# Isolation and Identification of Orange M2R and Green GS dye Degrading Bacteria from Textile Sludge (Soil) Samples and Determination of Optimum Growth Conditions

**A DISSERTATION SUBMITTED TO BRAC UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF BACHELOR OF SCIENCE IN BIOTECHNOLOGY**


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DECLARATION

I hereby certify that this thesis project entitled **"Isolation and Identification of Orange M2R and Green GS Dye Degrading Bacteria from Textile Sludge (Soil) Samples and Determination of Optimum Growth Conditions"** is submitted by me, Fahim Ahmed Alif (ID – 11136013), to the Department of Mathematics and Natural Sciences under the supervision of Ms. Romana Siddique, Department of Mathematics and Natural Sciences, BRAC University, Dhaka. This dissertation was done as a part of my requirement for the degree of BS in Biotechnology. I also declare that this work is entirely based on the original results I have found. Materials and knowledge that I have consulted from the published works accomplished by other researchers have been properly cited and acknowledged within the text of my work.

Dated: April 7, 2016

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# Abstract

Environmental pollution has been confessed as one of the major troublesome of the 21st century. Most severely damaged part of this planet is its precious unique water bodies and in this deterioration textile and dyeing industries playing a major role as releasing intensely complex effluent containing highly toxic and long persistent Azoic dyes with extreme physiochemical condition. The existing physical and chemical effluent treatment methods are not cost effective. As those treatments generate huge amount of sludge and to dispose the sludge is a major problem. On the contrary Biological treatment using acclimatized microorganisms could remove 99-100% dye colour from wastewater. Hence nowadays most of the research is concentrated on biotransformation of textile azo dyes by adapted organisms. Modern bio-treatment offers a cheaper and environmentally friendly alternative for color removal of textile effluents which is very fascinating. So this research is to find out some highly effective Azo dye degrading bacterial isolates from effluent disposal areas soil.

The bacterial inoculums were isolated from effluent soil samples and then applied for azo dye decolourization into flasks containing azo dyes as a sole carbon source (1g/L) with trace amounts of yeast extract, glucose, peptone and some others essential salts and incubated for 5 days to observe their decolourizing ability. The decolourization was inferred from the decrease in the optical density of the dye effluent.

The bacterial strains identefied in the study were *Entorococcus termitis, Entorococcus camelliae, Bacillus farraginis, Bacillus muralis, Paenibacillus macerans, Bacillus decolorationis, and Macrococcus brunensis*. Out of these isolates *Entorococcus termitis, Bacillus farraginis, Paenibacillus macerans, Bacillus decolorationis* emerged out to be most potent decolourizer, being selected for further studies. *Bacillus farraginis* was identified as the best decolourizer of OM2R (Orange M2R) dye that decolourized 98% of the dye and *Paenibacillus macerans* showed maximum decolourization on GGS(Green GS) dye that decolourized 97% of the dye. The effect of pH, NaCl, temperature and initial concentration of dye was studied with an aim to determine the optimal conditions required for maximum decolourization. The research showed different decolourization rate with varying parameters. The optimum pH for decolourization of OM2R and GGS dye was 7.0, the optimum NaCl concentration for decolourization was 2%, initial dye concentration was 1% and the temperature was $37^0$C for optimum decolourization by the selected isolates. The findings are well acclimatized and have potentials for bioremediation in textile waste effluent treatment plants.

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| Abbreviations | Description |
|---|---|
| NA | Nutrient Agar |
| MIU | Motility, Indol, Urease test |
| MR | Methyl Red |
| VP | Voges-Proskauer |
| rpm | Rotation per minute |
| mL | milliliter |
| g/L | Grams per liter |
| SM | Salt media |
| OM2R | Orange 2R |
| GGS | Green GS |

# Chapter 1: Introduction

# Chapter 1: Introduction

The world around us is getting polluted so very rapidly. One of the reasons may be the side effect of the advancement in science and technology. Science and technology lead our life to more comfort and easy as well as towards the destruction. Nuclear reactor to food factory, everywhere we are producing an immense amount of hazardous by product and most of them are through untreated in to the open environment. There is a saying that says "The world is so small so that we will meet again". Likewise we can't keep ourselves safe from those highly volatile biochemical and organic reactive molecules. Most of the human civilizations have been destroyed for the reason they were concern about but for ignoring as a reason of terminator.

In this 21$^{st}$ century industrialization have boom every corner of the world and textile industry is on the top of all, very reasonably. Its use thousands of tons of synthetic dyes (Azo) annually. A big portion of that dye goes to water bodies untreated. Those carcinogenic and recalcitrant molecules penetrate in to the ecosystem and harming every member of the system. Human used up the polluted water directly for daily necessaries like irrigation, bathing and drinking. As a result diseases like cancer, malfunction, infertility, genetic mutation near to be epidemic. So it is the high time to think about the optimum solution for this threat to humanity and all other creatures.

## 1.1 Textile industry: an overview

Textile production was one of the first areas where industrial processes developed in the early-nineteenth century. The textile industry in Bangladesh accounts for 45 percent of all industrial employment and contributes 5 percent to the total national income. The industry employs nearly 4 million people, mostly woman. Despite the significant economic contribution of the textile industries in Bangladesh, it has brought with it a range of environmental problems, mostly pollution of water resources of the country .Textile industry consumes large quantities of water for various processes and discharge equally large volumes of wastewaters containing variety of pollutants and colouring matters like azo dye.

It is estimated that over 2, 80,000 tons of textile dyes are discharged in industrial effluent every year, worldwide. Therefore, pollution from these discharge contaminated with dyestuff is becoming alarming (Pandey et al., 2007; Jin et al., 2007). This sector placed in number one for the water pollution of Bangladesh.

## 1.2 Characteristics of textile waste water

Textile wastewater is highly coloured which mainly block the penetration of sunlight thereby retarding the growth of aquatic animals and plants; it also contains the dissolved toxic substance and carcinogens (Walsh 1980, Chung 1992).Effluent discharged from the textile industries has variable characteristics in terms of pH, dissolved oxygen, organic, and inorganic chemical content, etc. Together with industrialization, awareness towards the environmental problems arising due to effluent discharge is of critical importance. Pollution caused by dye effluent is mainly due to durability of the dyes in wastewater (Jadhav et al., 2007).

## 1.3 Ecological aspects

Azo dyes are widely known dyestuff used in industries and hence commonly released in the environment (Chang et al., 2001). About 3500 dyes are in practical use. Azo dyes contribute 84%, of which sulphonated azo dyes predominate. About 10-15% (128 tons/day globally) of the dyes are lost at various finishing steps of the printed cloths. Besides dyes, the wastewater contains acids/alkalis, common salt (NaCl), heavy metals, sulphides, chlorine and mineral oils. As a result, the dye wastewaters are extremely toxic to both aquatic fauna and flora, crop plants, including human beings (Sharma et al., 1999).

## 1.4 Textile fabric and dyeing processes.

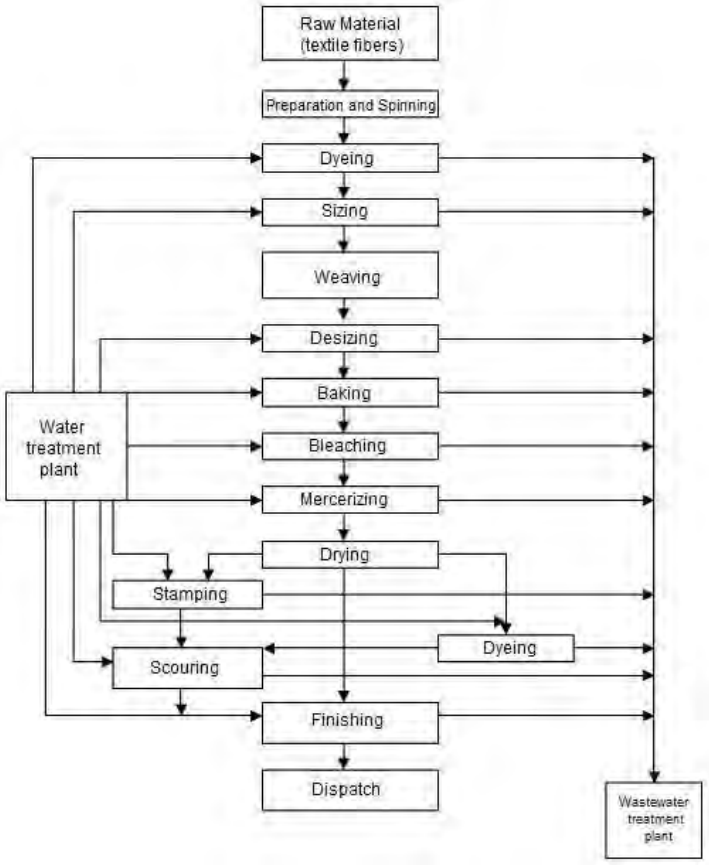



Figure 1.4 – Stages of a textile industry (Braile and Cavalcanti, 1993).

In the figure 1.4a process diagram of a textile industry is shown, we can see that an initial dyeing after spinning and another dyeing step after drying is present in textile industry. After both of the steps dye containing waste water is directly through to waste water treatment plant.

## 1.5 Azo dyes: an overview

Mauveine, the first synthetic dye, was discovered in 1856 by William Henry Perkin. Since then, thousands of new synthetic dyes have been produced .Dyes can be divided in 20-30 different groups regarding their chromophores. The most important are azo (monoazo, diazo, triazo, polyazo), anthraquinone, phthalocyanine and triarylmethane dyes (Figure 1.6). Azo dyes represent about 70 % on weight basis of total annual world production (dos Santos et al., 2003).

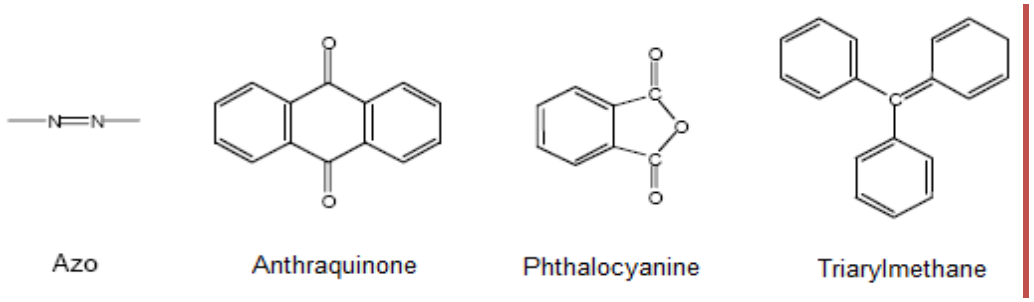



**Figure 1.5:** The most important chromophores (dos Santos et al., 2005).

Azo compounds are distinguished by the double azo bound between two nitrogen atoms (Figure.1.5).Each nitrogen atom is bound to another group, usually an aromatic group. There are dyes with one, two, three and even four azo groups in the molecule. The aromatic rings usually have chloride, hydroxyl, sulphate or nitro groups attached to increase solubility in water and enhance interactions with the substrate (Hunger, 2003). Azo dyes are widely used(more than I million tons worldwide) in the textile dyeing process due to the superior fastness for the fabric, high photolytic stability and resistance to microbial degradation and cost-effectiveness of their synthesis, stability and variety of colors available in comparison to natural dyes (Stolz, 2001).

## 1.6 Dye removal treatment

Currently there are several methods that can be used in the removal of dyes from textile industrial effluents. However, due to the variety of existent dyes and to the effluents complexity all methods don't have the same efficiency and the combination of various methods may be required, since each method has its limitations. Existing methods can be divided into three categories: physical, chemical and biological. Physical methods like Coagulation/Flocculation, Adsorption, Membrane filtration, Ion exchange are applied but expensive. Chemical methods like Fenton's reagent, Ozone, Photochemical, Sodium hypochlorite (NaOCl), Electrolysis and Wet air oxidation (WAO) are not cost effective and produce toxic byproduct.

### 1.6.1 Biological methods

Biological treatment is the most economic and eco-friendly process due to least running cost, no hazardous chemicals are required and very low non-toxic sludge are produced.

Biotreatment offers a cheaper and environmentally friendly alternative for color removal in textile effluents. Biological methods involve the use of bacteria, fungi and algae.

Bacterial degradation has been mainly applied in the removal of azo dyes. The azo dyes generally resist to aerobic degradation. However its degradation was observed in anaerobic conditions, but aromatic amines are formed as final product, which despite having no colour, can be toxic, mutagenic or carcinogenic (Isik and Sponza, 2007).Under these anaerobic conditions it is not possible to degrade the aromatic amines formed, which in turn are only degraded in aerobic environment. Thus, to achieve a complete degradation of azo dyes a method that combines anaerobic treatment of the dyes with the mineralization of aromatic amines under aerobic conditions should be applied (Carvalho et al., 2008; Lin et al., 2010).

## 1.7 Objective

As Bangladesh stands among the leading textile exporting countries, textile industries use large quantity of water in its production processes and highly persistent azo dye and toxic waste waters are discharged into water body without any kind of treatment because of the high expense of those treatment operation. Biological treatment is the most economic and eco-friendly process due to least running cost, no hazardous chemicals are required and very low non-toxic sludge are produced. In this method waste water is treated by microorganisms mainly bacteria. The use of microorganisms to remove contaminants from wastewater is highly effective and widespread. In biological approach it is very crucial to use highly effective microorganisms those are more compatible and effective to degrade the target product.

So this research was to find out some highly effective dye degrading bacteria from effluent soil sample and optimization of physiochemical condition for their optimum growth.

# Chapter 2: Materials and Methods

## 2.1 Place of research

The study was performed at Microbiology and Biotechnology Research Laboratory under Department of Mathematics and Natural Sciences, BRAC University.

## 2.2 Soil samples collection

Soil samples were aseptically collected from textile effluent disposal area, in Savar. Sterile plastic containers were used to carry the soil samples .The soil samples were stored in sterile plastic bags at 4°C for later use (Islam *et al.*, 2013). Low temperature was used to keep the microorganism viable (T. Teeresa, 1999).

**2.2 Table:** List of soil samples that were collected for the study

| Soil samples | Location |
|---|---|
| A | Savar |
| B | Hemaitpur |
| C | Aminbazar |
| D | Savar |


Figure 2.2: Four different soil samples A, B, C and D were used.

## 2.3Azo dye

Azo dyes were collected from Mitford dye market in Dhaka .Sterile poly pack was used for carrying to lab. Then samples were preserved in room temperature. For the confirmation of sterility, 0.5 gm of the Azo dye(OM2R, GGS) were inoculated on SM broth and incubated for 24 hours at 37ºC before the start of every experiment. Lack of growth on plates ensured the Azo dye to be uncontaminated.

## 2.4Isolation and selection

Isolation of bacteria from soil samples through dilution technique was performed after culturing the isolates in SM broth with Azo dye.

### 2.4.1 Suspension preparation

1gm of soil sample was taken from sample A, B, C and D and then homogenously suspended with 100 ml of 0.85% NaCl solution by vortexing.

### 2.4.2 Media preparation

Table 2.4.2: The composition of SM broth for 1000ml

| **Ingredients** | **Amount** |
|---|---|
| Glucose | 10 g/L |
| Di potassium Hydrogen Phosphate | 0.6 g/L |
| Peptone, | 10 g/L |
| Potassium dy Hydrogen Phosphate | 1.9 g/L |
| Magnesium Sulphate | 1 g/L |
| Yeast Extract | 1 g/L |

Then adjust the pH to 6.0 to 6.4.

Thus the final media contained all important nutrients and buffers except any carbon source

### 2.4.3 Inoculation of samples in dye containing media

The suspension of each soil (effluent) sample was individually applied to 1% dye containing SM broth media to saw the dye degrading capability of those samples. As SM broth does not contain any carbon source but the dye as a sole carbon source. So only those bacteria will grow that have the capability to use up dye (OM2R and GGS) as a carbon source**.**

For each soil sample 2 conical flasks were prepared contained 200ml SM broth and 1% of 1ml dye .One was kept as a control another conical flask was inoculated with prepared soil sample suspension.

These steps were followed for every sample A, B, C and D.As the research was performed with two different dyes. So everything remain same just the dye got changed, so eight media with OM2R dye and another eight media with GGS dye were prepared.

For OM2R dye

| | | | | |
|---|---|---|---|---|
| **Soil Samples** | A | B | C | D |
| **Control** | AC | BC | CC | DC |
| **Inoculated** | A1 | B1 | C1 | D1 |

For GGS dye

| | | | | |
|---|---|---|---|---|
| **Soil Samples** | A | B | C | D |
| **Control** | AC | BC | CC | DC |
| **Inoculated** | A1 | B1 | C1 | D1 |

The same procedure followed for GGS dye containing SM broth media.

**2.4.4 Dilution technique was done to isolate pure culture**

After 5 days each conical flask were compared with the control and decolourization was observed .As each of the sample was (A, B, C and D) contained mixture of different types of microorganism in high concentration so dilution technique up to 10-4 and 10-5 were performed and then plated on nutrient agar plates through spread plate technique and incubated for 24 hours at 37°C to obtain soil isolates. The different bacterial colonies based on their morphology were selected in a way to ensure no two colonies displayed the same characteristics. Selected isolates were then enriched in nutrient agar media plated by four ways streaking method and incubated for 24 hours at 37°C to obtained better growth**.** Then plates were sealed with parafilm, refrigerated at 4°C and were frequently sub cultured.

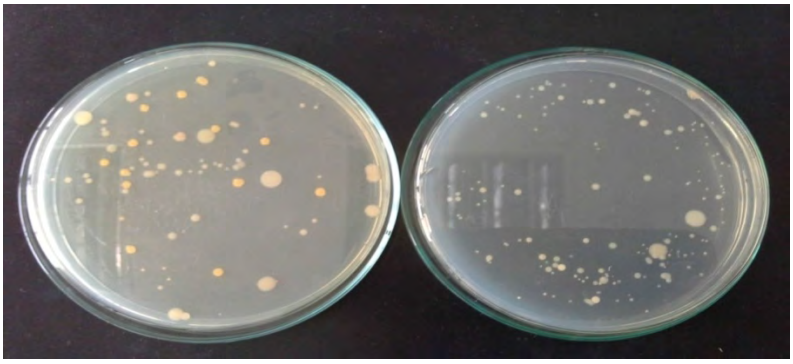


**Figure2.4.4: Diluted plates of $10^{-5}$ for single colony isolation.**

**Table 2.4.4(I):** Four isolates based on their different morphology were selected From OM2R dye containing conical flask and used throughout the study

| Soil samples | Number of isolates | Designation of selected isolates |
|---|---|---|
| A | 1 | AO |
| B | 1 | BO |
| C | 1 | CO |
| D | 1 | DO |

**Table 2.4.4(II):** Four isolates based on their different morphology From GGS dye containing conical flask were selected and used throughout the study.

| Soil samples | Number of isolates | Designation of selected isolates |
|---|---|---|
| A | 1 | AG |
| B | 1 | BG |
| C | 1 | CG |
| D | 1 | DG |

All of the isolates from OM2R and GGS dye containing media theoretically have the dye degrading ability at different scale. As those isolates were in mix culture form so we don't know there individual performance that's why each isolates were enriched in nutrient broth media for 24 hours at $37^0$C then inoculated (always added 200µl of bacterial inoculums in 50ml of dye containing media) in 1% dye (both OM2R and GGS) containing SM broth media to evaluate individual Azo dye degrading ability by analyzing the OD (absorbance) of each sample.

Then from, initial four OM2R dye degrading bacteria best two were chosen. Similarly best two were selected from GGS dye degrading group of isolates.

**2.4.5 Colony forming unit was measured for each isolates.**

Colony forming unit was measured to see the viable bacterial count of selected bacterial isolates to understand their cellular performance in dye decolourization. So one milliliter of enriched selected isolates were individually serially diluted into tubes containing 9 ml of water or saline (normal saline= 0.9%).Dilution was done up to $10^{-5}$ times. Then only 0.1 ml onto the plate from the tube was transferred to count cfu/ml (colony forming units per milliliter)

**Table 2.4.5 Colony forming unit of different isolates**

| Isolates designation | Colony number at 0.1ml from $10^{-5}$ times dilution tube | cfu/ml(colony forming units per milliliter) |
|---|---|---|
| AO | 14 | 14000000 |
| BO | 11 | 11000000 |
| CO | 09 | 9000000 |
| DO | 07 | 7000000 |
| AG | 06 | 6000000 |
| BG | 13 | 13000000 |
| CG | 08 | 8000000 |
| DG | 03 | 3000000 |

## 2.5 Incorporating variations in growth conditions

Optimum growth conditions for the isolates were identified applying different physiochemical state in time of growth. Parameters were tested like dye concentration, pH of the media, NaCl concentration and incubation temperature.

### 2.5.1Decolourizationof dye at different concentration

Best four selected isolates later cultured in 50ml SM broth with three different dye concentration 1%, 3% and 5% at 37 $^0$C for a period of 5 days with corresponding dye OM2R and GGS.

### 2.5.2 Effect of pH on decolourization of dye

Isolates were cultured in individual 50 ml SM broth media whose pH was adjusted to pH 5, pH 6, pH 7 and pH 8. The flasks were incubated at 37$^0$C for 5days with corresponding dye OM2R and GGS and then OD was measured.

### 2.5.3 Effect of different concentration of sodium chloride

Experiment was repeated by culturing best four isolates individually in 50 ml SM broth with corresponding dye (OM2R and GGS) in various sodium chloride concentrations of 2%, 4%, 6% and 8% .The flasks were incubated at 37$^0$C for 5 days and then OD was measured.

### 2.5.4 Effect of different temperature in dye degradation

Four different temperatures 30$^0$C, 37$^0$C, 45$^0$C and 55$^0$C were setup for each four isolates individually in 50 ml SM broth media with corresponding dye (OM2R and GGS) for 5 days and then OD was measured.

**2.6 Biochemical test were done to identify the isolates**

Name of the biochemical tests which were done in this project

1. Catalase
2. Oxidase
3. Casein hydrolysis
4. Simmon citrate
5. Nitrate reduction test
6. Starch hydrolysis test
7. MIU test (motility in dole urease)
8. Methyl red test
9. Indole test
10. Vogas-proskuras test.
11. Aerobic growth test
12. Growth in 7% NaCl
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
23. Arabinose test

Bacterial isolates used in this study were identified based on:

1. Morphological characteristics of colony

2. Staining and microscopic visualization

3. Biochemical tests

Gram staining and biochemical tests were performed on the bacterial isolates according to Microbiology Laboratory Manual (Cappuccino & Sherman, 2005). Presumptive identification was performed through using ABIS Online software.

### 2.6.1 Staining

### 1) Gram staining

Procedure: A small amount of a single bacterial colony was transferred onto saline drop over a slide and heat fixed. The slide was then flooded with crystal violet and left to stand for 30 seconds. After a 10 seconds wash with tap water, mordent iodine was applied to flood the slide for 30 seconds. The slide, washed with tap water, was flooded with 95% acetone as a decolorizing agent. Then immediately the slide was counterstained with safranin for 30 seconds and rinsed again. The dried slide was observed under microscope.

Inference: Gram positive cells would appear violet while Gram negative cells would appear red.

### 2.6.2 Motility test

Procedure: 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 of isolates was determined by picking up bacterial colony with sterile needle and stabbing semisolid agar (MIU agar base) in test tubes. After overnight growth at $37^0$C, diffusion of cloudiness was observed.
Inference: Motile bacteria would migrate readily through the semisolid agar, away from the line of stab, and create cloudiness upon incubation. Non motile bacteria would grow, but only along the line of stab inoculation.

### 2.6.3 Enzyme tests

### 1) Indole utilization test

Procedure: Indole test was executed to identify isolates capable of degrading tryptophan and produce indole. Bacteria were inoculated from fresh plates in individual tubes with peptone water containing tryptophan. After overnight incubation at $37^0$C, a few drops of Kovac's reagent were added.

Inference: A positive test would produce a red layer on top of the agar, whereas the presence of original yellow or brown layer would confer a negative test result.

### 2) Urease test

Procedure: Urease test was performed to identify bacteria that are capable of hydrolyzing urea. The test was accomplished by inoculating well-isolated bacterial colony into urea base containing urea and phenol red. Change in color of the agar, incubated at $37^0$C, was observed after 24 hours and every day for a period of 6 days (for slow hydrolyser).

Inference: Urease positive bacteria would produce ammonia which would turn phenol red pink. The culture medium would remain yellow for bacteria unable to hydrolyze urea.

### 3) Citrate utilization test

Procedure: The citrate test screens a bacterial isolate for the ability to utilize citrate as its carbon and energy source (Difco**.** 1998 and Mac Faddin, J. F. 2000).Bacterial colonies from fresh agar plate were streaked on the slope of Simmons' citrate agar (Oxoid ltd, England) and incubated at $37^0$C for 24 hours.
Inference: 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 Bacteria with the ability to utilize citrate would turn the agar blue through creating alkaline condition. Bacteria unable to utilize citrate would show no change in the agar.

### 4) Oxidase test

Procedure: Oxidase test identifies bacteria that are able to produce cytochrome c oxidases. A small piece of filter paper was soaked with a few drops of freshly prepared Kovac's reagent (tetra-methyl-p-phenylenediamine dihydrochloride). With the aid of a toothpick single bacterial colony was transferred on to the soaked paper. Change in color of the treated paper was observed within 5-10 seconds.

Inference: Oxidase positive bacteria would turn the soaked paper dark purple by oxidizing Kovac's reagent. Oxidase negative bacteria would display no change.

## 5) Catalase test

Procedure: This test was used for the detection of the enzyme catalase present in bacterial isolates. 1 drop hydrogen peroxide (3%) was placed on a slide with the aid of a dropper. A small amount of bacterial colony was transferred with a sterile loop onto the applied hydrogen peroxide (H2O2).

Inference: Catalase positive bacteria breakdown H2O2 into water and oxygen and would give off effervescence of bubbles. Catalase negative bacteria would not produce bubbles.

## 6) Starch hydrolysis test

Procedure: Starch hydrolyzing bacteria 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. This test was performed to determine the presence of alpha-amylase activity in bacterial isolates. Bacterial isolate was streaked back and forth across starch agar plate. After overnight incubation at $37^0$C, the plate was flooded with iodine reagent with the aid of a dropper.

Inference: Iodine reagent changes the color of starch to blue-brown. If these enzymes were produced by the bacteria during incubation period, it would hydrolyze starch by breaking the glycosidic linkages between glucose subunits the starch around its growth, creating halos in the middle of the plate. This would suggest a starch hydrolysis positive result. Absence of clear halo would determine a negative test result and non-starch utilizing bacteria.

## 7) Nitrate reduction test

Procedure: 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).Single colony of bacteria was inoculated into nitrate broth containing Durham tube, with the aid of sterile loop. Following overnight incubation at $37^0$C, 5 drops of sulfalinic acid and 5 drops of alpha-naphthyl amine reagents were added.

Inference: On addition of the reagents, a bright pink or red color would appear for the strains having the ability to reduce nitrate to nitrite. Bubble formation in Durham tubes would confer the farther reduction of nitrite to nitrogen gas. A negative test result would give no change when reagents are added. Farther addition of a pinch of zinc to the negative test tubes would provide a pink color.

### 2.6.4 Fermentation tests

### 1) Carbohydrate fermentation test

Procedure: Single bacterial colony was aseptically suspended into individual glucose, sucrose and lactose broth containing phenol red. The tubes had inverted Durham tubes for observation of gas formation. The tubes were incubated for 48 hours at $37^0$C for slow fermenters.

Inference: The ability of bacteria to ferment a particular sugar would turn the broth yellow in color through acid formation. Slow fermenters would change it to a yellowish orange color, while non-fermenters would let the broth retain its original red color.

### 2) Methyl red (MR) test

Procedure: Methyl red test was performed to determine the ability of isolate to carry out mixed acids fermentation when supplied glucose. Bacterial colonies from fresh plates were inoculated into individual potassium phosphate broth (MR-VP broth) containing peptone, dextrose and potassium phosphate. These were incubated overnight at $37^0$C. A few drops of methyl red reagent were added to test the pH of the broth.

Inference: If the organism produced sufficient acid to overcome the phosphate buffer, red color would be produced on the addition of MR reagent to indicate a positive test. Prevalence of original yellow color would indicate a negative test.

### 3) Voges-Proskauer (VP) Test

Procedure: The VP test identifies organism that use the butylene glycol pathway and produce acetoin. Bacteria to be tested were inoculated into MR-VP broth and incubated overnight at $37^0$C. Few drops of Barritt's reagent A was added to the broth and slightly shaken to disperse the cloudiness. An equal amount of Barritt's reagent B was next added and the tubes allowed standing for 15 min.

Inference: A positive test result would be indicated by appearance of pinkish-red color, whereas a negative test would show no change in color.

### 4) Arabinose test

Procedure: Bacterial colony was inoculated in L-arabinose broth with sterile loop. After overnight incubation at $37^0$C, gas formation and change in color of the broth was observed.

Inference: The ability of a strain to ferment arabinose would change the red broth into yellow in color. Non arabinose fermenter would display no change.

## 5. Fructose test

Procedure: Bacterial colony was inoculated in fructose broth with sterile loop. After overnight incubation at $37^0$C, change in color of the broth was observed.

Inference: The ability of a strain to ferment fructose would change the red broth into yellow in color. Non fermenters would create an alkaline condition and turn the broth bright pink.

## 6. Galactose test

Procedure: Bacterial colony was inoculated in galactose broth with sterile loop. After overnight incubation at $37^0$C, gas formation and change in color of the broth was observed.

Inference: The ability of a strain to ferment arabinose would change the red broth into yellow in color. Non fermenters would create an alkaline condition and turn the broth bright pink

## 7. Glucose test

Procedure: Bacterial colony was inoculated in glucose broth with sterile loop. After overnight incubation at $37^0$C, gas formation and change in color of the broth was observed.

Inference: The ability of a strain to ferment glucose would change the red broth into yellow in color. Non fermenters would create an alkaline condition and turn the broth bright pink

## 8. Lactose test

Procedure: Bacterial colony was inoculated in lactose broth with sterile loop. After overnight incubation at $37^0$C, gas formation and change in color of the broth was observed.

Inference: The ability of a strain to ferment lactose would change the red broth into yellow in color. Non fermenters would create an alkaline condition and turn the broth bright pink

## 9. Maltose test

Procedure: Bacterial colony was inoculated in maltose broth with sterile loop. After overnight incubation at $37^0$C, gas formation and change in color of the broth was observed.

Inference: The ability of a strain to ferment maltose would change the red broth into yellow in color. Non fermenters would create an alkaline condition and turn the broth bright pink.

### 10. Manitol test

Procedure: Bacterial colony was inoculated in manitol broth with sterile loop. After overnight incubation at $37^0$C, gas formation and change in color of the broth was observed.

Inference: The ability of a strain to ferment manitol would change the red broth into yellow in color. Non fermenters would create an alkaline condition and turn the broth bright pink

### 11. Sucrose test

Procedure: Bacterial colony was inoculated in sucrose broth with sterile loop. After overnight incubation at $37^0$C, gas formation and change in color of the broth was observed.

Inference: The ability of a strain to ferment sucrose would change the red broth into yellow in color. Non fermenters would create an alkaline condition and turn the broth bright pink

### 12. Treshalos test

Procedure: Bacterial colony was inoculated in treshalos broth with sterile loop. After overnight incubation at $37^0$C, gas formation and change in color of the broth was observed.

Inference: The ability of a strain to ferment treshalos would change the red broth into yellow in color. Non fermenters would create an alkaline condition and turn the broth bright pink

### 2.6.5 Selective and differential media tests

### 1) Salt tolerant test

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

Procedure: With the aid of sterile inoculating loop, single bacterial colony from fresh plate was transferred to 6.5%, 7%, 10% and 15% NaCl in nutrient agar plat. The plats were incubated at $37^0$C. Observation was made at 24 hours and every day for a period of 3 days if no change occurred.

Inference: High salt tolerant bacteria would grow after incubation, giving a positive result. Bacteria would be unable to grow and make the negative test result.

## 2.7 Preservation of bacteria

3 mL T1N1 agar was inoculated through stabbing each bacterium from nutrient agar plate. The vial was incubated for 5 hours to allow the bacteria to acquire log phase. 200 µL of sterile glycerol was next added and the vial sealed with parafilm and stored at room temperature.

# Chapter 3: Results

In this study four different isolates for OM2R dye degradation and another four different isolates for GGS dye degradation were selected from four different effluent soil samples. Isolates were individually cultured for 5 days in SM broth containing Azo dye (OM2R and GGS) as a sole carbon source. The ability of each isolates to degrade in the presence of corresponding azo dye (OM2R and GGS) was determined by observing the optical density after degradation. Higher dye degrading isolates were shown low absorbance and vice versa. Through this technique two isolates for OM2R and another two isolates for GGS dye degradation were selected.

**Decolourization (%) percentage of isolates was measured by using the following equation:**

After the measurement of Initial OD and Final OD, values were placed in this equation.

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

Visible lights wavelength is from 400 to 700 nm. The visible green light has a wavelength of about 510nm and the visible orange light has a wavelength of about 590nm been used in this experiment.

## 3.1Azo dye degrading ability of different isolates

Four isolates AO, BO, CO and DO that were isolated from effluent soil samples then inoculated for 5 days in 100 ml SM broth media that contain 1% OM2R azo dye at room temperature to decolourized OM2R azo dye. In each100 ml SM broth inoculum amount was 50µl individually .Then 7ml of each culture was centrifuged for 5 minutes at 8000rpm. Then using spectrophotometer the absorbance of supernatant of cultured media was measured to identify the highest dye degrading bacteria.

**Table 3.1(I):** Absorbance of 1% OM2R dye containing SM broth after decolourization by selected isolates.

| Isolates | Control | Absorbance at 590nm |
|---|---|---|
| **AO** | 0 | 0.008 |
| **BO** | 0 | 0.031 |
| **CO** | 0 | 0.001 |
| **DO** | 0 | 0.023 |

Best two (AO and CO) were selected for further study comparing the absorbance value of the OM2R degrading isolates. Low absorbance means high degradation. Initial OD value for 1% was 0.120 and was turned in to auto zero.


**Figure3.1 (I):** Decolourization of OM2R dye by AO, BO, CO and DO isolates.

Same procedure was applied for GGs dye degrading bacterial isolates.

**Table 3.1(II):** Absorbance of 1% GGS dye containing SM broth after decolourization by selected isolates.

| Isolates | Control | Absorbance at 510nm |
|---|---|---|
| **AG** | 0 | 0.017 |
| **BG** | 0 | 0.011 |
| **CG** | 0 | 0.009 |
| **DG** | 0 | 0.038 |

Best two (BG and CG) were selected for further study comparing the absorbance value of the GGS dye degrading isolates. Initial OD value for 1% was 0.160 and was turned in to auto zero.


**Figure 3.1(II):** Decolourization of GGS dye by AG, BG, CG and DG isolates.

## 3.2 Optimization of different parameters for OM2R decolourizing bacteria.

### 3.2.1 Effect of different concentration of Azo dye (OM2R) on decolourization of selected isolates.

The AO and CO isolates were cultured in six 50 ml SM broth containing 1%, 3% and 5% OM2Rdyeindividually at $37^0$C for 5 days then OD of each sample supernatant was taken after centrifugation at 8000rpm.

**Table3.2.1 (I): Absorbance data of decolourization by AO and CO isolates when cultured for five days in SM broth with varying dye concentration**

| Day | Absorbance at 590nm | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | control | | 1% | | 3% | | 5% | |
| | AO | CO | AO | CO | AO | CO | AO | CO |
| 1 | 0 | 0 | 0.031 | 0.020 | 0.041 | 0.029 | 0.047 | 0.038 |
| 2 | 0 | 0 | 0.025 | 0.011 | 0.036 | 0.022 | 0.036 | 0.030 |
| 3 | 0 | 0 | 0.019 | 0.009 | 0.024 | 0.015 | 0.030 | 0.021 |
| 4 | 0 | 0 | 0.013 | 0.007 | 0.020 | 0.011 | 0.024 | 0.020 |
| 5 | 0 | 0 | 0.009 | 0.003 | 0.018 | 0.008 | 0.021 | 0.016 |

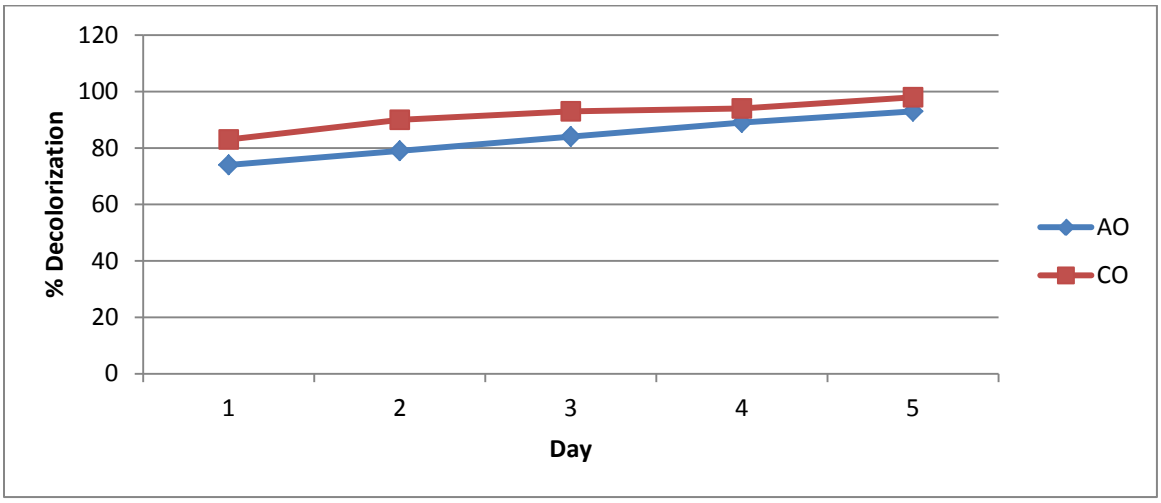
Initial OD value for 1%, 3% and 5% were consecutively 0.120, 0.138 and 0.165.Those value were turned in to auto zero. (AO and CO were the designation of selected isolates)

**Table 3.2.1(II): Calculated dye decolourization % by AO and CO at 1%, 3% and 5% OM2R dye concentration.**

| Day | 1% | | 3% | | 5% | |
|---|---|---|---|---|---|---|
| | AO | CO | AO | CO | AO | CO |
| 1 | 74 | 83 | 70 | 78 | 72 | 77 |
| 2 | 79 | 90 | 74 | 84 | 78 | 82 |
| 3 | 84 | 93 | 82 | 89 | 82 | 87 |
| 4 | 89 | 94 | 86 | 92 | 85 | 88 |
| 5 | 93 | 98 | 87 | 94 | 87 | 90 |

(AO and CO were the designation of selected isolates)

**Graph 3.2.1 (I): Decolourization of OM2R at 1% dye concentration by the isolates AO and CO through 5 consecutive days incubated at $37^0$C.**




(AO and CO were the designation of selected isolates)

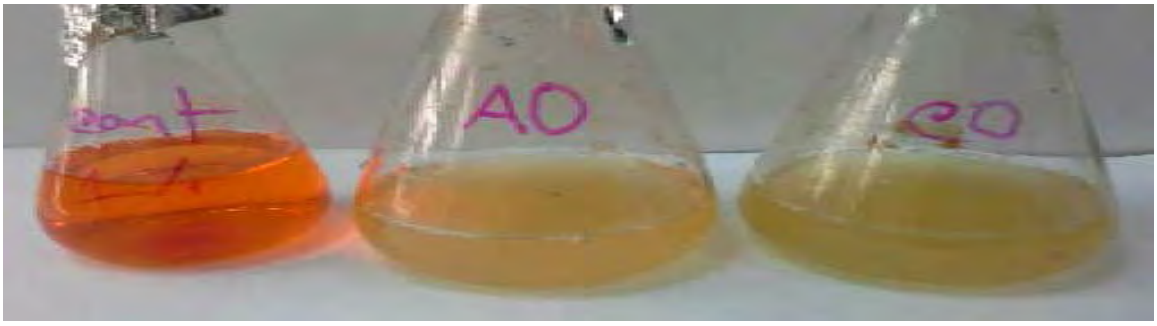
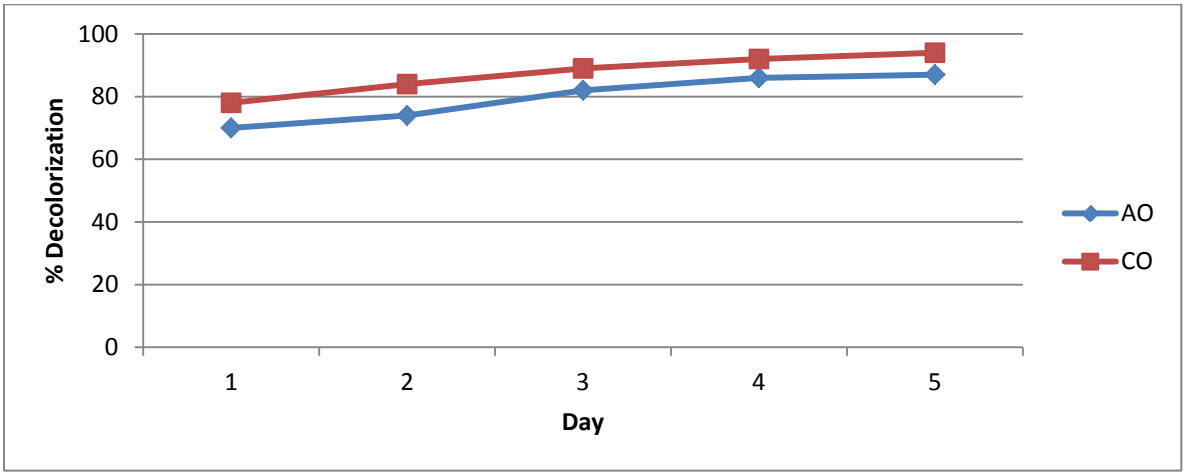



**Figure 3.2.1(I):** Decolourization of OM2R at 1% dye concentration by the isolates AO and CO when incubated for 5 days at $37^0$C.

**Graph 3.2.1 (II): Decolourization of OM2R at 3% dye concentration by the isolates AO and CO through 5 consecutive days incubated at $37^0$C.**




(AO and CO were the designation of selected isolates)

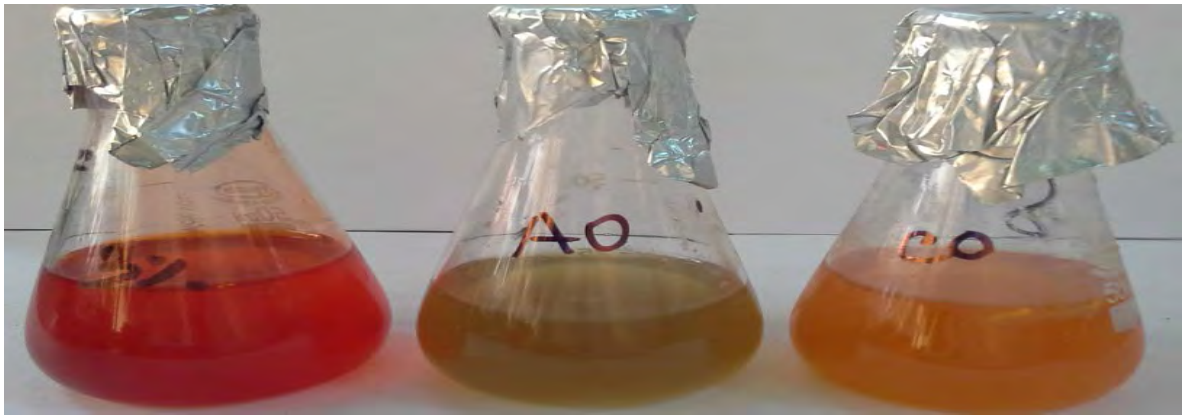
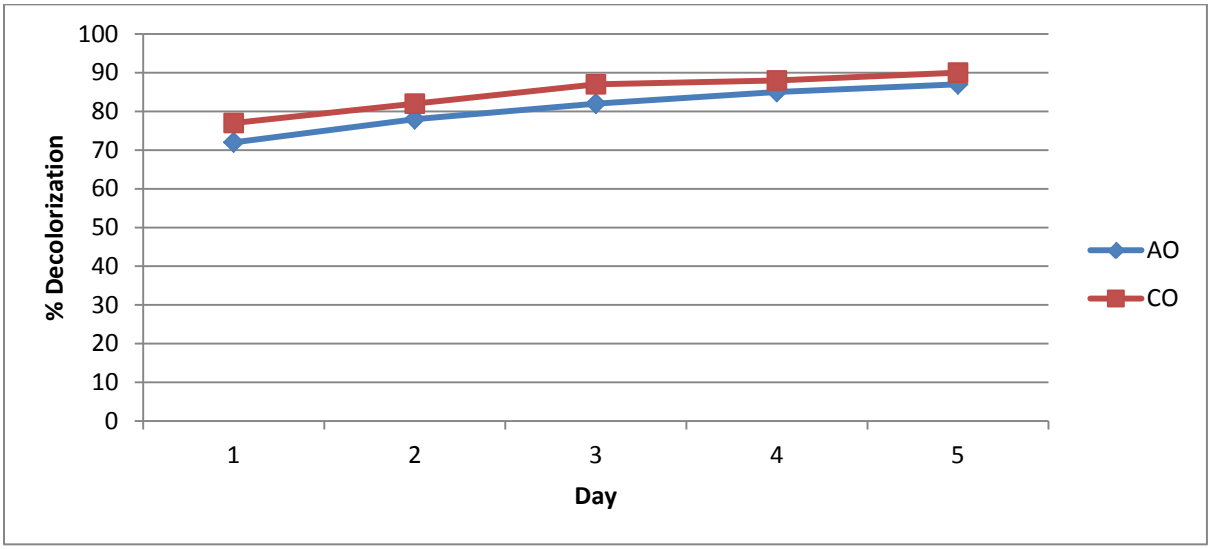



**Figure 3.2.1(II):** Decolourization of OM2R at 3% dye concentration by the isolates AO and CO when incubated for 5 days at $37^0$C.

**Graph 3.2.1 (III): Decolourization of OM2R at 5% dye concentration by the isolates AO and CO through 5 consecutive days incubated at $37^0$C.**




(AO and CO were the designation of selected isolates)

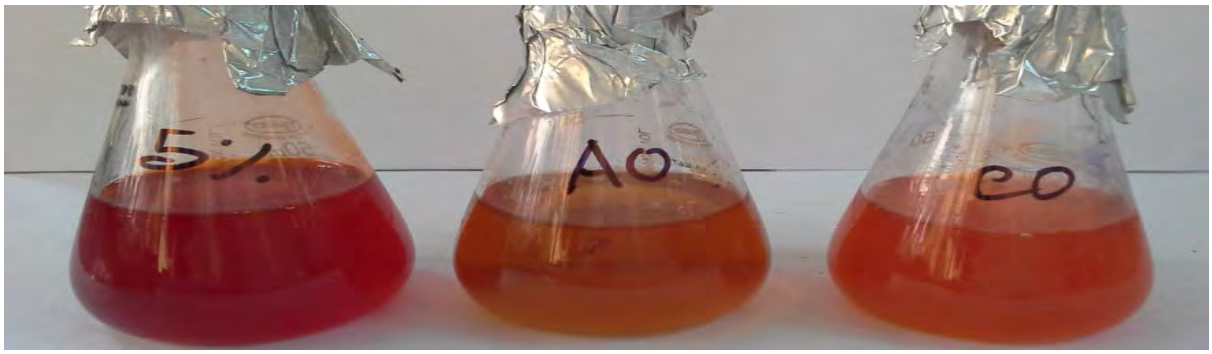



**Figure 3.2.1(III):** Decolourization of OM2R at 5% dye concentration by the isolates AO and CO when incubated for 5 days at $37^0$C.

### 3.2.2: Effect of different pH on decolourization of OM2R dye by the isolates AO and CO when incubated for 5 days at $37^0$C.

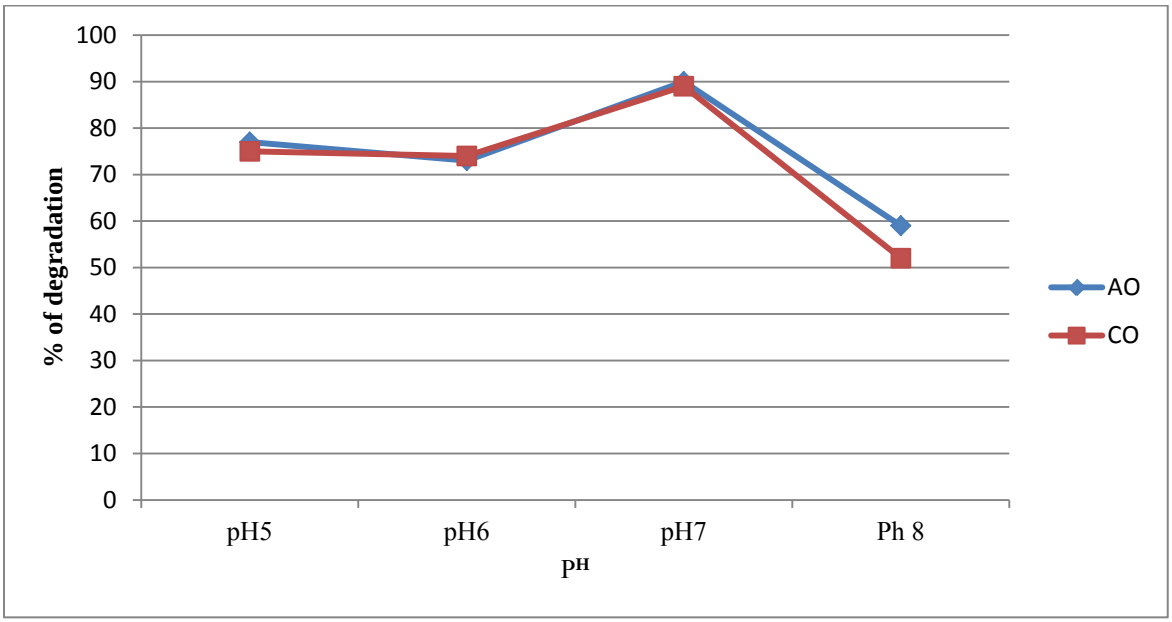
**Table 3.2.2: Absorbance data of decolourization by the isolates AO and CO after five days cultured in OM2R dye containing SM broth with varying pH**

| Day | Absorbance at 590nm | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | control | | 5 | | 6 | | 7 | | 8 | |
| | AO | CO | AO | CO | AO | CO | AO | CO | AO | CO |
| $5^{th}$ | 0 | 0 | 0.031 | 0.034 | 0.040 | 0.038 | 0.018 | 0.020 | 0.051 | 0.059 |
| Decolourization (%) | | | 77% | 75% | 73% | 74% | 90% | 89% | 59% | 52% |

(AO and CO were the designation of selected isolates)

Initial OD value for pH 5, pH 6, pH 7 and pH 8 were consecutively 0.138, 0.150, 0.175 and 0.123.Those values were turned in to auto zero.

**Graph3.2.2: Effect of different pH on decolourization of OM2Rdye by the isolates AO and CO when incubated for 5 days at $37^0$C.**




(AO and CO were the designation of selected isolates)

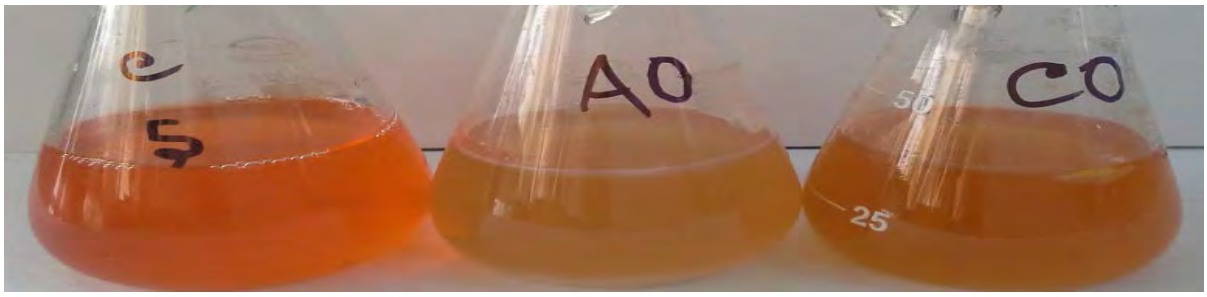



**Figure 3.2.2 (I):** Effect of pH 5 on decolourization of the OM2R dye by isolates AO and CO when incubated for 5 days at $37^0$C.


**Figure 3.2.2 (II):** Effect of pH 6 on decolourization of the OM2R dye by isolates AO and CO when incubated for 5 days at $37^0$C.

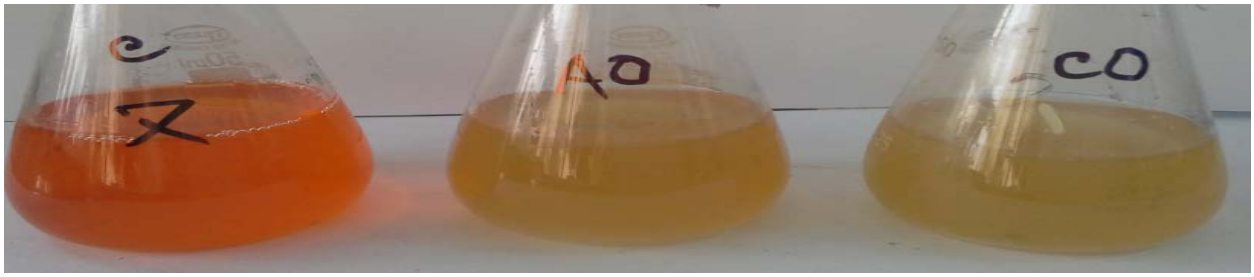



**Figure 3.2.2 (III):** Effect of pH 7 on decolourization of the OM2R dye by isolates AO and CO when incubated for 5 days at $37^0$C.

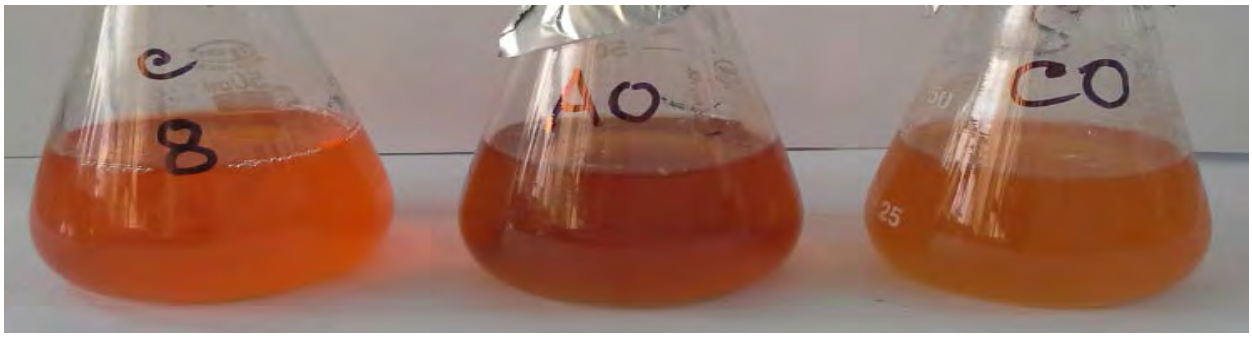



**Figure 3.2.2 (VI):** Effect of pH 8 on decolourization of the OM2R dye by isolates AO and CO when incubated for 5 days at $37^0$C.

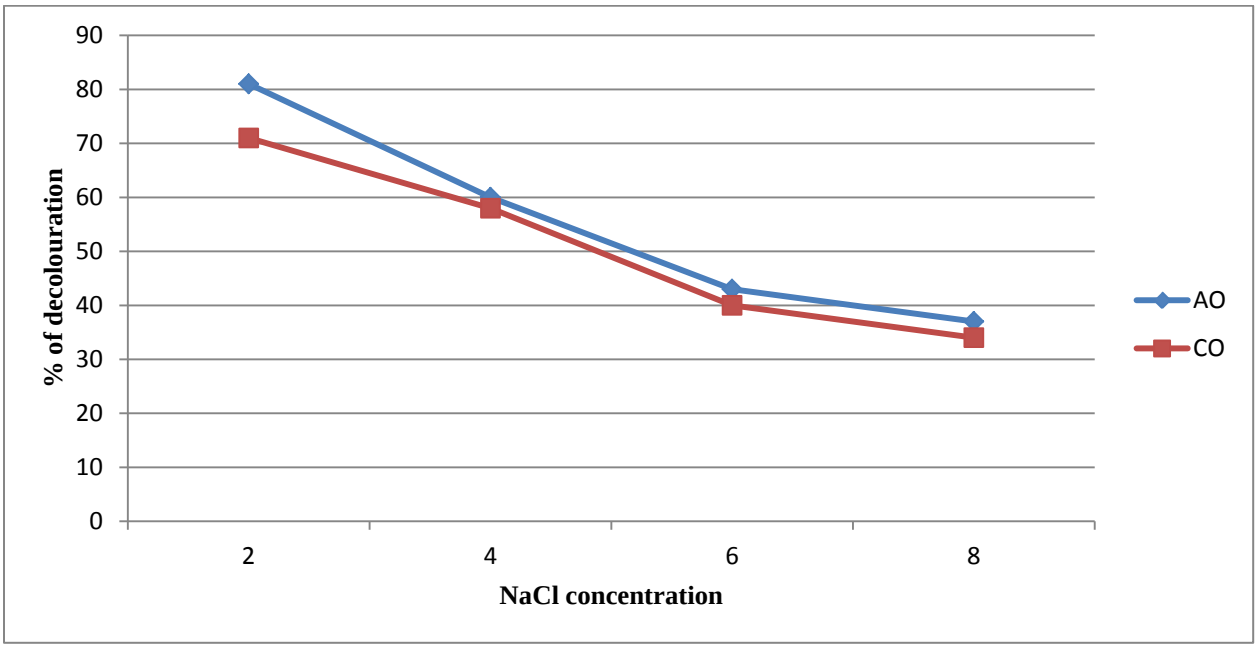
### 3.2.3: Effect of different NaCl concentration on decolourization of OM2R dye by the isolates AO and CO when incubated for 5 days at $37^0$C.

Table 3.2.3: Absorbance data of decolourization **by** AO and CO isolates when incubated for five days in SM broth with varying NaCl concentration.

| Day | Absorbance at 590nm | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | control | | 2% | | 4% | | 6% | | 8% | |
| | 0 | 0 | AO | CO | AO | CO | AO | CO | AO | CO |
| 5th | 0 | 0 | 0.031 | 0.049 | 0.054 | 0.057 | 0.072 | 0.076 | 0.075 | 0.079 |
| Degradation (%) | | | 81 | 71 | 60 | 58 | 43 | 40 | 37 | 34 |

Initial (OD) value of 2%, 4%, 6% and 8% sodium chloride were0.170, 0.136, 0.126 and 0.120. (AO and CO were the designation of selected isolates)

**Graph3.2.3: Decolourization of OM2R at varying NaCl concentration by the isolates AO and CO.**




(AO and CO were the designation of selected isolates)

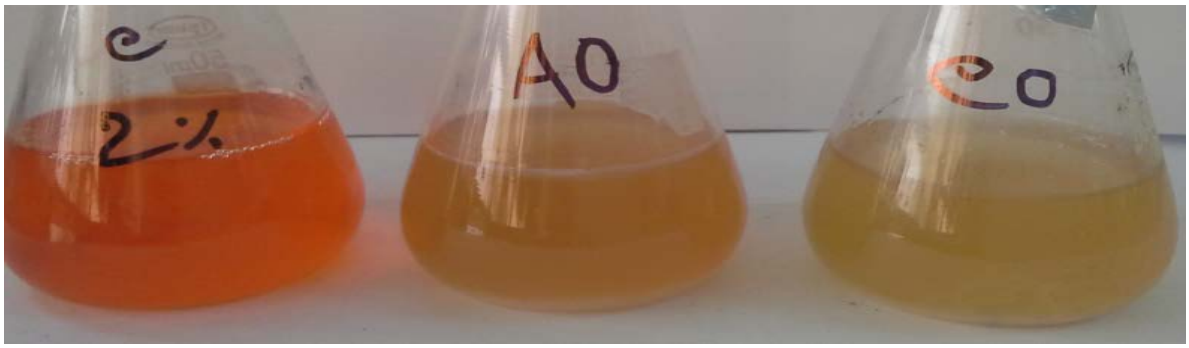



**Figure 3.2.3 (I):** Effect of 2% NaCl on decolourization of the OM2R dye by isolates AO and CO when incubated for5 days at $37^0$C.

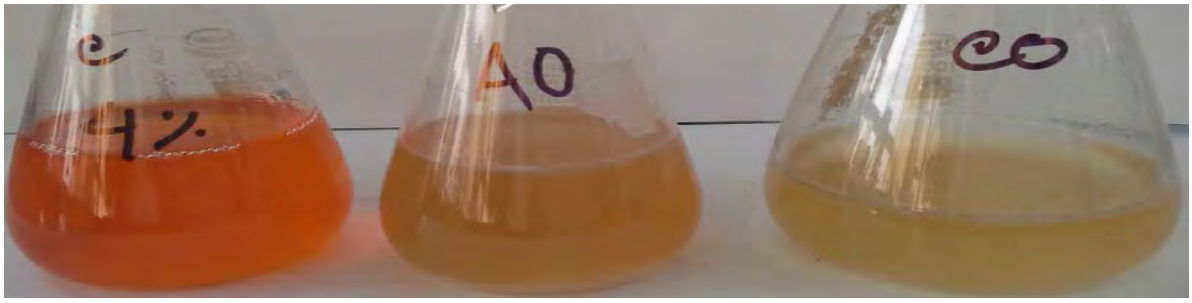



**Figure 3.2.3 (II):** Effect of 4% NaCl on decolourization of the OM2R dye by isolates AO and CO when incubated for 5 days at $37^0$C.

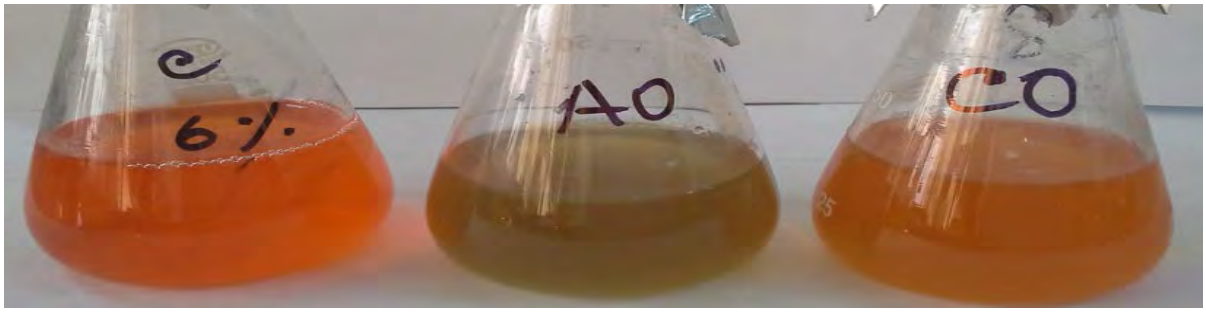



**Figure 3.2.3 (III):** Effect of 6% NaCl on decolourization of the OM2R dye by isolates AO and CO when incubated for 5 days at $37^0$C.

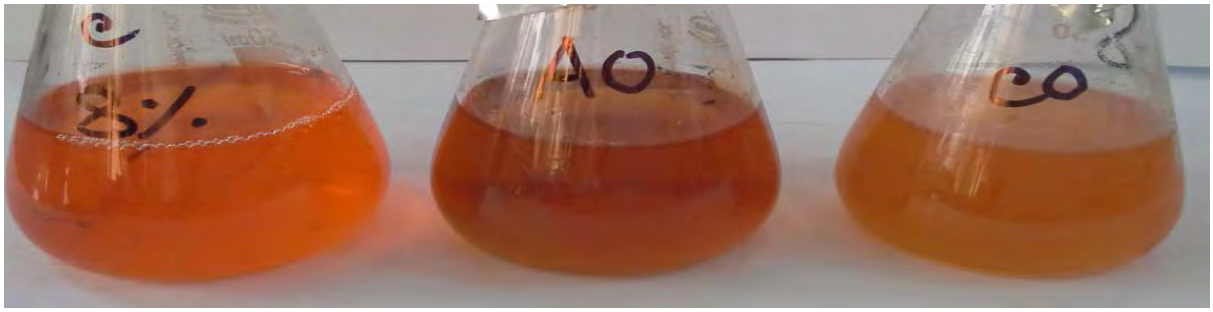



**Figure 3.2.3 (IV):** Effect of 8% NaCl on decolourization of the OM2R dye by isolates AO and CO when incubated for 5 days at $37^0$C.

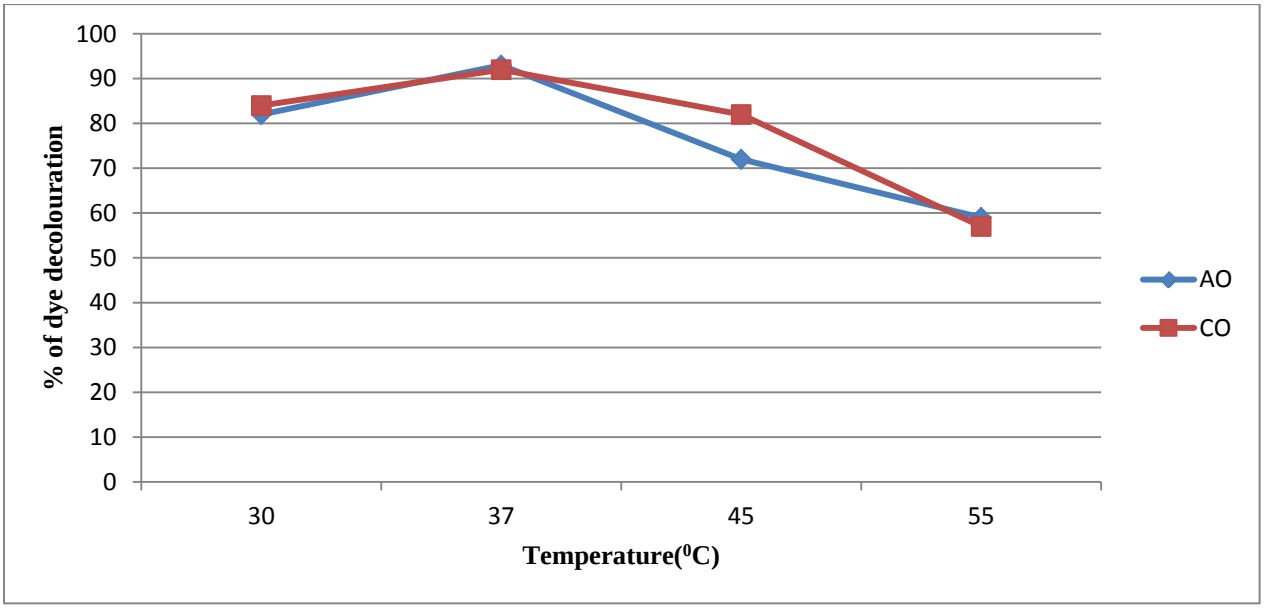
**3.2.4Effect of different temperature on decolourization of OM2R dye by the isolates AO and CO when incubated for 5 days at $37^0$C.**

**Table 3.2.4: Absorbance data of decolourization by AO and CO isolates after five days cultured in SM broth contain 1% OM2R dye with varying temperature$30^0$C, $37^0$C, 45 $^0$C and $55^0$C.**

| Day | Absorbance at 590nm | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | control | | $30^0$C | | $37^0$C | | $45^0$C | | $55^0$C | |
| | | | AO | BO | AO | BO | AO | BO | AO | BO |
| $5^{th}$ | 0 | 0 | 0.025 | 0.022 | 0.008 | 0.010 | 0.040 | 0.025 | 0.053 | 0.055 |
| decolourization (%) | | | 82 | 84 | 93 | 92 | 72 | 82 | 59 | 57 |

Initial (OD) value of $30^0$C, $37^0$C, $45^0$Cand $55^0$Cwere0.136, 0.121, 0.143 and 0.130(AO and CO were the designation of selected isolates)

**Graph 3.2.4: Decolourization of OM2R at varying temperature by the isolates AO and CO.**




(AO and CO were the designation of selected isolates)

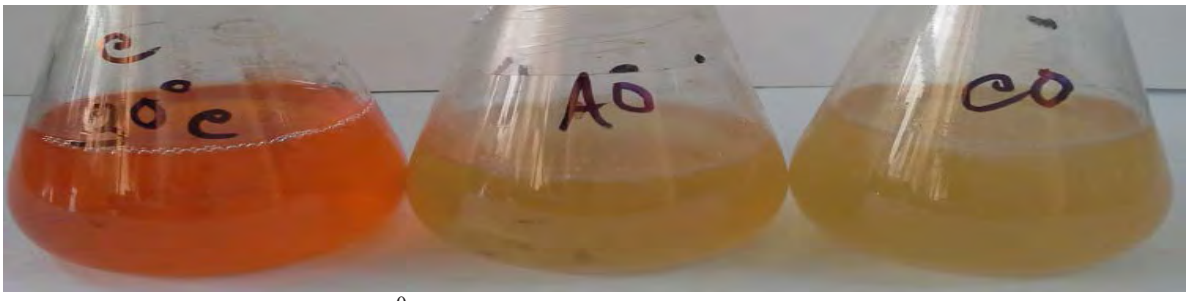



**Figure 3.2.4 (I):** Effect of $30^0$C on decolourization of the OM2R dye by isolates AO and CO when incubated for 5 days.


**Figure 3.2.4 (II):** Effect of $37^0$C on decolourization of the OM2R dye by isolates AO and CO when incubated for 5 days.

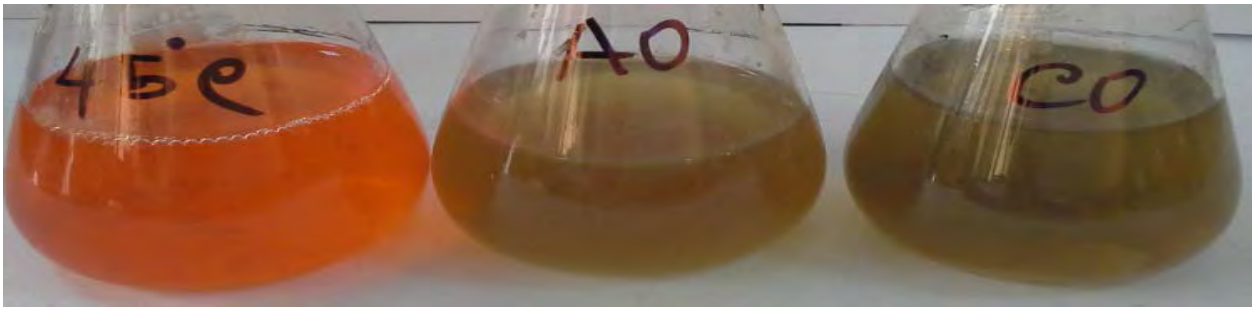



**Figure 3.2.4 (III):** Effect of $45^0$C on decolourization of the OM2R dye by isolates AO and CO when incubated for 5 days.

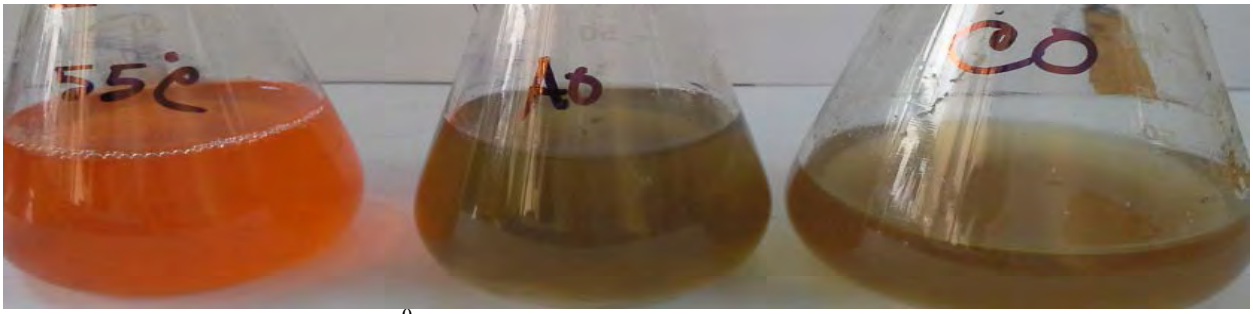



**Figure 3.2.4 (IV):** Effect of $55^0$ C on decolourization of the OM2R dye by isolates AO and CO when incubated for 5 days.

## 3.3 Optimization of different parameters for GGS dye decolourizing bacteria.

### 3.3.1Effect of different concentration of Azo dye (GGS) on decolourization by selected isolates

The BG and CG isolates were cultured in six 50 ml SM broth containing 1%, 3% and 5% GGS dye individually at $37^0$C for 5 days then OD of each sample supernatant was taken after centrifugation at 8000rpm.

Isolates ID-BG and CG Dye –GGS

**Table3.3.1 (I): Absorbance data of decolourization by BG and CG isolates when cultured for five days in SM broth with varying dye concentration at $37^0$C.**

| Day | Absorbance at 510nm | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | control | | 1% | | 3% | | 5% | |
| | BG | CG | BG | CG | BG | CG | BG | CG |
| 1 | 0 | 0 | 0.025 | 0.033 | 0.031 | 0.042 | 0.079 | 0.098 |
| 2 | 0 | 0 | 0.018 | 0.026 | 0.023 | 0.030 | 0.066 | 0.078 |
| 3 | 0 | 0 | 0.013 | 0.019 | 0.019 | 0.022 | 0.051 | 0.062 |
| 4 | 0 | 0 | 0.009 | 0.015 | 0.016 | 0.017 | 0.044 | 0.054 |
| 5 | 0 | 0 | 0.005 | 0.011 | 0.010 | 0.013 | 0.039 | 0.049 |

(BG and CG were the designation of selected isolates)

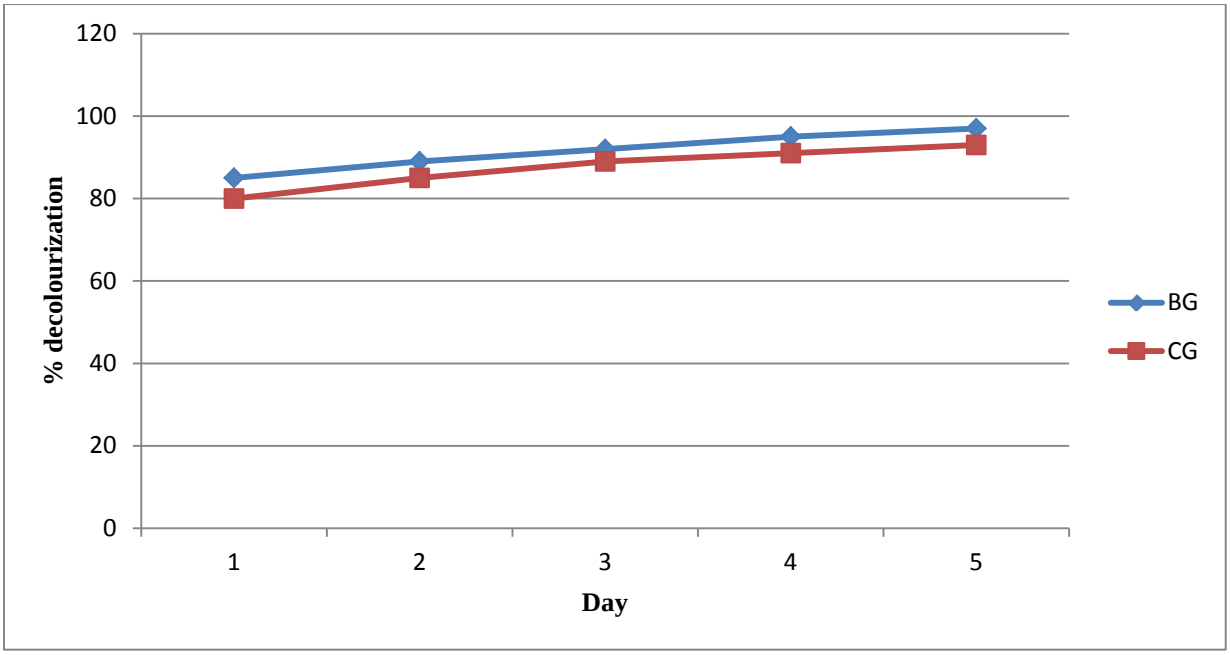
**Table 3.3.1(II): Calculated dye decolourization % by BG and CG at 1%, 3%and 5% (GGS) dye concentration.**

| Days | 1% | | 3% | | 5% | |
|---|---|---|---|---|---|---|
| | BG | CG | BG | CG | BG | CG |
| 1 | T | 80 | 83 | 76 | 63 | 54 |
| 2 | 89 | 85 | 87 | 83 | 69 | 63 |
| 3 | 92 | 89 | 89 | 86 | 76 | 71 |
| 4 | 95 | 91 | 91 | 90 | 80 | 75 |
| 5 | 97 | 93 | 94 | 93 | 81 | 77 |

(BG and CG were the designation of selected isolates)

Initial OD value for 1%, 3% and 5% were consecutively 0.164, 0.178 and 0.215. Those value were turned in to auto zero.

**Graph 3.3.1 (I): Effect of 1% GGS dye on decolourization by the isolates BG and CG through 5 consecutive days when incubated at $37^0$C.**




(BG and CG were the designation of selected isolates)

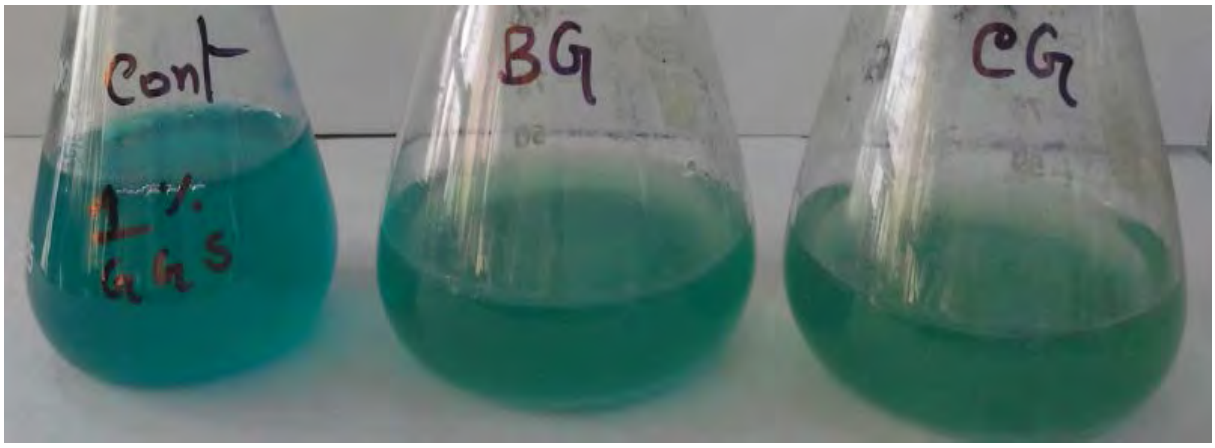
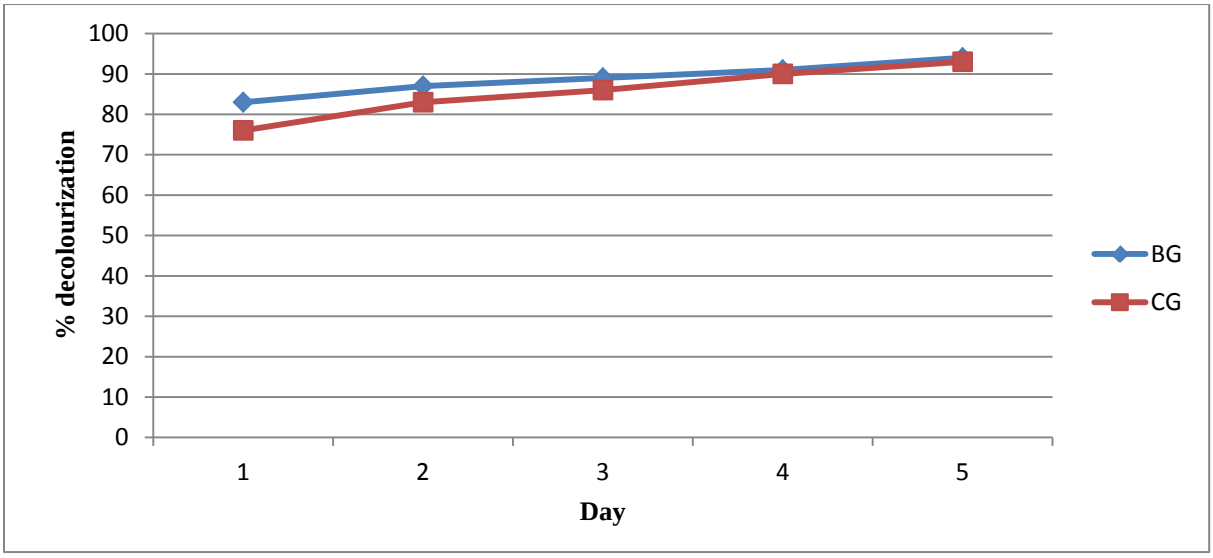



**Figure 3.3.1(I):** Decolourization of GGS dye at 1% dye concentration by the isolates BG and CG when incubated for 5 days at $37^0$C

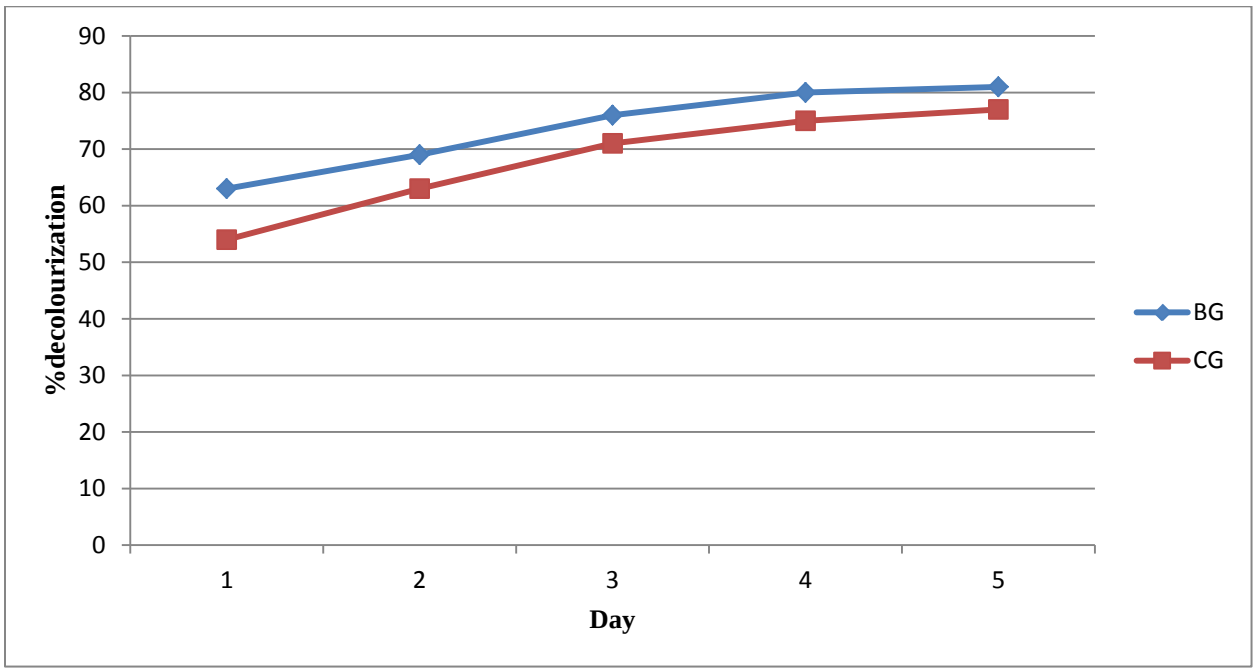
**Graph 3.3.1 (II): Effect of 3% GGS dye on decolourization by the isolates BG and CG through 5 consecutive days incubated at $37^0$C.**




(BG and CG were the designation of selected isolates)


**Figure 3.3.1(II):** Decolourization of GGS dye at 3% dye concentration by the isolates BG and CG when incubated for 5 days at $37^0$C

**Graph 3.3.1 (III): Effect of 5% GGS dyeon decolouriztion by the isolatesBG and CG through 5 consecutive days incubated at $37^0$C.**




(BG and CG were the designation of selected isolates)

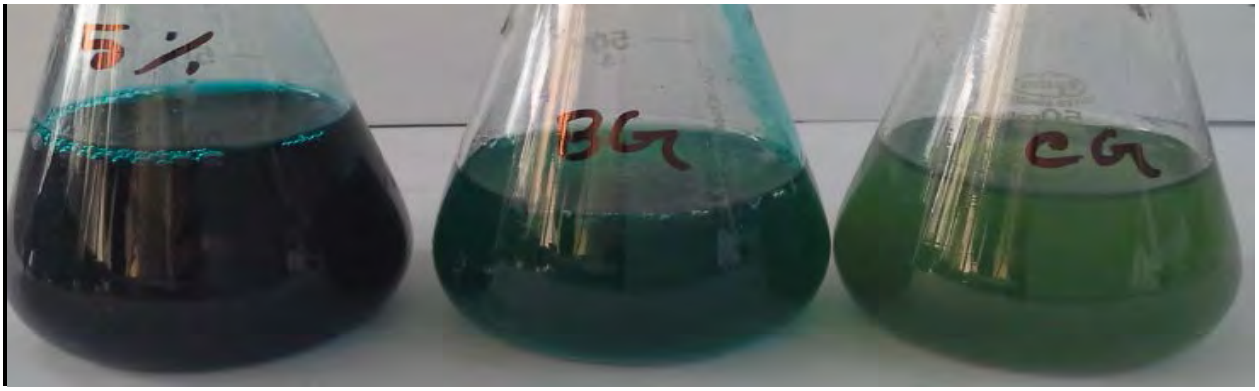



**Figure 3.3.1(iii):** Decolourization of GGS dye at 5% dye concentration by the isolates BG and CG when incubated for 5 days at $37^0$C

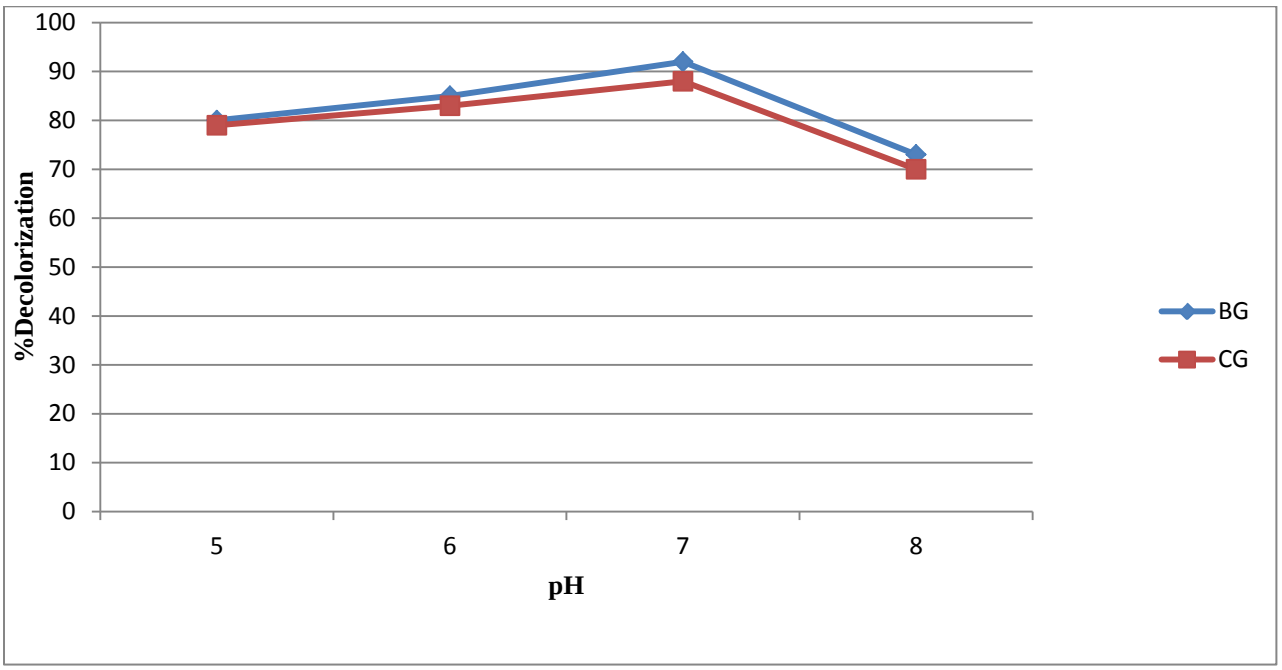
### 3.3.2: Effect of different pH on decolourization of GGS dye by the isolates BG and CG when incubated for 5 days at $37^0$C.

**Table 3.3.2: Absorbance data of decolourization by BG and CG isolates when incubated for 5 days in SM broth containing 1% of GGS dye with varying pH 5, pH 6, pH 7 and pH 8.**

| Day | Absorbance at 510nm | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | control | | 5 | | 6 | | 7 | | 8 | |
| | BG | CG | BG | CG | BG | CG | BG | CG | BG | CG |
| $5^{th}$ | 0 | 0 | 0.042 | 0.044 | 0.055 | 0.061 | 0.051 | 0.080 | 0.089 | 0.098 |
| decolourization (%) | 0 | 0 | 80 | 79 | 85 | 83 | 92 | 88 | 73 | 70 |

Initial value for pH 5, pH 6, pH 7 and pH 8 were consecutively 0.209, 0.361, 0.676 and 0.329.Those values were turned in to auto zero. (BG and CG were the designation of selected isolates)

**Graph 3.3.2: Decolourization of GGS dye at varying pH by the isolates BG and CG.**




(BG and CG were the designation of selected isolates)


**Figure 3.3.2 (I):** Effect of pH 5 on decolourization of the GGS dye by isolates BG and CG when incubated for 5 days at $37^0$C.

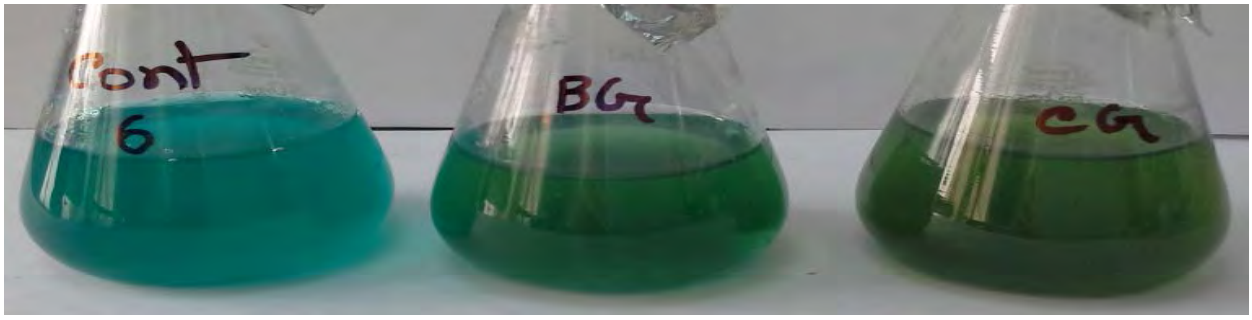



**Figure 3.3.2 (II):** Effect of pH 6 on decolourization of the GGS dye by isolates BG and CG when incubated for 5 days at $37^0$C.

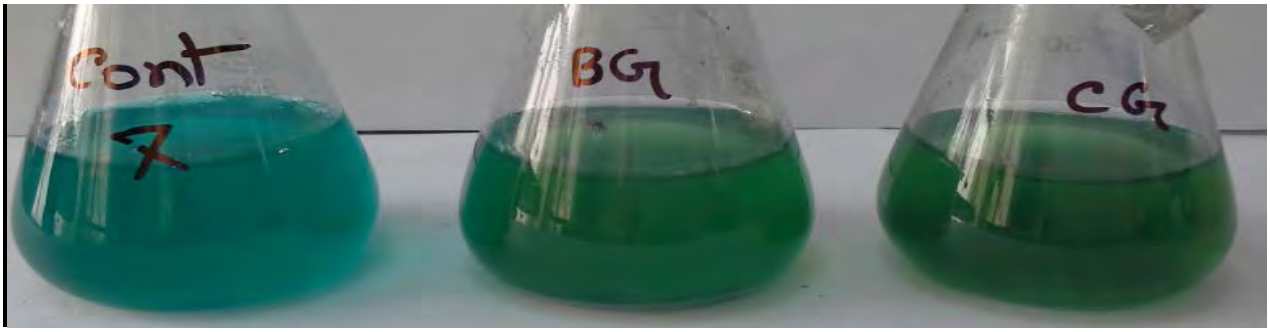



**Figure 3.3.2 (III):** Effect of pH 7 on decolourization of the GGS dye by isolates BG and CG when incubated for 5 days at $37^0$C.

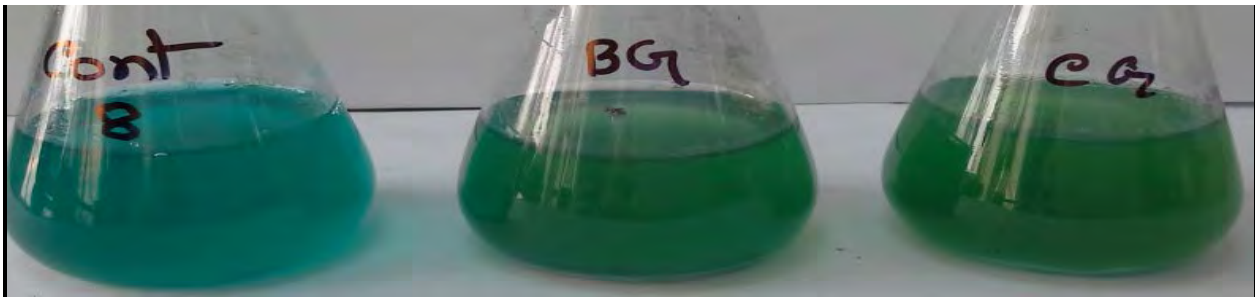



**Figure 3.3.2 (IV):** Effect of pH 8 on decolourization of the GGS dye by isolates BG and CG when incubated for 5 days at $37^0$C.

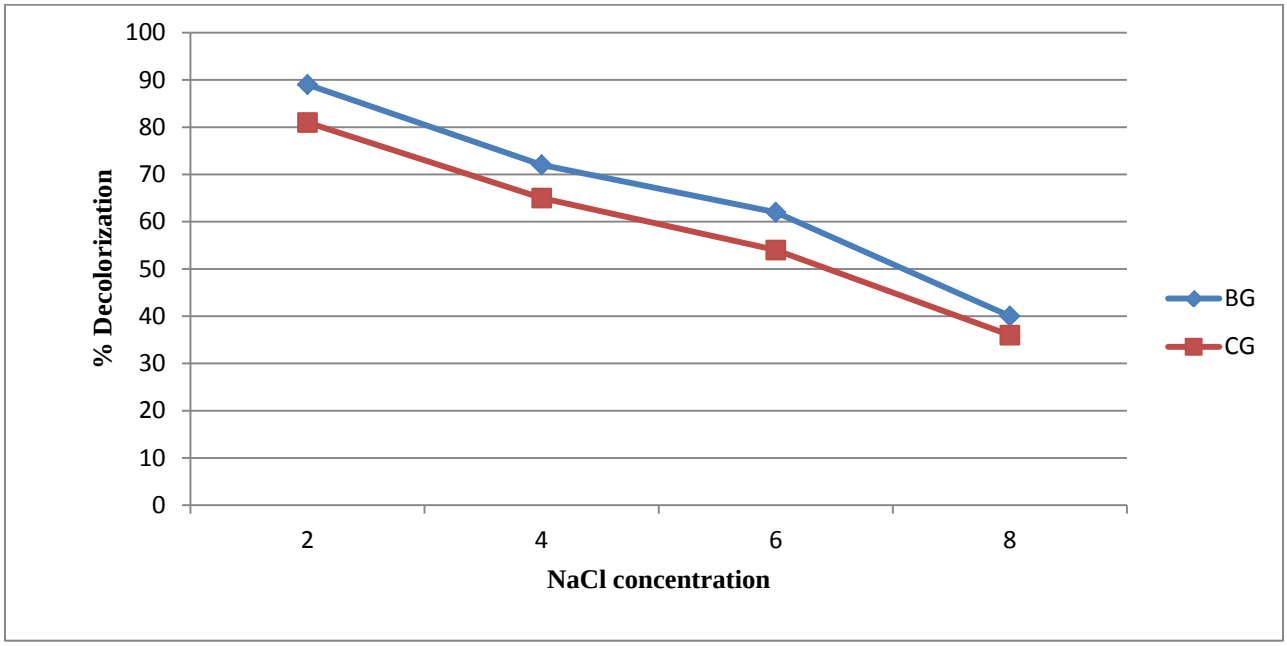
**3.3.3: Effect of different NaCl concentration on decolourization of GGS dye by the isolates BG and CG when incubated for 5 days at 37 $^0$C.**

**Table3.3.3: Absorbance data of decolourization by BG and CG isolates when incubated for five days in SM broth contained 1% of GGS dye with varying NaCl concentration.**

| Day | Absorbance at 510nm | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | control | | 2% | | 4% | | 6% | | 8% | |
| | | | BG | CG | BG | CG | BG | CG | BG | CG |
| 5$^{th}$ | 0 | 0 | 0.019 | 0.035 | 0.045 | 0.058 | 0.070 | 0.086 | 0.099 | 0.105 |
| decolourization (%) | 0 | 0 | 89 | 81 | 72 | 65 | 62 | 54 | 40 | 36 |

Initial (OD) value of 2%, 4%, 6% and 8% sodium chloride were 0.191, 0.171, 0.186 and 0.164.(BG and CG were the designation of selected isolates.

**Graph3.3.3**: **Decolourization of GGS dye at varying NaCl concentration by the isolates BG and CG.**




(BG and CG were the designation of selected isolates)

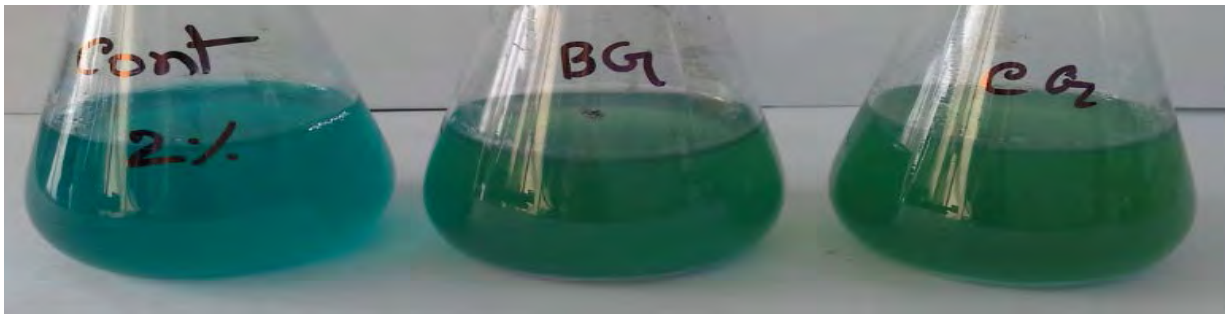



**Figure 3.3.3 (I):** Effect of 2% NaCl on decolourization of the GGS dye by isolates BG and CG when incubated for 5 days at $37^0$C.

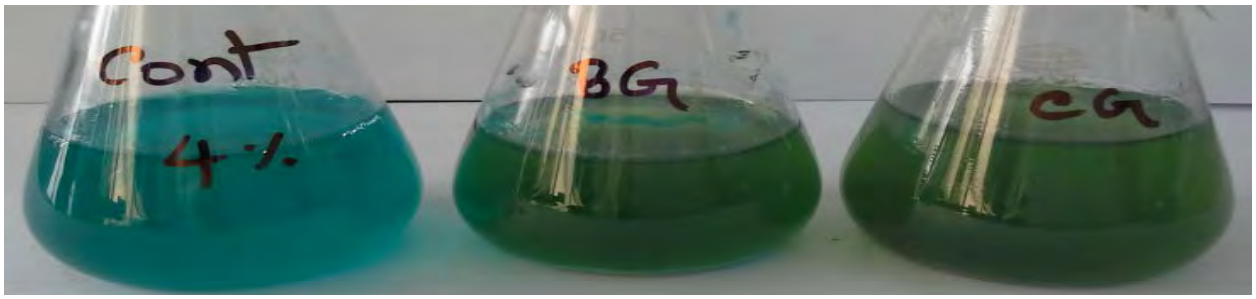



**Figure 3.3.3 (II):** Effect of 4% NaCl on decolourization of the GGS dye by isolates BG and CG when incubated for 5 days at $37^0$C.

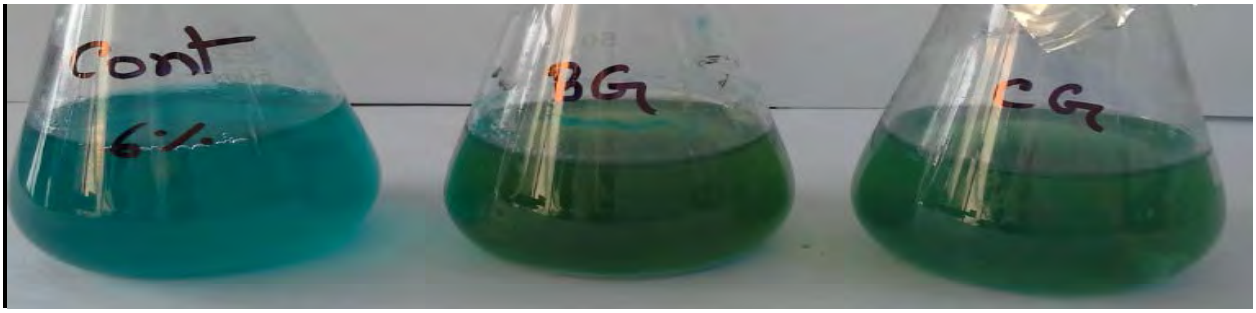



**Figure 3.3.3 (III):** Effect of 6% NaCl on decolourization of the GGS dye by isolates BG and CG when incubated for 5 days at 37 $^0$C.

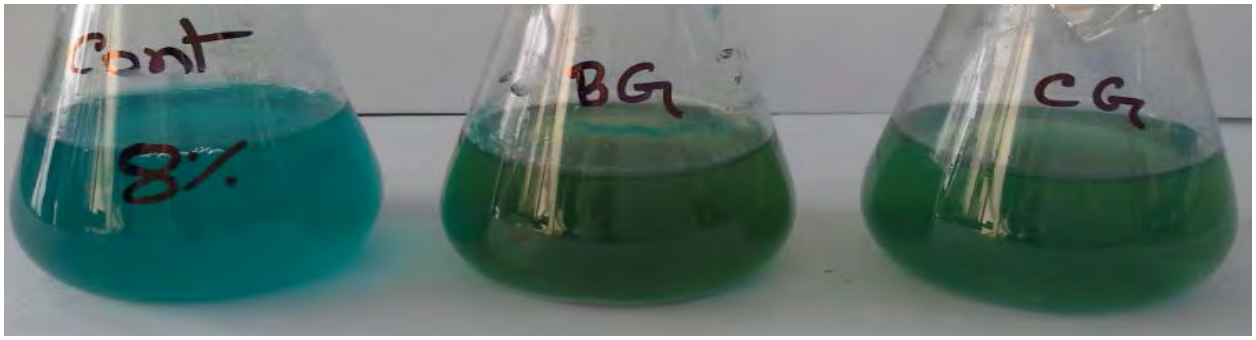



**Figure 3.3.3 (IV):** Effect of 8% NaCl on decolourization of the GGS dye by isolates BG and CG when incubated for 5 days at 37 $^0$C.

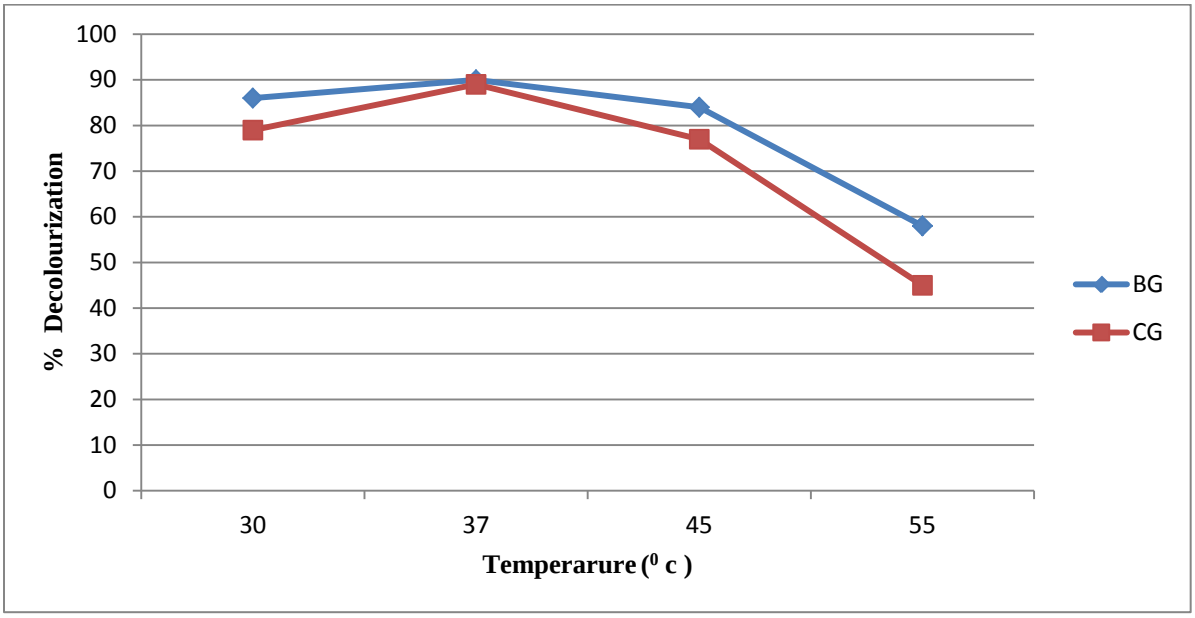
**3.3.4Effect of different temperature on decolourization of GGS dye by the isolates BG and CG when incubated for 5 days at $37^0$C.**

**Table 3.3.4: Absorbance data of decolourization by BG and CG isolates after five days cultured in SM broth contain 1% GGS dye with varying temperature of $30^0$C, $37^0$C, $45^0$C and $55^0$C.**

| Day | Absorbance at 510nm | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | control | | $30^0$C | | $37^0$C | | $45^0$C | | $55^0$C | |
| | BG | CG | BG | CG | BG | CG | BG | CG | BG | CG |
| $5^{th}$ | 0 | 0 | 0.031 | 0.045 | 0.025 | 0.028 | 0.035 | 0.049 | 0.075 | 0.093 |
| Decolourization (%) | 0 | 0 | 86 | 79 | 90 | 89 | 84 | 77 | 58 | 45 |

Initial (OD) value of $30^0$C, $37^0$C, $45^0$C and $55^0$C were 0.216, 0.256, 0.214 and 0.170 (BG and CG were the designation of selected isolates)

**Graph 3.3.4: Decolourization of GGS dye at varying temperature by the isolates BG and CG.**




(BG and CG were the designation of selected isolates)

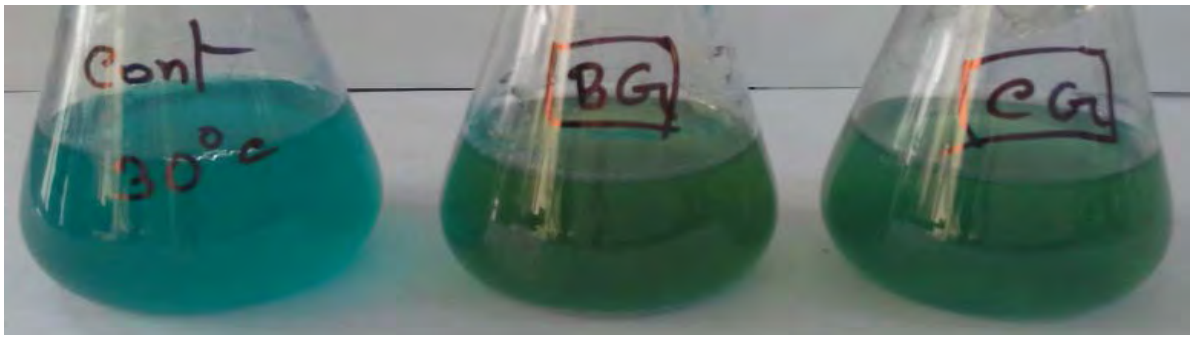



**Figure 3.3.4 (I):** Effect of $30^0$C on decolourization of the GGS dye by isolates BG and CG when incubated for 5 days.

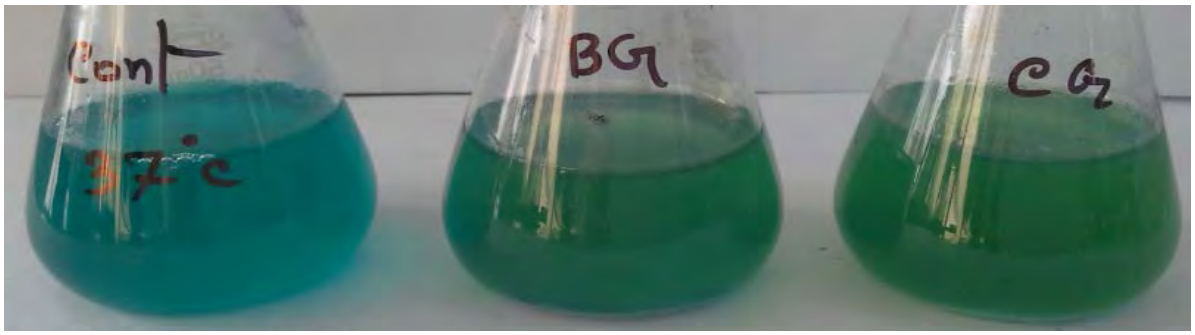



**Figure 3.3.4 (II):** Effect of$37^0$C on decolourization of the GGS dye by isolates BG and CG when incubated for 5 days.


**Figure 3.3.4 (III):** Effect of $45^0$C on decolourization of the GGS dye by isolates BG and CG when incubated for 5 days.

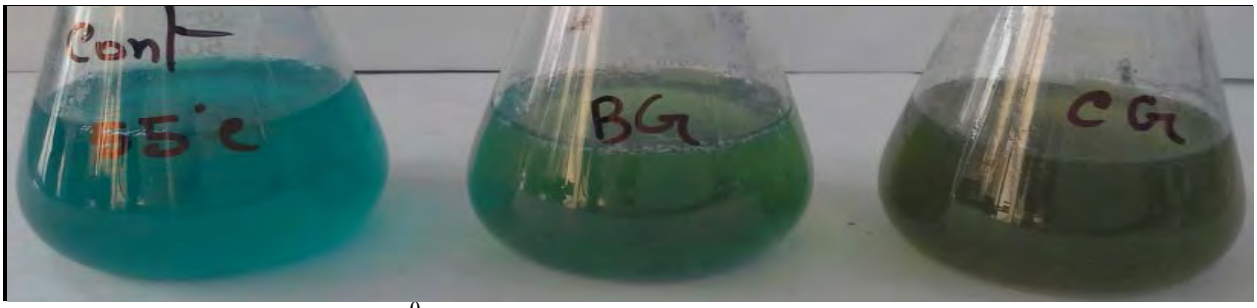



**Figure 3.3.4 (IV):** Effect of $55^0$Con decolourization of the GGS dye by isolates BG and CG when incubated for 5 days.

### 3.4 Identification tests:

All eight isolates were cultured in nutrient agar plate and analyzed for morphological characteristics accordingly. Microscopic identification of grams status was done after gram staining. Biochemical tests were also performed and then assumptive identification of isolates was done through the use of ABIS software online.

**The identification tests pictures**

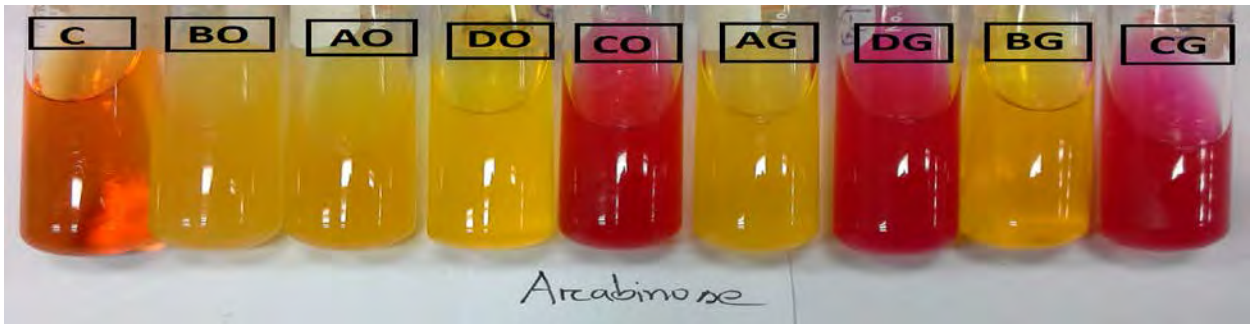



**Figure 3.4:** Arabinose fermentation test. The first one from the left was the control the reddish orange colour. Then chronologically the isolates were BO, AO, DO, CO, AG, DG, BG and CG. Arabinose positive bacteria turned media in to yellow and negative bacteria kept media unchanged.

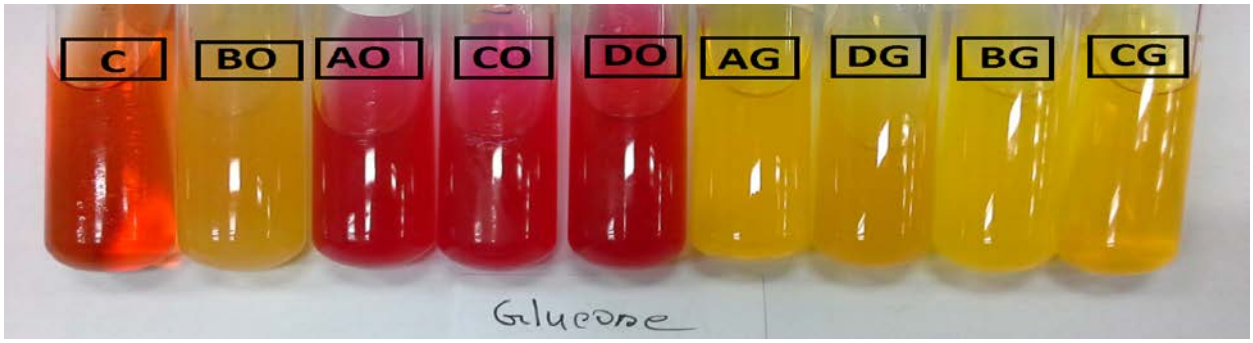



**Figure 3.5:** Glucose fermentation test. The first one from the left was the control the reddish orange colour. Then chronologically the isolates were BO, AO, DO, CO, AG, DG, BG and CG. Glucose positive bacteria turned media in to yellow and negative bacteria turned media in to bright pink colour.

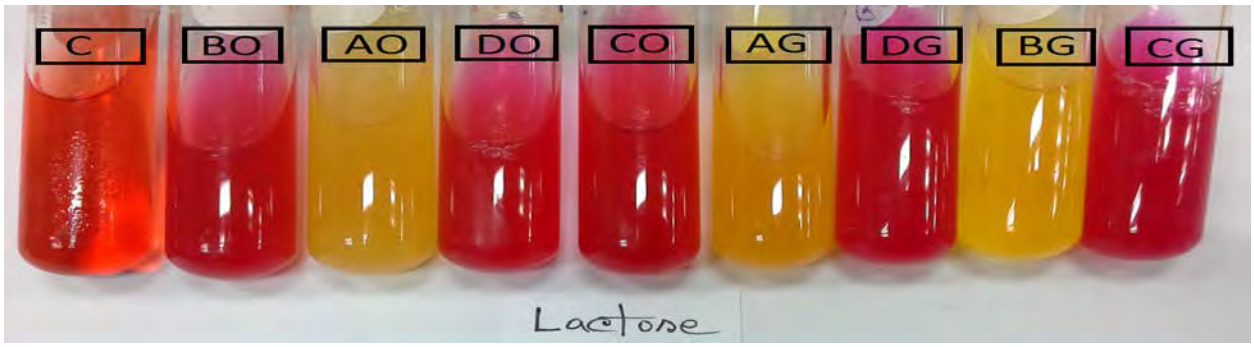



**Figure 3.6:** Lactose fermentation test. The first one from the left was the control the reddish orange colour. Then chronologically the isolates were BO, AO, DO, CO, AG, DG, BG and CG. Lactose positive bacteria turned media in to yellow and negative bacteria turned media into bright pink colour.

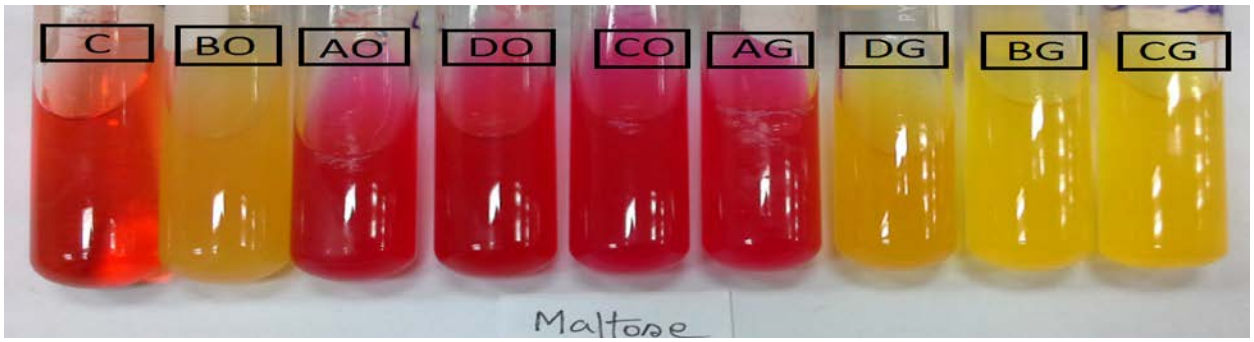



**Figure 3.7:** Maltose fermentation test. The first one from the left was the control the reddish orange colour. Then chronologically the isolates were BO, AO, DO, CO, AG, DG, BG and CG. Maltose positive bacteria turned media in to yellow and negative bacteria turned media into bright pinkcolour.


**Figure 3.8:** Sucrose fermentation test. The first one from the left was the control the reddish orange colour. Then chronologically the isolates were BO, AO, DO, CO, AG, DG, BG and CG. Sucrose positive bacteria turned media in to yellow and negative bacteria turned media in to bright pink colour.

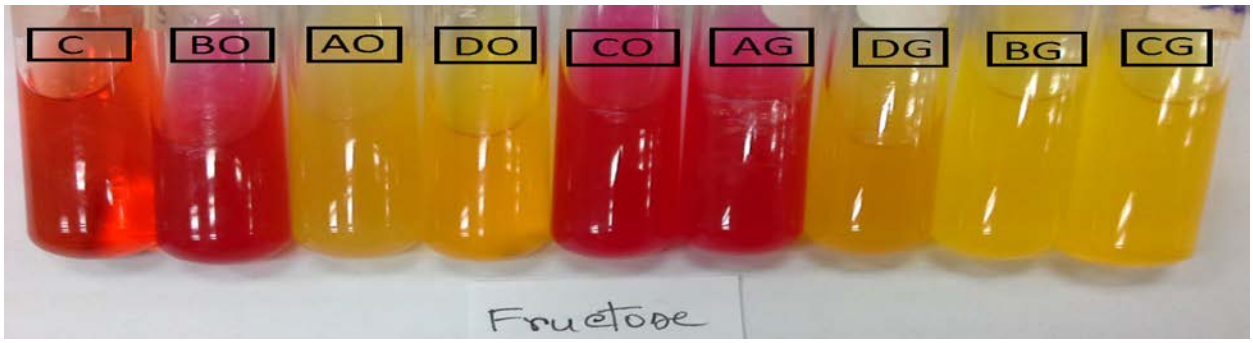



**Figure 3.9:** Fructose fermentation test. The first one from the left was the control the reddish orange colour. Then chronologically the isolates were BO, AO, DO, CO, AG, DG, BG and CG. Fructose positive bacteria turned media in to yellow and negative bacteria turned media in to bright pink colour

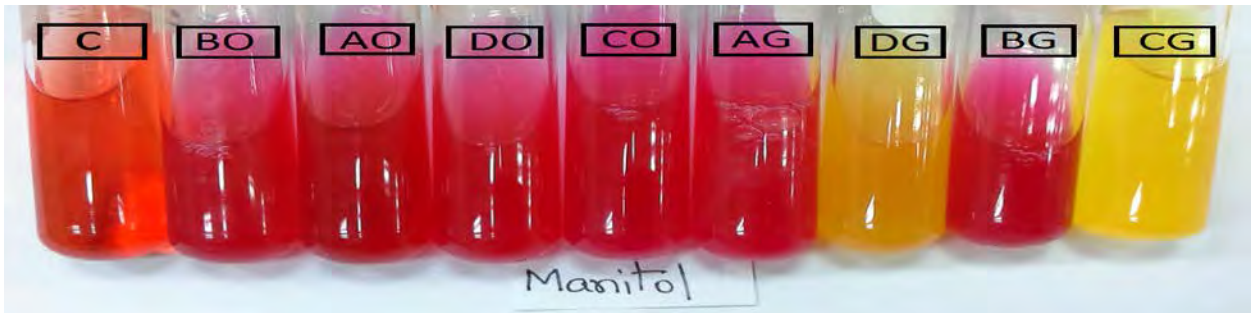



**Figure 3.10:** Manitol fermentation test. The first one from the left was the control the reddish orange colour. Then chronologically the isolates were BO, AO, DO, CO, AG, DG, BG and CG. Manitol positive bacteria turned media in to yellow and negative bacteria turned media in to bright pink colour.


**Figure 3.11:** Trehalose fermentation test. The first one from the left was the control the reddish orange colour. Then chronologically the isolates were BO, AO, DO, CO, AG, DG, BG and CG. Trehalose positive bacteria turned media in to yellow and negative bacteria turned media in to bright pink colour.


**Figure 3.12:** Galactose fermentation test. The first one from the left was the control the reddish orange colour. Then chronologically the isolates were BO, AO, DO, CO, AG, DG, BG and CG. Galactose positive bacteria turned media in to yellow and negative bacteria turned media into deep red colour.

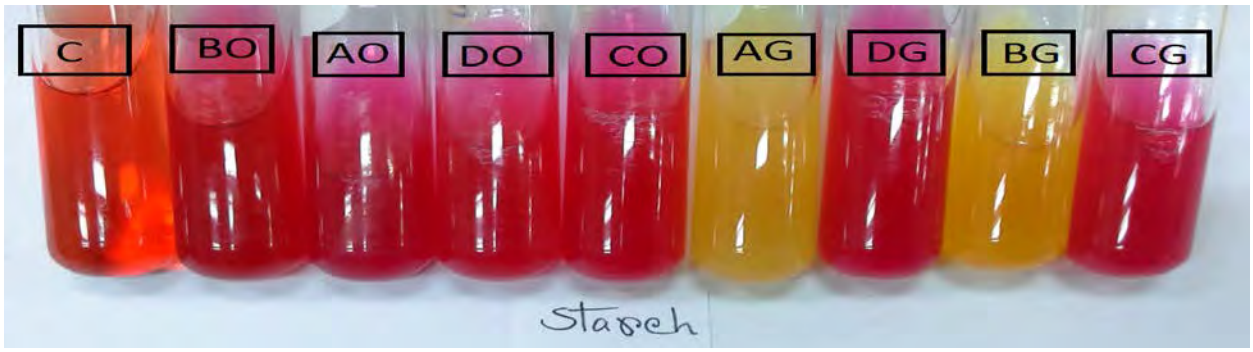



**Figure 3.13:** Starch fermentation test. The first one from the left was the control the reddish orange colour. Then chronologically the isolates were BO, AO, DO, CO, AG, DG, BG and CG. Starch positive bacteria turned media in to yellow and negative bacteria turned media in to bright pink colour.

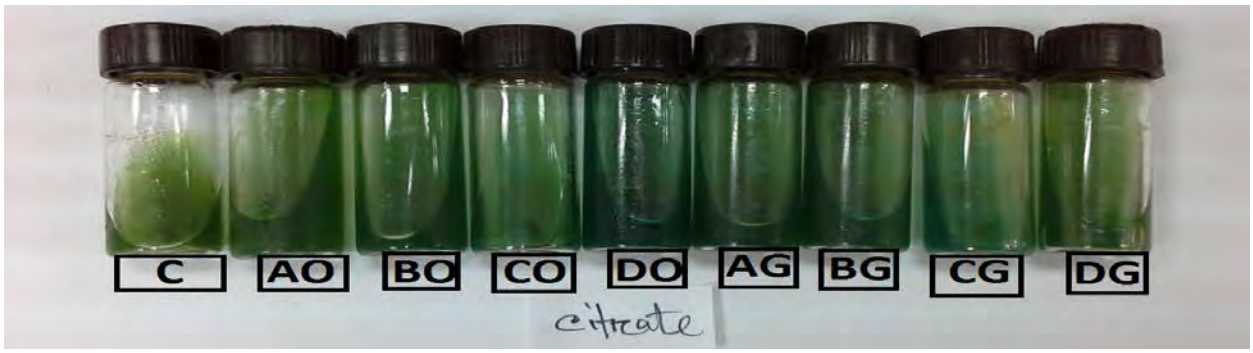



**Figure 3.14:** Simmons's citrate utilization test was performed. All the isolates showed negative results as no colour changed in agar. Positive bacteria turned the media into blue as creating alkaline condition. The first one from the left is the control and then chronologically AO, BO, CO, DO, AG, BG, CG and DG.

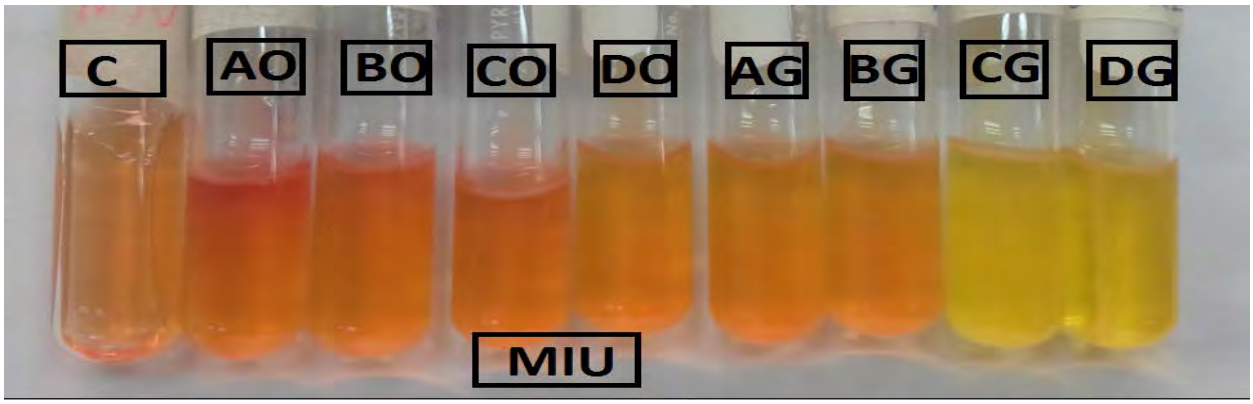



**Figure 3.15:** Motility test was performed. First one from the left was control then chronologically AO, BO, CO, DO, AG, BG, CG and DG. Only the CG showed motility.


**Figure3.16:** Closer view of CG motile bacteria.

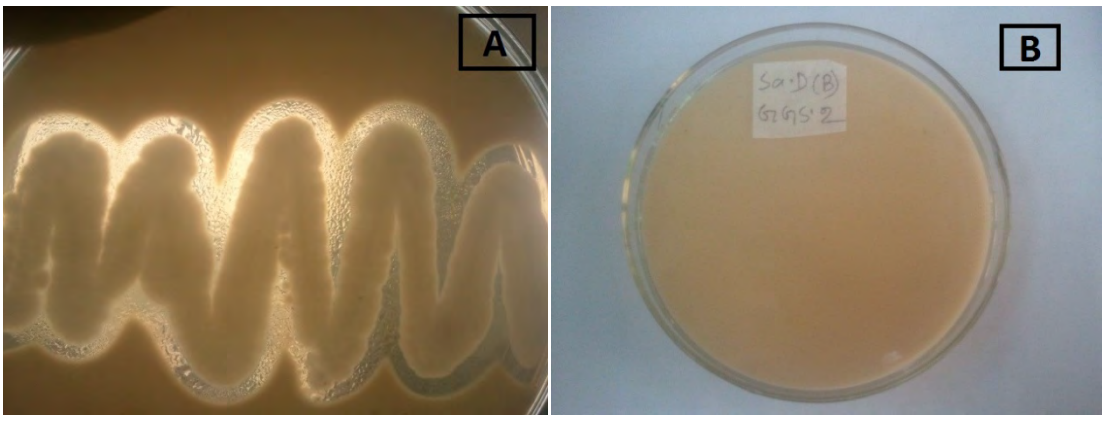



**Figure 3.17:** Casein hydrolysis test. All the isolates were negative except CG .Clear zone in caption A by CG isolates and all others were unable to hydrolyzed casein like caption B.

**Figure 3.18:** Starch hydrolysis test. All the isolates AO, BO, CO, DO, AG, BG, CG and DG were starch negative.

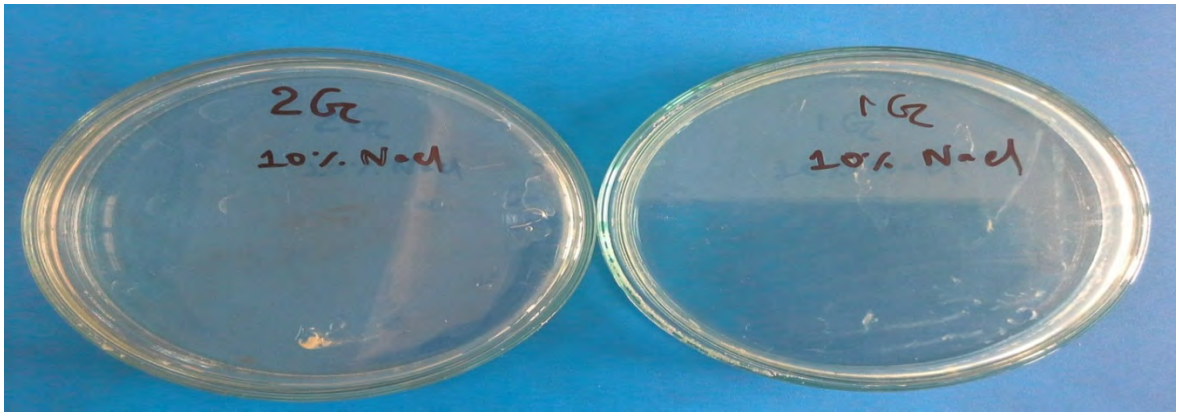



**Figure 3.19:** All the isolates AO, BO, CO, DO, AG, BG, CG and DG were unable to grow at 10% NaCl agar media.

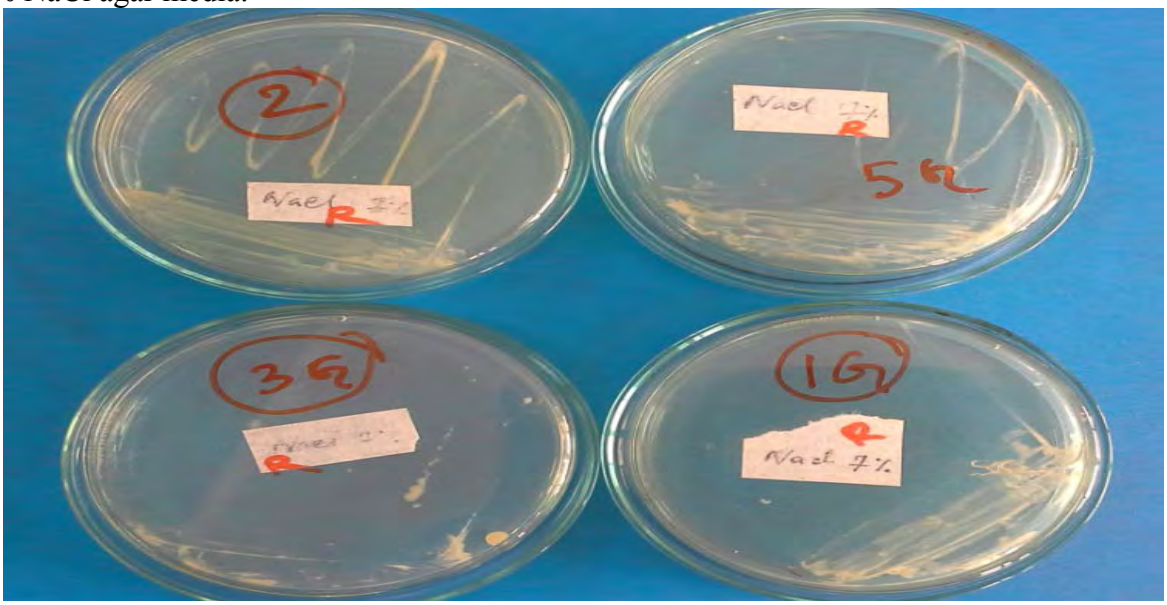



**Figure 3.20:** All the isolates AO, BO, CO, DO, AG, BG, CG and DG were able to grow at 7% NaCl agar media.

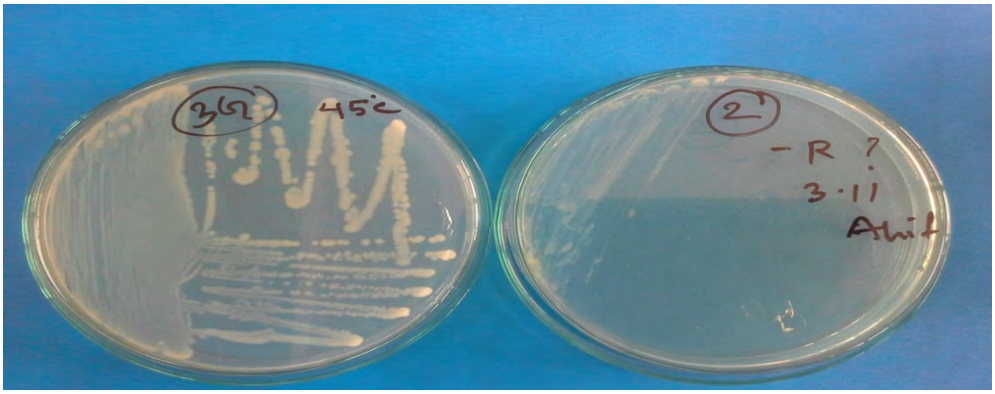



**Figure 3.21:** All the isolates AO, BO, CO, DO, AG, BG, CG and DG were grown in $45^0$C temperatures.

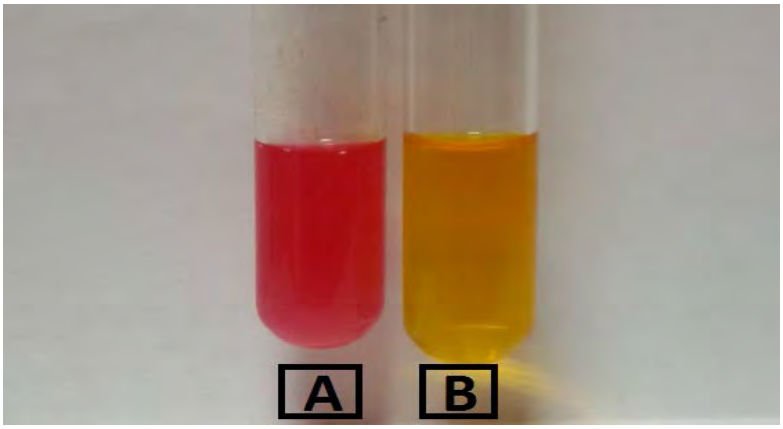



**Figure 3.22:** Methyl red test .B. Right one is negative did not change its colour after addition of reagent methyl red, but A. The left one turned red as pH was at or below 4.4 for the fermentation of glucose.

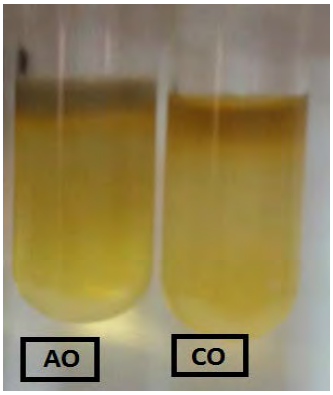



**Figure 3.23:** Voges-Proskaure test. All the isolates were VP positive like AO and CO isolates.

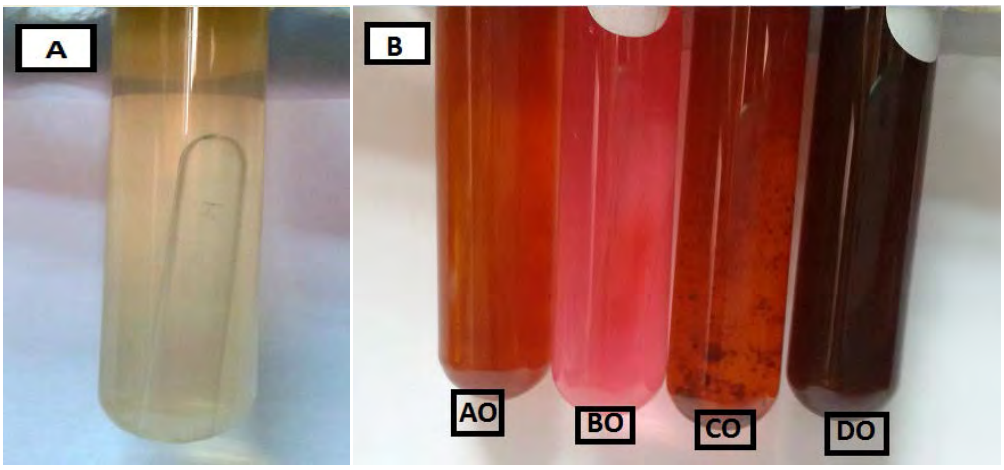



**Figure 3.24:** Nitrate reduction test. A. Nitrate broth control transparent and clear. B. All the isolates were nitrate positive turned the media to deep red colour like AO, BO, CO and DO.

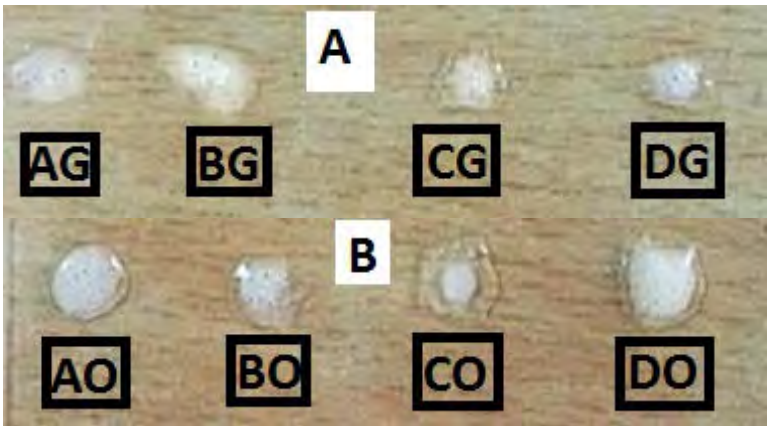



**Figure 3.25:**Catalase test.A & B. All eight isolates were catalase positive.

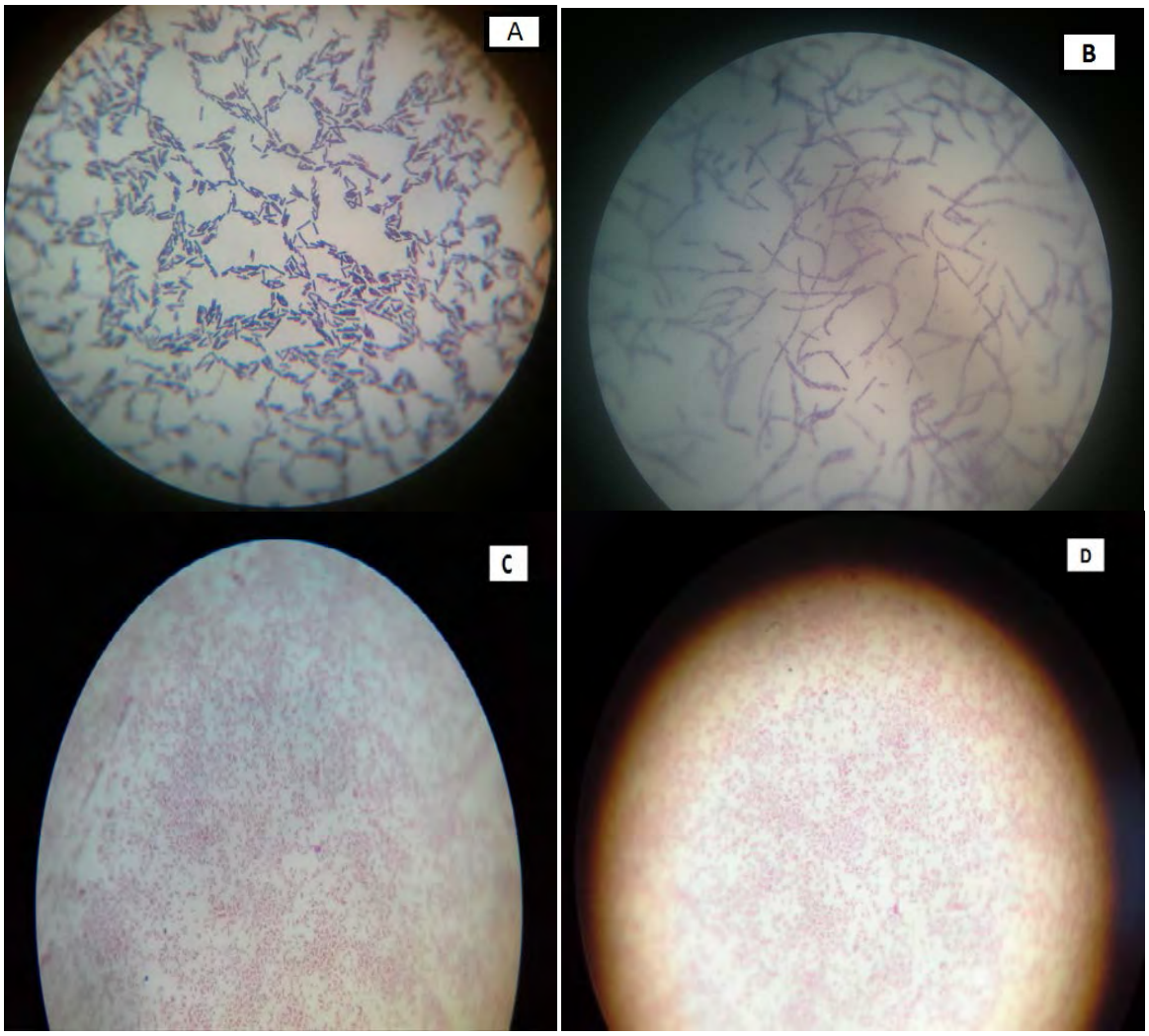



**Figure 3.26(I): Gram staining of selected isolates. A.** The isolates AG stained purple stained or gram positive with cellular morphology of long rods. **B**. The isolates BG stained purple stained or gram positive with cellular morphology of filaments rods. **C&D**. The isolates AO and CO stained red/pink with cellular morphology of small rods.

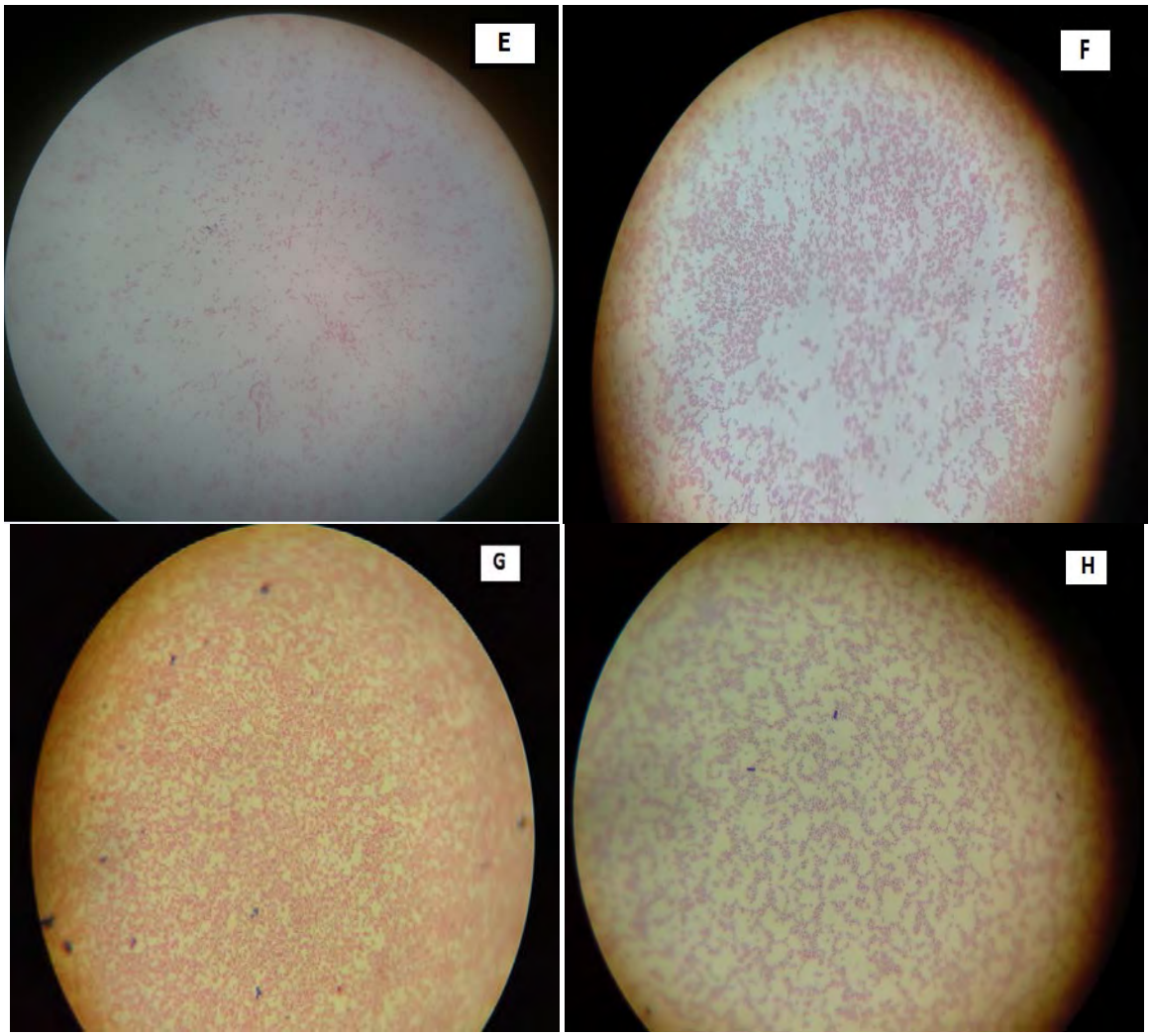



**Figure 3.26(II): Gram staining of selected isolates. E.** The isolate CG stained red/pink with cellular morphology of small rods. **F&G.** The isolates BO and DO stained red/pink with cellular morphology of coccid. **H**. The isolate DG stained purple with cellular morphology of coccid.

**Table 3.4.1: Gram's staining results of eight isolated strains**

| Isolates designation | Gram's staining | |
|---|---|---|
| | Gram's | Morphology |
| AO | -ve | Rod |
| BO | -ve | Coccid |
| CO | -ve | Rod |
| DO | -ve | Coccid |
| AG | +ve | Rod |
| BG | +ve | Rod |
| CG | -ve | Rod |
| DG | +ve | Coccid |

**Table 3.4.2(I): Biochemical test results of eight isolated strains.**

| Isolates designation | Indol | Methyl-red test (MR) | Voges - Proskauer test (VP) | Motility | Simmon citrate | Starch hydrolysis | Casin hydrolysis | Nitrate reduction |
|---|---|---|---|---|---|---|---|---|
| AO | - | + | - | - | - | - | - | + |
| BO | - | - | - | - | - | - | - | + |
| CO | - | + | - | - | - | - | - | + |
| DO | - | + | - | - | - | - | - | + |
| AG | + | - | - | - | - | - | - | + |
| BG | - | + | - | - | - | - | - | + |
| CG | - | + | - | + | - | - | + | + |
| DG | - | - | - | - | - | - | - | + |

**Table 3.4.2(II): Biochemical test results of eight isolated strains.**

| Isolates designation | Oxidase | Catalase | Urease | Arabinose | Glucose | Lactose | Maltose | Sucrose |
|---|---|---|---|---|---|---|---|---|
| AO | + | + | - | + | - | + | - | - |
| BO | + | + | - | + | + | - | + | + |
| CO | + | + | - | - | - | - | - | - |
| DO | + | + | - | + | - | + | - | - |
| AG | + | + | - | + | + | + | - | - |
| BG | + | + | - | + | + | + | + | + |
| CG | + | + | - | - | + | - | + | + |
| DG | + | + | - | - | + | - | + | - |

**Table 3.4.2(III): Biochemical test results of eight isolated strains.**

| Isolates designation | Fructose | Manitol | Trehalose | Galactose | Starch | Growth at 7% Nacl | Growth at 10% Nacl | Growth at 6.5 % Nacl | $45^0$ C | Growth at 15 % Nacl | Aerobic growth |
|---|---|---|---|---|---|---|---|---|---|---|---|
| AO | + | - | - | + | - | + | - | + | + | - | + |
| BO | - | - | + | - | - | - | - | + | + | - | + |
| CO | - | - | - | + | - | + | - | + | + | - | + |
| DO | + | - | - | + | - | + | - | + | + | - | + |
| AG | - | - | - | + | + | + | - | + | + | - | + |
| BG | + | + | + | - | + | + | - | + | + | - | + |
| CG | + | + | + | + | - | + | - | + | + | - | + |
| DG | + | + | + | + | - | + | - | + | + | - | + |

Data input in ABIS software online was individually done for each isolates according to their gram staining result.


**Figure 3.28:** ABIS software online page.

## Table 3.5: Isolates identified through ABIS software online were

| Isolates designation | Predicted name of the isolates |
|---|---|
| AO | *Entorococcus termitis* |
| BO | *Entorococcus camelliae* |
| CO | *Bacillus farraginis* |
| DO | *Unknown texon* |
| AG | *Bacillus muralis* |
| BG | *Paenibacillus macerans* |
| CG | *Bacillus decolorationis* |
| DG | *Macrococcus brunensis* |

## 3.5 Identified bacterial strain and their decolorizing ability at different physiochemical condition

1. *Entorococcus termitis* and *Bacillus farraginis* were very impressive in OM2R dye decolourization.

*Entorococcus termitis* performed best at 1% OM2R dye concentration that decolourized 93% of dye. Optimum physiochemical conditions for dye decolourization was 2% NaCl at which condition it decolourized 81% of dye, at pH 7 it decolourized 90% of dye and at temperature $37^0$C it decolourized 93% of dye.

*Bacillus farraginis* performed best at 1% OM2R dye concentration that decolourized98% of dye. Optimum physiochemical conditions for dye decolourization were 2% NaCl at which condition it decolourized 71% of dye, at pH 7 itdecolourized89% of dye and at temperature $37^0$C it decolourized 92% of dye.

2. *Paenibacillus macerans* and *Bacillus decolorationis* were very impressive in GGS dye decolourization.

*Paenibacillus macerans* also performed best at 1% GGS dye concentration that decolourized 97% of dye. Optimum physiochemical conditions for dye decolourization were 2% NaCl at which condition it decolourized 89% of dye, at pH 7 itdecolourized92%of dye and at temperature $37^0$C it decolourized 90% of dye.

*Bacillus decolorationis* as well performed best at 1% GGS dye concentration that decolourized 93% dye .Optimum physiochemical conditions for dye decolourization was 2% NaCl at which condition it decolourized 81% of dye, at pH 7 it decolourized 88% of dye and at temperature $37^0$C it decolourized 89% of dye.

# 4 Discussions and Conclusion

## 4.1 Azo dye degrading ability of different bacteria

Eight bacteria that had been isolated from different soil samples were identified as *Entorococcus termitis, Bacillus farraginis, Bacillus muralis, Paenibacillus macerans, Bacillus decolorationis, and Macrococcus brunensis*. Most of the isolates identified were *Bacillus* species.

Dye decolourizing ability of different bacterial isolates was investigated independently. When all isolates were cultured in SM broth media containing 1% of dye for 5 days at $37^0$C, where CO (*Bacillus farraginis )*showed the highest dye decolourization capacity on OM2R that was 98% of dye degradation and another isolate AO*(Entorococcus termitis)* showed 93% of dye degradation .On GGS dye degradation BG*(Paenibacillus macerans)*showed better decolourization that was 97% of dye decolourization and CG *(Bacillus decolorationis)*showed 93% of dye decolourization. Further research was carried on utilizing the isolate AO *(Entorococcus termitis*) and CO *Bacillus farraginis* that showed maximum degradation in 1% of OM2R dye.

## 4.2 Effect of different concentration of dye on decolourization

Percentage decolourization of OM2R dye by AO (*Entorococcus termitis)* and CO *(Bacillus farraginis)* was found to vary with different concentrations (1-5g/l) when studied for120 hours at $37^0$C. Maximum decolourization rate was 98% of 1% OM2R dye by CO *(Bacillus farraginis*) however as concentration of dye increased up to3% and 5% the decolourization rate was decreased respectively to 94% and 90%. It was because of the toxic nature of azo dyes. The Percentage decolourization was found to be decreasing with the increase of dye concentration as evident from (Table 3.2.1(ii)).Similar pattern of result was observed for isolates AO (*Entorococcus termitis)*. At 1% OM2R dye concentration the decolourization percentage was 93% of dye and at 3% and 5% dye concentration decolourization was dramatically same that was 87% of dye.

In the case of GGS dye decolourization a maximum decolourization rate was observed 97% of dye by BG *(Paenibacillus macerans)* and then 93% of dye by CG *(Bacillus decolorationis)* at 1% dye concentration. Both of the isolates were similarly effective at 3% dye concentration, decolourization rate was respectively 94% and 93% of dye. However at 5% dye concentration the degradation rate was found to be a slump for both of the isolates that was respectively 81% and 77% of dye (Table 3.3.1(ii)).Normally the dye concentration in the effluent varies within a range of 0.1-0.2 g l-1 (O'Neill et al., 1999).In comparison with this range our dye concentration is much higher (1-5g/l) so the isolates showed pretty well efficiency in dye decolourization.

## 4.3 Effect of different pH on dye decolourization

pH is one of the important abiotic factor that effect on the growth and metabolic homeostasis. The effect of pH was studied at pH values 5.0, 6.0, 7.0 and 8.0.The temperature was fixed at $37^0$C.At pH 7.0 both the AO (*Entorococcus termitis)* and CO (*Bacillus farraginis)* gave maximum decolourization respectively 90% and 89% of dye. Dye decolourization rate was almost similar over the pH range of 5.0 and 6.0 that was around 75% by both of AO (*Entorococcus termitis*) and CO *(Bacillus farraginis)*. A swift reduction in the decolourization was observed at pH 8 by AO(*Entorococcus termitis*) and CO*(Bacillus farraginis)* respectively 59% and 52% of dye evident from (Graph 3.2.2).Those results suggest that acidic pH values may influence the stability of the enzyme causing denaturation. Chang et al. (2001) found that azoreductase performance was affected by pH, with 2.5 times better dye reduction at pH 7-9 than below pH 7. These findings corresponded well to the best decolourization found between pH 7-9.5 (Saratale et al., 2011).

In (Graph 3.3.2) it was observed that the maximum decolourization rate was attained at pH 7.0 by BG (*Paenibacillus macerans)* 92% of dye and CG *(Bacillus decolorationis)* 88% of dye. Majority of the azo dye reducing bacterial species reported so far were able to reduce the dye at pH near 7(Chang JS *al*., 2001; Kalme S *al.,* 2007; Suzuki T *al.,* 2001). The requirement of near neutral pH for optimum growth had been reported in several studies (Espeche*et al.*, 1994; Kwapisz*et al.,* 2008; Shukor*et al.,* 2008).The results indicate that a pH increase from 5.0 to 7.0 enhanced the decolourization of GGS dyes. At pH 5 the decolourization rate was 80% and 79% of dye respectively by BG *(Paenibacillus macerans)* and CG (Bacillus decolorationis). A little improvement was observed at pH 6 that was 85% of dye by BG *(Paenibacillus macerans )* and 83% of dye by CG *(Bacillus decolorationis)*.At pH 8.0 the decolourization rate decreased dramatically that was 73% and 70% respectively by BG*(Paenibacillus macerans)* and CG *(Bacillus decolorationis)*. It was observed that better decolourization rate was around pH 6 and pH 7 for both of OM2R and GGS dye by the selected isolates.

## 4.4 Effect of different concentration of NaCl on Azo dye degradation

Percentage decolourization of OM2R by selected isolates was found to vary with different concentration (2-8g/l) of NaCl when studied for120 hours at $37^0$C. Maximum decolourization of OM2R by AO (*Entorococcus termitis)* and CO*(Bacillus farraginis)* was observed 81% and 71% respectively at 2% NaCl but the Percentage decolourization was found to be decreasing with the increase of NaCl concentration as evident from (Graph 3.2.3).The decolourization attained by AO(*Entorococcus termitis)* at $37^0$C for 4%, 6% and 8% NaCl was 60%, 43%, and 37% and for CO( *Bacillus farraginis)* it was 58%, 40% and 34% respectively. Kargi el at. (1996) mention that high salt concentrations (>1% salt) are known to cause plasmolysis and/or loss of cell activity.

Similarly At 2% NaCl concentration the degradation percentage of GGS dye were 89% and 81% respectively by BG (*Paenibacillus macerans)* and CG (Bacillus decolorationis). The decolourization attained by BG (Paenibacillus macerans) at $37^0$C for 4%, 6% and 8% NaCl was 72%, 62%, and 40% and for CG (Bacillus decolorationis), it was 65%, 54% and 36% respectively as evident from (Graph 3.3.3).

## 4.5 Effect of different temperature on dye degradation

To determine the optimum temperature for dye decolourization a temperature range of $30^0$C to $55^0$C was examined. As seen in Figure the optimum temperature for OM2R dye decolourization was $37^0$C for both of the AO *(Entorococcus termitis)* and CO *(Bacillus farraginis)* attained a maximum decolourization of 93% and 92%of dye respectively. Angelova et al.(2008) found that the azo bond reduction rate rose with an increased temperature, a maximum rate around 40 $^0$C, 3-5 times faster than at $20^0$C. At $30^0$C and $45^0$C the degradation rate for OM2R by AO *Entorococcus termitis* was 82% and 72% of dye and for CO *(Bacillus farraginis)* it was 84% and 82% of dye respectively. Then a low decolourization of 59% and 57% of dye was detected at $55^0$C by AO (*Entorococcus termitis)* and CO *(Bacillus farraginis) isolates* respectively. Temperatures above $55^0$C were not studied since results shown that the increase from $37^0$C to 45 $^0$C promoted a marginal decrease in dye decolourization as evident from (Graph 3.2.4).

The optimum temperature for GGS dye decolourization was $37^0$C for both of the BG (*Paenibacillus macerans)* and CG *(Bacillus decolorationis)* attained a maximum decolourization of 90% and 89% of dye respectively. In case of BG at $30^0$C and $45^0$C the decolourization percentage was 86% and 84% of dye respectively whereas by CG it was 79% and 77% of dye respectively. No improvement in dye decolourization was observed at temperatures above $45^0$C. Where a low decolourization of 58% and 45% of dye was detected at $55^0$C by BG (*Paenibacillus macerans)* and CG *(Bacillus decolorationis)* isolates respectively as evident from (Graph3.3.4).BG (*Paenibacillus macerans)* has a broad range of compatibility from $30^0$C to $45^0$C. Within the optimal values of temperature, the lowest temperature was selected as the optimum temperature since this leads to lower energy costs

## 4.6 Conclusion

Color removal of textile and dye stuff plant with a continuous discharge of great quantity of remaining dyes to the environment has been a major concern in waste water treatment. Traditional waste water treatment requires extensive cost and chemicals input that make the treatment inefficient and have the potential environmental threat. Hence, economical and eco-friendly techniques using bacteria can be applied for fine tuning of waste water treatment. Biotreatment offers easy, cheaper and effective alternative for color removal of textile dyes. Thus, by this present study we strongly concluded that the bacterial isolates like *Entorococcus termitis, Entorococcus camelliae, Bacillus decolorationis,* and *Paenibacillus macerans* species were a good microbial source for textile effluent treatment, in biological degradation of textile dye. However, potential of culture need to be demonstrated for its application in treatment of real dye bearing waste waters using appropriate bioreactors.

An increased knowledge of biological processes, complexity of wastewaters and the use of analytical methods would lead to new questions and further research. More biochemical tests, 16S rRNA and plasmid profiling could be done for the confirmation of selected isolates. The genetic information can be used for the genetic manipulations for more compatible, productive and stable trait in order to increase the system performance.

In future, the obtained bacterial isolates can be used as microbial consortium to remove textile dyes from complex textile effluent and hens a greener solution to environmental pollution. Furthermore if we can isolate the genes that are responsible for dye degradation and can develop genetically modified bacteria will be more robust and can commercialize.

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## Appendix I

## Media composition

The following media was used throughout the study. All components were autoclaved at 121°C, 15 psi for 15 minutes unless mentioned otherwise

### 01. Phenol red arabinos broth

| Ingredients | Amounts |
|---|---|
| Arabinose | 5.0 |
| Phenol red | 0.018 |
| Sodium chloride | 5.0 |
| Trypticase | 10.0 |

### 02. Phenol red fructose broth

| Ingredients | Amounts |
|---|---|
| Fructose | 5.0 |
| Phenol red | 0.018 |
| Sodium chloride | 5.0 |
| Trypticase | 10.0 |

### 03. Phenol red galactosebroth

| Ingredients | Amounts |
|---|---|
| Galactose | 5.0 |
| Phenol red | 0.018 |
| Sodium chloride | 5.0 |
| Trypticase | 10.0 |

### 04. Phenol red gulcose broth

| Ingredients | Amounts |
|---|---|
| Gulcose | 5.0 |
| Phenol red | 0.018 |
| Sodium chloride | 5.0 |
| Trypticase | 10.0 |

**05. Phenol red maltose broth**

| Ingredients | Amounts |
|---|---|
| Maltose | 5.0 |
| Phenol red | 0.018 |
| Sodium chloride | 5.0 |
| Trypticase | 10.0 |

**06. Phenol red mannitol broth**

| Ingredients | Amounts |
|---|---|
| Mannitol | 5.0 |
| Phenol red | 0.018 |
| Sodium chloride | 5.0 |
| Trypticase | 10.0 |

**07. Phenol red lactose broth**

| Ingredients | Amounts |
|---|---|
| Lactose | 5.0 |
| Phenol red | 0.018 |
| Sodium chloride | 5.0 |
| Trypticase | 10.0 |

**08. Phenol red starch broth**

| Ingredients | Amounts |
|---|---|
| Starch | 5.0 |
| Phenol red | 0.018 |
| Sodium chloride | 5.0 |
| Trypticase | 10.0 |

**09. Phenol red sucrose broth**

| Ingredients | Amounts |
|---|---|
| Sucrose | 5.0 |
| Phenol red | 0.018 |
| Sodium chloride | 5.0 |
| Trypticase | 10.0 |

**10. Phenol red trehalose broth**

| **Ingredients** | **Amounts** |
|---|---|
| Trehalose | 5.0 |
| Phenol red | 0.018 |
| Sodium chloride | 5.0 |
| Trypticase | 10.0 |

11. **MIU agar (HiMedia, India)**

| **Ingredients** | **Amounts** |
|---|---|
| Agar | 2.0 g/L |
| Casein enzyme hydrolysate | 10.0 g/L |
| Dextrose | 1.0 g/L |
| Phenol red | 0.010 g/L |
| Sodium chloride | 5.0 g/L |
| Urea* | 5 mL (40%) per 95 mL media |

* Sterile urea solution added to cooled autoclaved rest of the media

**12. MR-VP broth**

| **Ingredients** | **Amounts(g/L)** |
|---|---|
| Dextrose | 5.0 |
| Peptone | 7.0 |
| Potassium phosphate | 5.0 |

**13. Nitrate broth**

| **Ingredients** | **Amounts(g/L)** |
|---|---|
| Beef extract | 3.0 |
| Peptone | 5.0 |
| Potassium nitrate | 5.0 |

**14. Nutrient agar (Himedia, India)**

| **Ingredients** | **Amounts (g/L)** |
|---|---|
| Beef extract | 1.5 |
| Agar | 15.0 |
| Sodium chloride | 5.0 |
| Peptic digest of animal tissue | 5.0 |
| Yeast extract | 1.5 |

**15. Simmon's citrate agar (Oxoid, England)**

| Ingredients | Amounts (g/L) |
|---|---|
| Agar | 15.0 |
| Ammonium phosphate | 0.8 |
| Ammonium dihydrogen phosphate | 0.2 |
| Bactobromthymol blue | 0.08 |
| Magnesium sulfate | 0.2 |
| Sodium citrate | 2.0 |
| Sodium chloride | 5.0 |

**16. Starch agar**

| Ingredients | Amounts (g/L) |
|---|---|
| Agar | 15.0 |
| Beef extract | 0.75 |
| Peptic digest of animal tissue | 1.25 |
| Starch soluble | 2.0 |

**17. T1N1 soft agar**

| Ingredients | Amounts (g/L) |
|---|---|
| Agar | 0.42 |
| Sodium chloride | 0.3 |
| Tryptone | 0.6 |

**18. 6.5% NaCl agar**

| Ingredients | Amounts (g/L) |
|---|---|
| Beef extract | 1.5 |
| Agar | 15.0 |
| Sodium chloride | 5.0 |
| Peptic digest of animal tissue | 5.0 |
| Yeast extract | 1.5 |
| Sodium chloride | 6.5 |

**19. 10% NaCl agar**

| **Ingredients** | **Amounts (g/L)** |
|---|---|
| Beef extract | 1.5 |
| Agar | 15.0 |
| Sodium chloride | 5.0 |
| Peptic digest of animal tissue | 5.0 |
| Yeast extract | 1.5 |
| Sodium chloride | 10 |

**20. SM broth**

| **Ingredients** | Amounts(g/L) |
|---|---|
| Glucose | (10g/L) |
| Di potassium Hydrogen Phosphate | ( 0.6g/L) |
| Peptone, | (10g/L) |
| Potassium dy Hydrogen Phosphate | (1.9g/L) |
| Magnesium Sulphate | ( 1g/L) |
| Yeast Extract | (1g/L) |

The pH adjusted to 6.0 to 6.4.

## Appendix II
## Reagents

**1. Barritt's reagent**
Solution A .Solution B

**2. Carbol Fuchsin Stain (0.3%)**

**3. Crystal violet Stain (2%)**

**4. Iodine solution (Gram's)**

**5. Kovac's reagent**

**6. Malachite green (0.5%)**

**7. Methylene blue solution (1%)**

**8. Methyl red reagent**

**9. Oxidase reagent**

**10. Safranin**

## Appendix III

## Instruments

| **Instruments** | **Company** |
|---|---|
| Autoclave | SAARC |
| Centrifuge machine | Digisystem Laboratory instruments inc |
| Incubator | SAARC |
| Micropipette (10-100 µL) | Eppendorf, Germany |
| Micropipette (100-1000 µL) | Eppendorf, Germany |
| Microscope | Optima |
| pH meter, Model: E-201-C | Shanghai Ruosuaa Technology company, China |
| Pipette (5 mL, 10 mL) | Eppendorf, Germany |
| Refrigerator (4ºC), Model: 0636 | Samsung |
| Safety cabinet Class II Microbiological | SAARC |
| Shaking Incubator, Model: WIS-20R | Daihan Scientific, Korea |
| Spectrophotometer | UV mini 1240, UV VIS Spectrophotometer Shimadzu |
| Surgical Millipore syringe filter (0.22µm) | Millex-GS |
| Vortex Mixture | VWR International |
| Weighing balance | ADAM EQUIPMENT™, United Kingdom |