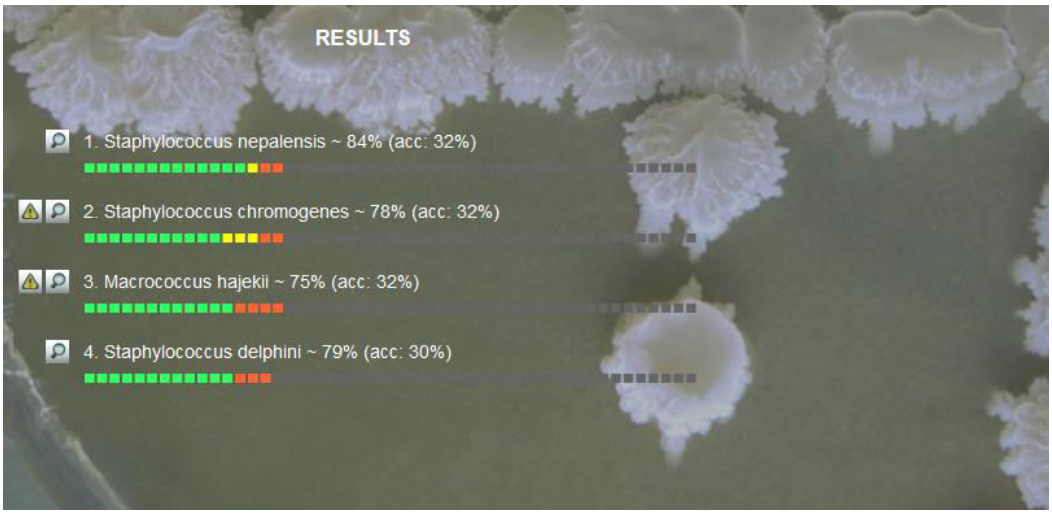


Figure 38: Results for colony L

1. *Staphylococcus nepalensis* ~ 84% (acc: 32%)
2. *Staphylococcus chromogenes* ~ 78% (acc: 32%)
3. *Macrococcus hajekii* ~ 75% (acc: 32%)
4. *Staphylococcus delphini* ~ 79% (acc: 30%)

And another bacterium was *Staphylococcus nepalensis*. Its percentage is little low. Because it's some biochemical tests did not matched with the software. But it might happen that it has some different characteristics. Because source of the bacterium has some effects on this tests. There were three more bacteria were shown in the results, but they were not picked up because their percentage were lower than the selected one. Though it was isolated first from goats lungs in the Himalayan region (20). Its grams staining photo is given bellow,

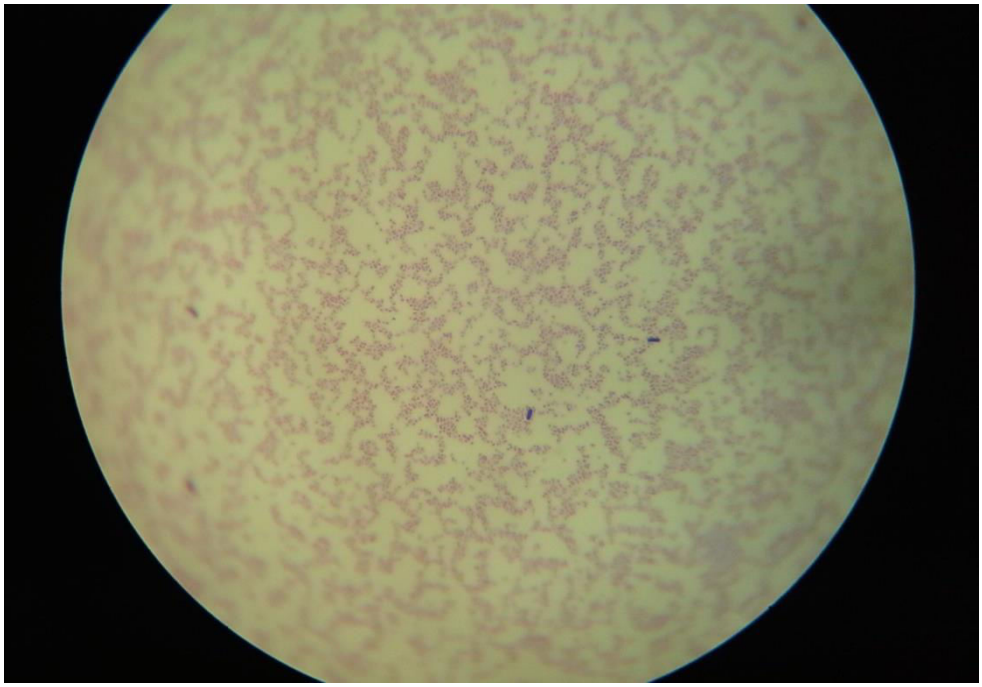


Figure 39**:** Grams staining photo of colony L (***Staphylococcus nepalensis***)

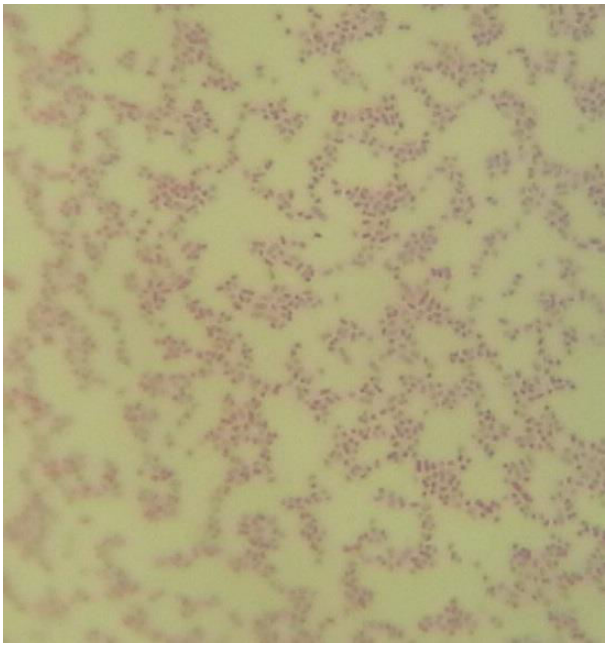


Figure 40: Zoomed view of colony L

To test its dye degrading capacity again it was given into higher concentration of dye, which was 5% solution of dye with media. But it could not break it. But it is efficient in low concentration dye.

In 1% Reactive Red dye it showed nice result,

Decolorization percentage of *Staphylococcus nepalensis*:

Initial OD = 0.592 nm

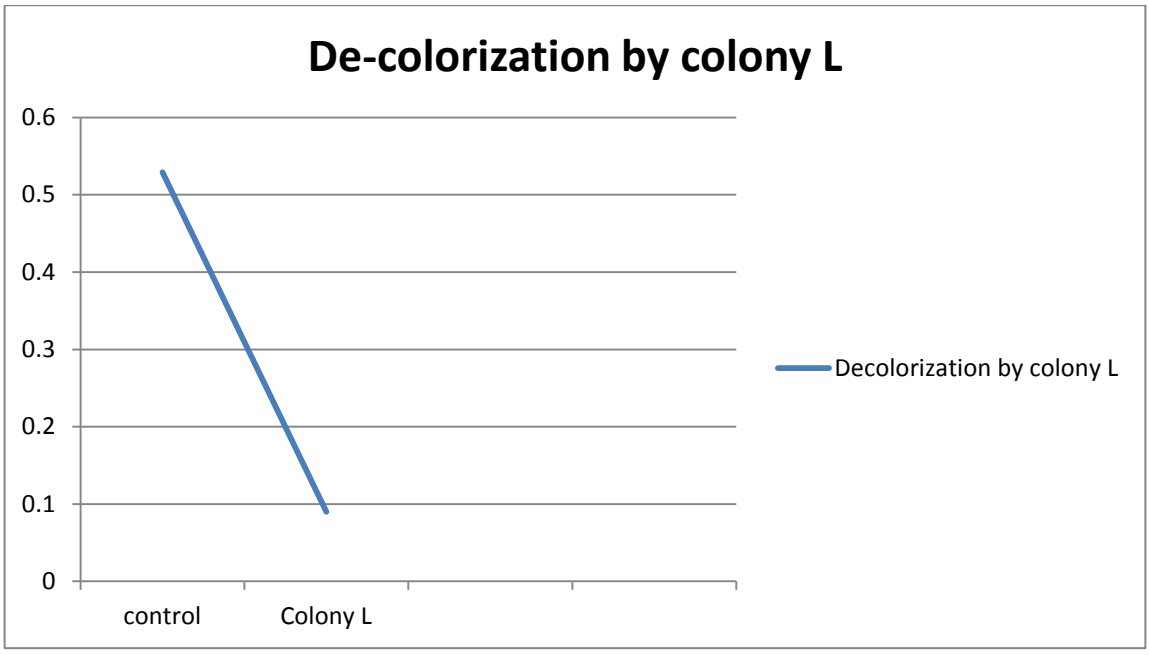
Final OD = 0.090 nm

Dye concentration = 1%

Decolorization% = {(Initial OD – Final OD) / Initial OD} * 100

= {(0.592 – 0.090) / 0.592} *100

= **85%** approximately.



Graph 4: *Staphylococcus nepalensis's* decolorization (Y axis is representing absorbance and X axis is representing Control to colony)

Other colonies:

But for other colonies, Colony M and Colony D, results were not found. Because biochemical results did not match with any bacterium.

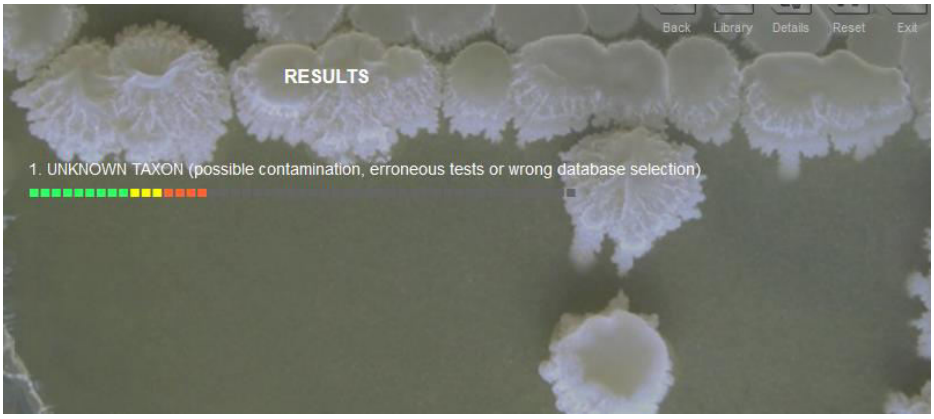


Figure 41: Results for colony M and D

So two identified bacteria from this project are *Brevibacillus laterosporus* and *Staphylococcus nepalensis*. These two bacteria were identified on the basis of biochemical tests, microscopic observation, software and their characteristics.

Chapter 4: Discussion & Conclusion

4. Discussion:

In this project two bacteria were ***Brevibacillus laterosporus*** and ***Staphylococcus nepalensis***. These identified two bacteria were assumed by the bio-chemical tests results and with the help of software. Because it is a rule. To be 100% sure Polymerase Chain reaction was needed to be done. Anyway this is a good result. The gram staining photo of ***Brevibacillus laterosporus*** and our isolated bacteria which was colony C gets matched. This bacterium is small rod in shape. And gram positive type. And biochemical results also get matched with the ***Brevibacillus laterosporus's*** properties. Though these same biochemical results three more bacteria were being showed by the software. But this was picked because its percentage was the highest. This was 96%. Other showed bacteria were ***Paenibacillus amylolyticus***, ***Brevibacillus agri***, and ***Paenibacillus chibensis***. But their percentage was lower because their some biochemical test results came unmatched with the biochemical results of this Colony C. And with the percentage, another number was showed that is accuracy 33%. What does it mean? This is the number of biochemical tests covered to find this bacterium out. In this project 33% biochemical tests were done to identify this bacterium. In the media composition that is SM broth, we used peptone and yeast extract. And it also capable of decolorization of Yellow 4G dyes. But its color changed into black.

Another isolated bacterium which was as ***Staphylococcus nepalensis***, it was also picked for the same reason. Its percentage was higher than the other three bacteria showed by the software. Its accuracy was 32%. But its percentage was little lower. It is 84%. It showed good result in low concentration of dye. These data are given on the graph. But the combination of these two bacteria can be very effective. If these two bacteria are given into dye polluted site, they will able to degrade higher concentration of dyes. And it would not take long to discolor the pollutants. At the beginning of the project soil was inoculated into the 10% concentration of dye.

And it was rapidly discolored. Because in that soil, different kinds of bacteria were present. And those degraded the dyes simultaneously.

Conclusion:

By these biochemical tests, microscopic observation, software and characteristics ***Brevibacillus laterosporus*** and ***Staphylococcus nepalensis*** are found. For further research these can be used**.** And obviously these two isolates have dye degrading capacity**.** ***Brevibacillus laterosporus*** has 90.8% Reactive Red dye degrading capacity in 37'C temperature (5% dye concentration) and ***Staphylococcus nepalensis*** has 85% Reactive Red dye degrading capacity in 37^0C temperature (1% dye concentration). These two bacteria can be used in future to reduce the environment pollution caused by the textile dyes. They can be an important finding in the sector of bioremediation. And this process would be very effective as biological process does not need specialized machines and special monitoring. It is a very cheap process than the other. If textile factories start to use it, their costing on the ETP treatment plant will decrease. And as biological process as no chance to create any secondary reactive products, so it is very safe. These two isolated bacteria can be a good source of future business, if the genes that are responsible for dye degradation, are being isolated and commercialized into the market. So genetically modified bacteria with those genes will be a great source of business.

Chapter 5: References

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Appendices:

Apparatus:

1. Conical flasks
2. Beaker
3. Measuring cylinder
4. Spatula
5. Vials
6. Test-tubes
7. Bunsen burner
8. Glass pipette
9. Micro pipette
10. Sprit lamp
11. Filter paper
12. Petridis
13. Glass slides
14. Falcon tubes
15. Aluminum Foil papers
16. Maskin tape
17. Autoclave tape
18. Marker
19. Durham tubes
20. Paraflim

Chemicals:

1. Peptone
2. Glucose
3. Yeast extract

4. Magnesium sulphate
5. Potassium di hydrogen phosphate
6. Di potassium hydrogen phosphate
7. Sodium chloride
8. Distilled water
9. Nutrient agar
10. Agar agar powder
11. MIU Agar
12. Nutrient broth
13. Urea
14. Cimon citrate
15. Starch agar
16. Skim milk agar
17. Beef extract
18. Trypticase
19. Phenol red
20. Ethanol
21. Potassium nitrate
22. Zinc powder
23. Lactose
24. Maltose
25. Mannitol
26. Sucrose
27. Fructose
28. Trehalos
29. Galactose
30. Savlon

Machineries:

1. Incubator

2. Laminar airflow
3. Shaker
4. Vortex machine
5. Refrigerator
6. Spectrophotometer
7. Wynn machine
8. pH meter
9. Centrifuge machine
10. Computer
11. Autoclave machine
12. Microscope

Sample soil is used to isolate bacteria. And three types of textile dyes were used in this total process. Name of the dyes are

1. Yellow G2
2. Yellow 4G
3. Reactive Red 3BX

Different types of reagents were used for the purpose to identify bacteria in the biochemical tests. Names of the reagents are given bellow.

For Grams staining:

1. Crystal violet
2. Grams iodine
3. 100% Ethanol
4. Safranin

For starch test:

1. Iodine

For Oxidase test:

1. 1% Kovacs oxidase reagent.

For Catalase test:

1. Hydrogen Per Oxide

For IMVIC test:

MR : Methyl Red

Voges-Proskauer :

1. Barritt's reagent A = 5% a napthol in absolute ethanol
2. Barritt's reagent B = 40% KOH in deionized water.