ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES
ADDIS ABABA INSTITUTE OF TECHNOLOGY
SCHOOL OF CHEMICAL AND BIO ENGINEERING

Treatment of Waste Water Effluent from Coffee Industry using Anaerobic Mixed Culture *Pseudomonas Florescence* and *Escherichia Coli* Bacteria and with Gypsum: A Case in Dilla-Ethiopia

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Addis Ababa, Ethiopia
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A thesis submitted to the School of Graduate Studies of Addis AbabaUniversity in partial fulfilment of the Requirements for the Degree of Master of Science in Biochemical Engineering

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This is to certify that the thesis is prepared by Fitsum Ashenafi, entitled: Treatment of Waste Water Effluent from Coffee Industry using Anaerobic Mixed Culture Pseudomonas Florescence and Escherichia Coli Bacteria and with Gypsum: A Case in Dilla- Ethiopia and submitted in partial fulfillment of the requirements for the Degree of Master of Science in Biochemical Engineering complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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DECLARATION

I declare that this thesis for the M.Sc. Degree at Addis Ababa University, hereby submitted by me, is my original work and has not previously been submitted for the degree at this or any other university, and that all resources of materials used in this thesis have been duly acknowledged.

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ACKNOWLEDGMENTS

First and foremost, I offer my thanks to God, who made this work possible. I express my deepest gratitude to my advisor Professor Eduardo Ojito Cespedes for his advice, and suggestions that greatly enriched this thesis. I have to express my sincere appreciation to the staff of Chemical and bioengineering and Biochemical engineering departments for their encouragement and concern. I want to give my greatest appreciation to institutes and staff of Ethiopian Biodiversity institute and Addis Ababa city government environmental protection and green development commission. I would like to thank Dilla University for providing sponsorship for my M.Sc. study. Finally, my heart felt appreciation and indebtedness goes to my family, my wife and my friends for their love, patience, support and encouragement throughout my study.

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LIST OF ACRONYMS AND ABRIVATIONS

ANOVA	Analysis Of Variance
BOD	Biological Oxygen Demand
BOD5	Biological Oxygen Demand over a five-day period
COD	Chemical Oxygen Demand
CWW	Coffee Waste Water
EBI	Ethiopian Biodiversity Institutes
MC	Mixed Culture
m.a.s.l	Mean Altitude above Sea Level
TDS	Total Dissolved Solid
TS	Total Solid
TSS	Total Suspended Solid
SNNPR	South Nations Nationalities & Peoples Regional
UASB	Up flow Anaerobic Sludge Blanket
WHO	Word Health Organization
WPC	Wet Processing of Coffee
WPCWWE	Wet Processing Coffee Wastewater Effluent

ABSTRACT

Coffee processing industries generate huge amount of wastewater. The effluent is characterized by a lot of sludge, high amount of organic load and acidic nature due to fermentation process. Due to this, it contains high value of COD, BOD, TS and other contaminants. This effluent when disposed in natural water, it contaminates aquatic animals and plants environment. So, care has to be taken to ensure the quality of the releasing waters should be reduced below the prescribed standards. There are many physical and chemical treatment methods available for the removal of pollutants but all these methods have problems associated with secondary effluent, hazardous and harmful end products, high energy consuming, non-economic etc. These problems can be overcome by the use of biological treatment methods which are simple, eco-friendly and efficient where complete removal of the pollutants is possible. Cognizant of this, the researcher was initiated to study the treatment of wastewater effluent from coffee industry in the specific area by using anaerobic *Pseudomonas florescence* and *Escherichia coli* bacteria as mixed culture and with gypsum (CaSO4.2H2O). In this study, the wastewater value of BOD5, COD, TS and neutralization of the PH reduction was achieved. The results show that the treatment reduced BOD5, COD and TS from its initial concentration of 320.26 mg/l, 1261 mg/l, and 3545 mg/l to 35.66 mg/l, 97 mg/l and 68 mg/l respectively. The treatment in the study shows 87.74% reduced of BOD5 load, 92.02% reduced of COD and 98.01% reduced of TS from the initial load using optimization of the treatment method. Therefore, it can be concluded that mixed culture bacteria of *Pseudomonas florescence* and *Escherichia coli* in combination with gypsum as a new effective treatment having potential for BOD5, COD and TS reduce from the effluent treated. The mixed culture of microorganisms is capable to reduce the contaminants that made the approach cost effective, time saving compared with other results found by other authors without mixed culture. The microorganisms included in the study can be applied for the treatment of effluents containing the multiple contaminants.

Key words: Effluent, Treatment, Anaerobic Microorganism, Mixed culture, Gypsum

CHAPTER ONE

INTRODUCTION

1.1. Background

World countries are continuously debating on the uprising issue of environmental pollution. The problem is still increasing at alarming rate in recent years threatening our ecosystem as well as the bio system. Consequently, pollution is occurring on unprecedented and covering at vast scale around the globe (Speth, 1988). Different research findings were reported on issues to alleviate or reduce the impact of pollution by devising different mechanism. Yet, according to (Solange et al., 2011) in Asrat et al. (2015, p. 1468) it is not enough to relieve from the negative impact of pollution.

The economy of developing countries like Ethiopia is dependent up on agricultural products. For their sustainable development, these countries should work on agro processing industries focused for relieving from poverty; so far pollution creates a risk on the sector. The impact of pollution in these countries is more severe than the rest of the world (Secretariat, 2003). According to (Asrat et al., 2015) pollutants in the wastewater from coffee industries affects the farm land and the ecosystem. One can deduce from the above is that; if the waste management system is not properly managed and then the agricultural efficiency can be in doubt. Environmental pollution due to improper waste management, especially coffee processing industry is making warning light for developing countries to tackle the problem and hence meet the millennium development goals (A. Beyene et al., 2013). Thus, no one would argue against establishing a proper treatment method to reduce the risk and the effect of the pollution to the environment.

In Ethiopia, coffee processing industry is one of the sources of contamination to water bodies which creates environmental pollution. Pollution of water bodies from these industries are critical issues (Asrat et al., 2015). In this regard, there are more than 400 wet coffee processing industries in Ethiopia which are located at the vicinity of rivers (Asrat et al., 2015). The total annual wet processing of coffee is estimated to be 52,000 tons coffee, in which there are about 5-15 liters of water are required to recover 1 kg of clean green coffee beans (Asrat et al., 2014). This is due to the actual volume of water used depending on pulping process, fermentation intensity and coffee bean transportation volume. The wastewater from these industries creates odor to town due to microbial decomposition, unclean river and streams as a result of sludge in wastewater effluent. In contrast, the waste can be used as a source of energy and fertilizer. Coffee processing industries wastewater generates huge amount organic with high levels of environmental pollutant (Rawel, 2016). Thus, peoples should clean up their environment, make it a better place, be good trustees of the Earth for future generations (Speth, 1988).

On the other hand, treatment methods of coffee processing wastewater are reduces pollution to the environment and has also economic benefit to nation. In the meantime, efforts have been through to develop methods for coffee waste treatment and management (Asrat et al., 2015). Alternatively, there are different methods of treatment of coffee processing wastewater that have more significant to treat the wastewater like chemical, radiation, physical and biological method but some treatment methods create inorganic residues. Nevertheless, these inorganic residues reduce the value of the coffee by percolating in to the roots coffee plant. Conversely, Ethiopian specialty coffee are those certified, using the standards of organic; hence the treatment method may affect these coffee (Tadesse, 2015). Thus, selecting best treatment method is a bit challenging and mandatory step while considering lesser impact in the environment is preferred.

However, using biological anaerobic treatment of coffee processing wastewater (CWW) treatment not only reduces emission of greenhouse gases to the environment but also treat contamination (A. Beyene et al., 2013). Consequently, to treat the wastewater we should need to curb these problems through innovative and eco-friendly techniques (Asrat et al., 2015). Hence, on the study by (A. Cruz Salomon et al., 2017) showed biological treatment advantageous than conventional method coffee processing. Coffee processing industry generates large quantities of wastewater requiring systematic treatment prior to disposal. Moreover, at hydraulic retention times (HRT) of 9 days was the one that greater chemical oxygen demand (COD) removal generated, so using a laboratory scale Expanded Granular Sludge Bed (EGSB) bioreactor can be a sustainable alternative to solve the environmental problems, compared to other conventional methods to coffee processing wastewater (CPWW) treatment (A. Cruz Salomon et al., 2017).

1.2. Statement of the Problems

Wet processing of coffee industries is mostly located around coffee farms and water bodies. The effects of waste water from this industry have significant relation to the coffee farm consequently to the product coffee bean. Nevertheless, coffee processing industries productions in Ethiopia have been increased in recent years due to market demand (Yinager, 2019). These industries produce huge amounts of wastes and discarded in to water bodies. These wastes have different pollutant substances which pollute the environment and bring health problem.

In SNNPR (Southern Nations, Nationality and Peoples Region) there are Sidama, Yirgachefe, Guji, Kochore and Wolayeta types of coffee, which have 22 % of the total production (Hernandez et al., 2015). According to the Ethiopian coffee authority report 2011 Yirgachefe coffee accounts for 6% (9,914 bags) (Yinager, 2019). In each of coffee processing industries there are about 2, 974,200 liter of wastes water were produced every year in Gedeo zone Dilla zuria districts. In these area, there is no proper guide line of coffee waste management and rule hence they uses primitive methods of wastewater disposal to the river and the streams. As result, the local communities in Gedeo zone and Dilla zuria district were affected by water borne disease (i.e cholera, typhoid, and dysentery). Moreover, coffee waste causes health problem such as spinning sensation, ear, eye and skin irritation, nausea and stomach pain (Asrat et al., 2014).

Yet, on dumping site of effluent uncontrolled biodegradation by microbial fermentation is taking place and it causes leachate which highly affects ground and surface water resources as well as emission of greenhouse gases. This on the other hand creates offensive odor due to microbial decomposition, unclean river and streams, lot of sludge around water bodies are clear indication of the severity of the pollution of water bodies in the area. The wastewater can grow microorganisms, which decompose biodegradable organic matter (S. Trinidad et al., 2017). Nevertheless, the wastes are used for production of bioethanol, biogas, biodiesel, bio fertilizer and other useful products (S. Trinidad et al., 2017). Thus, treatment of wastewater would reduce pollution to the environment as well as economic benefit to nation. Therefore, the study with the method will fill the gap by using anaerobic mixed culture microbes and gypsum powder so as to reduce pollution on water bodies.

1.3. Objective of the study

1.3.1. General objective

To treat wastewater effluent from wet processing of coffee industries using anaerobic mixed culture of *Pseudomonas florescence* and *Escherichia coli* bacteria and with gypsum ($CaSO_4.2H_2O$)

1.3.2. Specific objective

This study aimed:

- To characterization the raw wastewater effluent
- To evaluate treatment effect of the wastewater using anaerobic mixed culture *Pseudomonas florescence* and *Escherichia coli* bacteria
- To evaluate treatment effect of the wastewater using gypsum after treatment the microbial
- To evaluate the combined effect of treatment of wastewater using anaerobic mixed culture *Pseudomonas florescence* and *Escherichia coli* bacteria and gypsum
- To optimize parameters of the combined treatment method including BOD5, COD and TS for high removal efficiency

1.4. Significance of the study

Coffee industries waste water is affecting the community around the area. It creates pungent smell and odor; livestock and humans cannot be used at least for washing their cloth and bodies, animal drink, and other domestic use. When suspended solids in wastewater enters into water bodies microorganisms feed on them by attaching in to derbies and stones and creates odor and smell. This problem affects people to suffer health issue like common cold; the farmers affect their work and hard to live in the river area. Physical and chemical characteristics of waste water affected due to fermentation; in order to remove the mucilage from coffee fruit. However, using gypsum and biological treatment method waste water from these industries can be treated and reduce the above problem easily.

There are other methods that have more significant to treat the waste water like chemical, radiation, and others than physical and biological method but these methods create inorganic residues. These inorganic residues reduce the value of the coffee; after inorganic compounds treat the water it can enter in to the coffee plant as xenobiotic matter. Since, Ethiopian coffees mostly are known to the world as organic coffee. Thus, the treatment should not affect the product though residual effect. This study will improve waste treatment mechanism and solve the existing problem. Finally, the study finding with most important recommendations helps for the industries in the area to a collaborate action against pollution and helps a source of reference.

CHAPTER TWO

LITRATURE REVIEW

2.1. Introduction

Early historical references to coffee are evidenced that mid 1400 A.C by which time it was well established as a drink in Yemen, and widely grown. Though, amazing ceremonial drinking in Ethiopia, and the close relation between 'Kaffa' found in southwest Ethiopia and the Arab word for coffee, make it plausible but not proven that coffee was consumed here first (Crawford, 1852). Nevertheless, this plant now cultivated in more than 70 countries, primarily in the equatorial regions of the Americas, Southeast Asia, India, and Africa. On the other hand, the two most commonly coffee species are C. arabica and C. robusta (Bethyu, 2019).

2.1.1. Coffee bean processing

Coffee processing method can be divided in to two types; that are dry processing and wet processing method. Accordingly, wet processing produces parchment/ washed coffee whereas dry processing gives unwashed coffee (A. Beyene et al., 2013). However, these methods of coffee processing types release wastewater to the environment; yet, urgent intervention in the area of coffee factory for effluent management options should be dealt with top priority to avoid further needless damage to the environment (Dejen et al., 2015).

Dry processing method, the ripped and/or unripe coffee fruit is detached from the tree and speared to flat surface like mat in the ground to dry on the sun. Then, the moisture slowly decreases then the fruit changes it colors slowly from red/green to dark brown. At the end, the moisture decreases up to 11-12% after strong to medium sunshine about some days (Asrat et al., 2015). In drying process, the coffee cherry parts like the mucilage, the film, the pulp and the parchment unite to form one so that the coffee bean (seed) becomes easy to be detached by coffee pulped machine by mechanical hammer. Then, the final coffee bean will go out by screening process.

In wet processing, the first step is mechanical removing of the pulp using pulpier machine/ after removing any derbies to the ripped coffee cherry. After the pulp and the other parts of the coffee cherry are detached then the pulp is removed the bean with parchment kept under water from 24-72hrs. for the removal of the mucilage/ mesocarp layer/ (Asrat et al., 2015). During soaking in the water microbial fermentation process would have been develop since mesocarp layer contain different nutrient. Later, fermentation process not only for the removal of mucilage but also the coffee develops flavor and aroma. Yet, the parchment coffee would be found by continuous washing and by keeping under sunshine in mat / rack for drying up to 10-12% moisture content. However, in this process to produce one kilogram of dried coffee produce around 2.5kg of wet pulp and 12.4 kg of effluent (B Manoj Kumar et al., 2012).

2.1.2. World production of coffee

Now days, Brazil is the world's largest coffee producer in which in 2016, Brazil produced a staggering 2,595,000 metric tons of coffee beans which over 150 years (Gerry Carty G. O., 1997). Correspondingly, Brazil also distinguishes itself from other coffee producing nations in that Brazilians process coffee using the dry process (unwashed coffee), where the coffee cherries are dried in the sun rather than washed in a wet process (Simon, 2019).

Table 1: Coffee producing countries according to their ranks

Rank	Country	Coffee Production (Metric Tons)	Coffee Production (Pounds)
1	Brazil	2,592,000	5,714,381,000
2	Vietnam	1,650,000	3,637,627,000
3	Colombia	810,000	1,785,744,000
4	Indonesia	660,000	1,455,050,000
5	Ethiopia	384,000	846,575,000
6	Honduras	348,000	767,208,000
7	India	348,000	767,208,000
8	Uganda	288,000	634,931,000
9	Mexico	234,000	515,881,000
10	Guatemala	204,000	449,743,000

Source: (Szenthe, 2019)

On the other hand, the wastewater generated from wet coffee processing firm contains organic matter like pectin, proteins, and sugars (R. B. Mendoza and M. F. C. Rivera, 1998). Coffee pulp, one of the principal by-products of wet processed coffee constitutes almost 40% of the wet weight of the coffee berry, is rich in carbohydrates, proteins, amino acids, poly-phenols, minerals, and appreciable quantities of tannins, caffeine and potassium. Coffee pulp is generated to the extent of 40% in the fermentation of coffee berries poses many environmental problems in the coffee producing countries. Its disposal in nature without any treatment would have being causes savior environmental pollution due to decomposition of biodegradable wastes (Asrat et al., 2014).

2.1.3. Ethiopian production of coffee

Coffee in Ethiopia is the first export item; its share of selected commodities in 2017/18 year covers 29.5% (Yinager, 2019). According to a report by the Ministry of Trade (MoT) of Ethiopia as referred on title "The Ethiopian Commodity Exchange and the coffee market" referred in (Hernadez et al., 2015) Ethiopia, where one of largest in the producers in the world, the country produced an average of 300 thousand tons of coffee per year between 2000 through 2012. The report suggested that coffee producing regions in Ethiopia were Oromia and SNNP regions, which together roughly account for 97 % of the total national production.

Correspondingly, in the south-west part of the country, which covers Illubabor, Kelem, Jimma, Kaffa, Shaka, Bench Maji and Wollega (Nekemt), produces dry coffee and represents about 70% of the total production (Dejen et al., 2015). Whereas, West and East Hararghe (Harar) were produced both dry and washed coffee beans and account for the remaining 8% of the national production. On the other hand, the southern region part of Ethiopia, which includes Sidama, Yirgachefe, Guji, Kochore and Wolayeta, produces washed coffee beans and represents 22 % of the total production (Hernadez et al., 2015). However, these coffee processing industries produces very high pollution load wastewater because coffee processing industries are one of the significant consumers of water (Dejen et al., 2015).

2.2. Coffee waste

Coffee processing byproducts constitute a source of a serious environmental contamination problem in Ethiopia as well as other coffee producing countries (D.P. Navia et al., 2011). Wet and dry methods discard away 99% of the biomass generated by the coffee plants at different stages. This includes cherry wastes, coffee parchment husks, sliver skin, coffee spent grounds, coffee leaves, coffee pulp and wastewater (A. Kivaisi et al., 2010).

Organic waste products, such as mucilage and pulp represent a major source of environmental pollution and their disposal is usually done in the water resources closest to the processing sites, such as rivers and ponds. Moreover, pulp and mucilage consume the oxygen in water, resulting in the death of plants and animals due to the lack of oxygen or the increased acidity. However, the idea of using these products came from the need to minimize their negative environmental impacts and to satisfy the demand for resources suitable for ethanol production (D.P. Navia et al., 2011).

2.2.1. Coffee wastewater characteristics in Ethiopia

Coffee is the most important cash crop and largest export commodity, which account 90% of exports and 80% of total employment in Ethiopia. Ethiopia had been the origin of coffee since coffee plant was initially found in the Kaffa province (Bonga, Makira) of Ethiopia (ITC (International Trade Centre) UNCTAD/WTO 2002) (Secretariat, 2003). On the other hands, wastewater quality can be expressed by their physical, chemical, and biological characteristics. Physical parameters include color, odor, temperature, solids, turbidity, oil, and grease. Chemical parameters associated with the organic content of wastewater include the biochemical oxygen demand (BOD), chemical oxygen demand (COD), total organic carbon (TOC), and total oxygen demand (TOD) and inorganic chemical parameters include PH, acidity, nutrients and the like (Asrat et al., 2014).

A. Water Quantities

Depending on the processing technology applied, quantities of coffee wastewater is varying. In Ethiopia at present, wet coffee processing firms are more than 400, all of which are located at the near water especially in river. In which, these wet processed coffee will produce total annual coffee estimated to be 52,000 tons'. In the process it use at about 5-15 liters of water to recover 1 kg of clean green coffee beans (the actual volume usage of water depends on the pulping process, fermentation intensity and coffee bean transportation volume) (Asrat et al., 2014). In order to treat wastewater appropriately with reasonable costs, the amounts of wastewater must be minimized.

B. Organic Components

The main pollution in coffee wastewater stems from the organic matter set free during pulping the mucilage texture surrounding the parchment is partly disintegrated. Water is needed in pulping process followed by quickly fermenting of sugars from both pulp and mucilage components; which helps for the removal of mucilage from parchment. Since the pulp and mucilage consists to a large extend of proteins, sugars and the mucilage in particular of pectin's, i.e. polysaccharide carbohydrates (Asrat et al., 2015).

Wastewater from mechanical mucilage removers contains a certain amount of sugars (disaccharide carbohydrates). But, its apparent gel like texture comes from the segments of undigested mucilage and pectic substances; which have been removed from the parchment by mechanical means. In order to be biodegraded, the solid materials have to be fermented, acidified and hydrolysed by natural fermentation in a later tanker (Asrat et al., 2014).

During fermentation and acidification of sugars from by wastewater, pectin oligosaccharides get out of solution and float on the surface of the wastewater. The remaining highly resistant materials left in the effluent water are acids and flavanoid color compounds from coffee cherries. At around PH 7 and over, flavanoids turn wastewater into dark green to black color staining rivers downstream from coffee industries (Jan C. von Enden and Ken C. Calvert, 2010). However, flavanoids do not do any harm to the environment nor add significantly to the Biological Oxygen Demand (BOD) or Chemical Oxygen Demand (COD) (Jan C. von Enden and Ken C. Calvert, 2010).

The chemical characteristics of wet processing of coffee associated with the organic content of wastewater best expressed in their biochemical oxygen demand (BOD) and chemical oxygen demand (COD). This wastewater from wet coffee processing industries the value BOD is up to 20,000 mg/L. It should be reduced to standard discharge level that is less than 200 mg/L before let into natural waterways. Whereas, resistant organic materials which can only be broken down by chemical means indicated by the COD make up around 80% of the pollution load and are reaching 50.0 mg/l and more (Asrat et al., 2014). The material making up the high COD can be taken out of the water as precipitated the mucilage as solids. Other substances in small amounts found coffee wastewater are toxic chemicals like tannins, alkaloids (caffeine) and polyphenolics.

Nevertheless, the toxic substances mainly found in the disposed solids (suspended solids) of the coffee pulp (Jan C. von Enden and Ken C. Calvert, 2010).

C. Acidity

During the fermentation process in the sugars will ferment in the presence of yeasts to alcohol and CO2. However, the alcohol is quickly changed to vinegar or acetic acid in the fermented pulping water (Jan C. von Enden and Ken C. Calvert, 2010). The simplified chemical formula for fermentation of sugars by yeasts to ethanol is symbolized by the fructose to ethanol reaction:

$$C_6H_{12}O_6 = 2\ CH_3\ CH_2OH + 2\ CO_2$$

Sugar = 2 Ethanol + 2 Carbon dioxide

Ethanol is quickly broken down by bacteria into acetic acids. This complex enzymatic catalyzed reaction is simplified as

$$2\ CH_3\ CH_2OH + O_2 = 2\ CH_3\ COOH$$

2 Ethanol + Oxygen = 2 Acetic acid

Acidification of sugars will drop the PH up to 4. The digested mucilage will be precipitated out of solution and build three layer i.e. a thick crust on the surface of the wastewater, black on top and oily orange/brown color beneath. If not separated from the wastewater, these crusts quickly clog up waterways and further contribute to anaerobic conditions (Jan C. von Enden and Ken C. Calvert, 2010).

2.2.2. Coffee waste management in Ethiopia

In Ethiopia, knowing Agro-industrial residues/wastes with poor management created different problems happening on the environment as well as bio system. Though, several attempts have been made to manage the dispute; among remedial actions, coffee pulp solid waste is being renewed into compost, which farmer used in fertilizing their coffee farms. One of techniques currently to management wastewater used by the coffee industries is based on the use of ponds. However, the improved application by biotechnological means undertakes social, economic and industrial importance (Asrat et al., 2015).

As a consequence, severity in case of pollution by the coffee industries showed by different articles. Although the coffee wastewater coming from the coffee processing industries in Jimma zone is a valuable resource, it is released to the nearby water course without any treatment. Wastewater creates a severe threat to the aquatic ecosystem and downstream users. To solve the problem understanding the nature of the coffee processing wastewater is crucial for suggesting possible solution and operation of appropriate and effective treatment technologies. Therefore, the government bodies and stack holders in the area of coffee factory for effluent management options should be dealt with top priority to avoid further needless damage to the environment (Derejen et al., 2015).

On this regards, 400 wet coffee processing industries which estimated to be 52,000 tons' production produces surprising amount of waste; so the effect by wastewater pollution in Ethiopia needed at savior level. On the study to characterize wet coffee processing waste and determine total reducing sugar potential of coffee waste in the pulp juice and mucilage showed, the waste was determined and showed has high organic component, 66.5% and 90.2%, respectively. The effluent waste is acidic with PH 4.75 and 3.67, respectively. Thus, the study showed serious problems for environment because of their high BOD/COD values. Which shows the COD: BOD ratio for pulp juice was 1.76 and for mucilage was 1.7. Thus, the ratio is less than 5:1, which shows the wastes are bio-degradable (Asrat et al., 2014).

2.3. Treatment methods in coffee processing of wastewater and their drawbacks

Struggles have been made by different scholars in the world to improve an efficient and economic method for wet processing of coffee treatment of wastewater. The first recorded effort to treat such wastewater was made in Kenya in the 1950s'. The East African Industrial Research Organization (EAIRO) worked on it in Uganda in the early 1960s' while IDRC, Canada sponsored research projects in South America in the 1970s'. The first attempt to separate the solids and concentrate on the wastewater cleanup was made in Costa Rica during the 1980s'. However, it did not succeed in handling the mucilage problems and proved to be costly on a large scale (Calvert, 1997). In Papua New Guinea, an UASB (Up flow Anaerobic Sludge Blanket) reactor was used for treating coffee wastewater, along with a biological filter system. In spite of this, the flavonoid color compounds in coffee skins continued to discolor the rivers and other discharge areas (Calvert, 1997). But these processes are high energy consuming, non-economic and release effluents and wastewaters which require further treatment and thus are alarming for the environment. Also complete removal of the pollutants cannot be possible by the use of physical and chemical processes. Here are some of treatments of coffee processing wastewater treatment applied.

A. Treatment of coffee wastewater by Gamma radiation

Gamma radiation employed was the chemical treatment followed by the irradiation of the samples since no nuclear changes of the coagulant solution or wastewater samples were expected. Irradiation is a high cost treatment although has increased its applications nowadays (Y. Aguilera et al., 1988). The method is fast and effective and it does not generate any pollution. Weakness with the method was higher decontamination, due to a radiation activation of the coagulant and high cost treatment. However, the study in this thesis reduce rescue of contamination due to radiation and also cost cheaper in the treatment method.

B. Chemical coagulation

Chemical coagulation is a chemical reaction involving chemical destabilization of particles that forms larger particles though per kinetic flocculation. Coagulant is a chemical added to destabilize the colloidal particles in the wastewater to form flocs which will be settled down and separated from treated water (Elida Novita et al., 2013). The disadvantage in the treatment are high cost of the adsorbent and after use the disposal of chemical coagulant sedimentation creates problems; conversely, this study in thesis were oppositely treat the wastewater.

C. Treatment of coffee effluent by *Moringa oleifera* seed

The study use *Moringa oleifera* seed as a means to treat the coffee effluent. *Moringa oleifera* was effective in the removal of chemical impurities from the effluent. Likewise, *Moringa oleifera* was effective in the inhibition of bacterial contamination (R. Padmapriya et al., 2015). Since, the natural products are of low cost and are easily available they can be used to treat the wastewater. Drawback with the method was low reduction of DO, COD and BOD load. However, the treatment of wastewater effluent from coffee industry of this study fills the delimitation the above problem by making improved removal rate of COD and BOD.

D. Coffee industry wastewater recycling

Environmental problem creates great political and social pressure to reduce the pollution arising from industrial activities. Developed and underdeveloped countries are trying to adapt to this reality by modifying their processes so that their residues can be recycled. Now days, most large companies no longer consider residues as waste, but as a raw material for other processes (Michiel R. J. Doorn et al., 2006).

Once, the waste released more than 50% is considered a waste; it no longer has any commercial application, knowing that its chemical components could be exploited for the production of inputs and energy. Biotransformation using coffee by-products and residues will produce bio-ethanol, biogas and biodiesel by fermentation process. Thus, by products like biofuels offer greater energy security, lower emissions of greenhouse gases and particulate matter, rural development, reduced demand for oil, among others (S. Trinidad et al., 2017).

Consequently, the presence biodegradable matter in the waste and its demand of great quantities of oxygen to degrade confer a toxic nature. Despite wastewater can be considered as both a resource and a problem, there are few studies focusing on their use and profitable applications. Focusing on value adding in unused materials, finding alternative forms to use them would be useful to decrease their impact to the environment (Intizar et al., 2002).

Thus, generation of residues and by-products is inherent in any productive sector; moreover, agro-industrial and the food sectors produce large quantities of waste, both liquid and solid. Coffee is the second largest traded commodity in the world, after petroleum, and therefore, the coffee industry is responsible for the generation of large amount of residues and by product. The use of such wastes has been subject of several studies, but this concern did not exist in past decades when 77 million bags of green coffee were simply burned and released to the sea and in landfills (I.M. Solange et al., 2011).

2.4. Biological wastewater treatment

Biological/ biodegradation of wastewater treatment processes and other recent potential biological process which emphasis on the removal of organic matter (COD/BOD), NH_4-N and sulphides/sulphates (Sabumon, 2016). Biological/ biodegradation are the process of decaying or reduction of different organic materials and toxic metals to their nontoxic form with the help of microorganisms. There are various advantages associated with biodegradation such as process is a simple process, it is an eco-friendly and cost effective process that requires low capital and operating cost and being environmentally friendly process it produces no harmful end products. Accordingly, there are different biological treatment methods; they are aerobic, anaerobic and/ or anoxic method these three methods have also their own prone and cons.

Aerobic treatment method has poor settling of biological solids remains one of the most common operational problems. Yet, well-mixed reactor, suspended growth and a gas separator before settling tank is used to make settling easier as well as simple and inexpensive. In contrast to aerobic degradation, which is mainly a single species phenomenon, anaerobic degradation proceeds as a chain process, in which several sequent organisms are involved. Overall anaerobic conversion of complex substrates therefore requires the synergistic action of the micro-organisms involved. Generally, the uses of anaerobic and aerobic methods have advantages and disadvantages in which this is summarized in the following Table 2 below.

Table 2 : Comparison between anaerobic and aerobic treatment methods

Parameter	Aerobic treatment method	Anaerobic treatment method
Process principle	• Microbial reaction takes place in presence of molecules /free oxygen/ • Reaction products are carbon dioxide, water and excess biomass	• Microbial reaction takes place in absence of molecules /free oxygen/ • Reaction products are carbon dioxide, methane and excess biomass
Applications	Wastewater with medium to high organic impurities (COD<1000ppm) and for wastewater that are difficult to biodegradable wastewater e.g. municipal sewage, refinery wastewater etc.	Wastewater with medium to high organic impurities (COD>1000ppm) and easily biodegradable wastewater e.g. food and beverage wastewater rich in starch /sugar/ alcohol
Reaction kinetics	Relatively fast	Relatively slow
Net sludge yield	Relatively high	Relatively low (generally one fifth to one tenth of aerobic treatment processes)
Post treatment	Typically direct discharge or filtration disinfection	Invariably followed by aerobic treatment
Foot-Print	Relatively large	Relatively small and compact
Capital investment	Relatively high	Relatively low with pay back
Example technologies	Activated sludge e.g. Extended Aerobic Oxidation Ditch, MBR, Fixed Film Processes e.g. Trickling Filter /Biotower/, BAF, MBBR or Hybrid Processes e.g. IFAS	Continuously stirred tank reactor /digester, up flow anaerobic sludge blanket (UASB), ultra-high rate fluidized bed reactors e.g. EGSBTM, ICTM etc.

Source: (Mittal, 2011)

On the other hand, the use of both aerobic and anaerobic method is effective method to treat wastewater from coffee industries. For instant, treatment of wastewater is necessary to reduce the impact of nitrogen waste on human health, particularly in infants, pregnant women and other biotic ecosystems. The extent and purpose to which nitrification and denitrification are applied are dependent on the level of nitrogen removal required and the specific treatment method(s) used. Removal of nitrogen from wastewater effluents is vital by biological means which is typically categorized into the two processes of nitrification and denitrification.

Biological wastewater treatment has advantage than conventional method coffee processing. Coffee processing agro-industry generates large quantities of wastewater requiring systematic treatment prior to disposal. Study by (A. Cruz Salomon et al., 2017) showed chemical oxygen demand (COD) removal efficiency increases from 94 to 98% when the Hydraulic Retention

Time (HRT) increases from 3 to 9 days. While increasing hydraulic retention time from 7–9 days generated effluents capable to be dischargeable into water bodies with a permitted COD concentration according to World Health Organization (WHO) and Official Mexican Environmental Regulations permissible limits (A. Cruz Salomon et al., 2017). In the above study, at HRT of 9 days was the one that greater COD removal generated, so the EGSB bioreactor can be a sustainable alternative to solve the environmental problems, compared to other conventional methods to CPWW treatment (A. Cruz Salomon et al., 2017).

On the other hands, using yeast extract and nitrification six of these bacteria exhibited appreciable nitrification activity, yielding as much as 5.8 mM nitrite and little or no nitrate when grown in a mineral salts medium containing 7 mM pyruvic oxime and 0.05% yeast extract. These six active bacteria, four (*Pseudomonas* denitrificans, *Pseudomonas aeruginosa*, and two strains of *Pseudomonas fluorescence*) could grow on yeast extract (Domenic Castignett and Thomas C. Hollocher, 1984). Likewise, under varieties of PGPR (Plant growth promoting rhizobacteria) have been studied and some of them have been commercialized, including the species *Pseudomonas*, *Bacillus*, *Enterobacter*, *Klebsiella*, *Azobacter*, *Variovorax Azosprillum*, and *Serratia* (Pravin Vejan et al., 2016).

While, in the study on tannery effluents and it’s prospective on biological treatment processes and other recent potential biological processes are discussed. Aerobic treatment in the study was efficient in treating tannery effluent, it requires an extended aeration time at low organic loading rates and thereby increasing the overall treatment cost. While, anaerobic treatment is not effective because of sulphide inhibition problems (Sabumon, 2016).

2.4.1. Nitrogen cycle in wastewater treatment

Nitrogen cycle is important process in wastewater treatment in which nitrification and denitrification processed for reduction of nitrogen containing compounds. Nitrification is one of steps nitrogen cycle; where ammonia would be changed first to nitrate and then to nitrite. The other steps in the nitrogen cycle are denitrification the change from nitrite in to nitrate further to nitrogen dioxide then to nitric oxide finally to atmospheric nitrogen a shown in Figure 1.

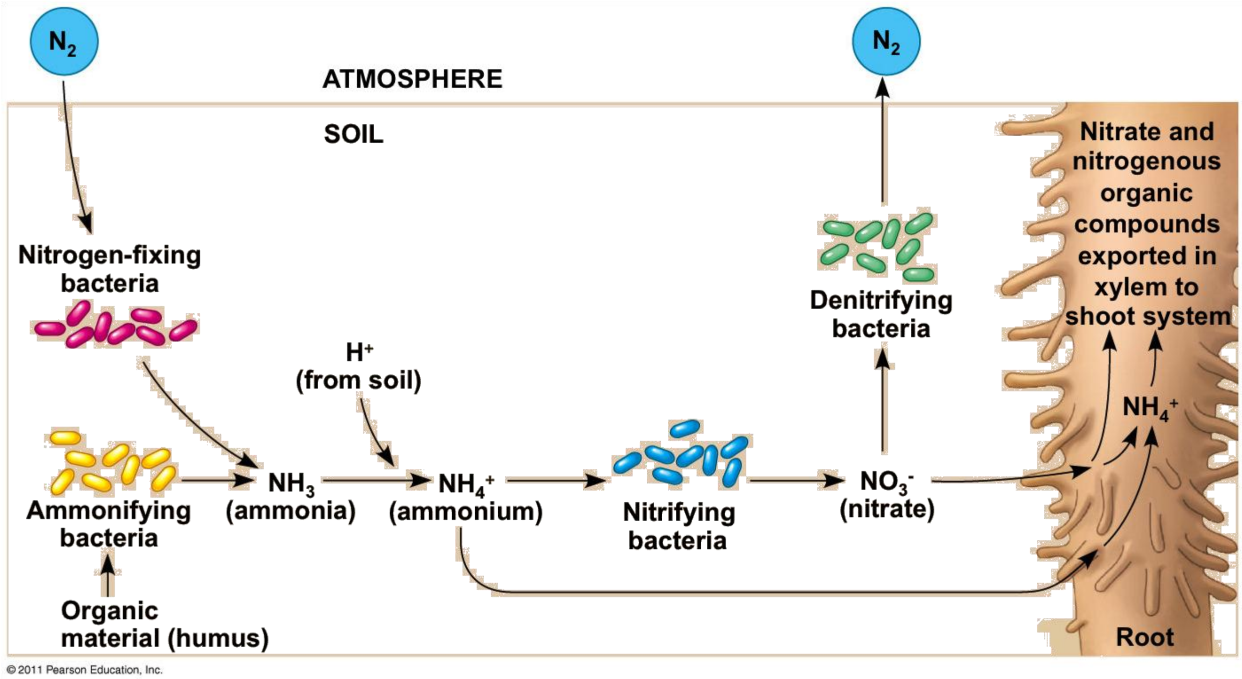

Figure 1: Over all steps in nitrogen cycle

Once, a nitrification process end which is nitrite formed then plants can assimilate nitrite. However, after the end of nitrification process denitrification process immediately starts. Denitrification process every steps is mandatory steps. One step could not start before the other and vice versa.

On the other hands, nitric oxide (NO) is one of the steps in the process which is very toxic to organisms including human being. Nitric oxide (nitrogen monoxide, NO) is a molecule of major importance in biological systems where it plays signaling, vasodilator and cytotoxic. However, bacteria's like *E. coli* unlike other bacteria's without denitrification process they will produce nitrogen. While NO is also an obligate intermediate in denitrification, the process by which certain bacteria sequentially reduce nitrate ion to dinitrogen. However, microbes without having nitric oxide of the "non-denitrifying" *Enterobacteriaceae*, including *Escherichia coli*, grown anaerobically with nitrate, were shown to produce up to one-twentieth of the NO produced by denitrifiers (Hazel Corker and Robert K. Poole, 2003).

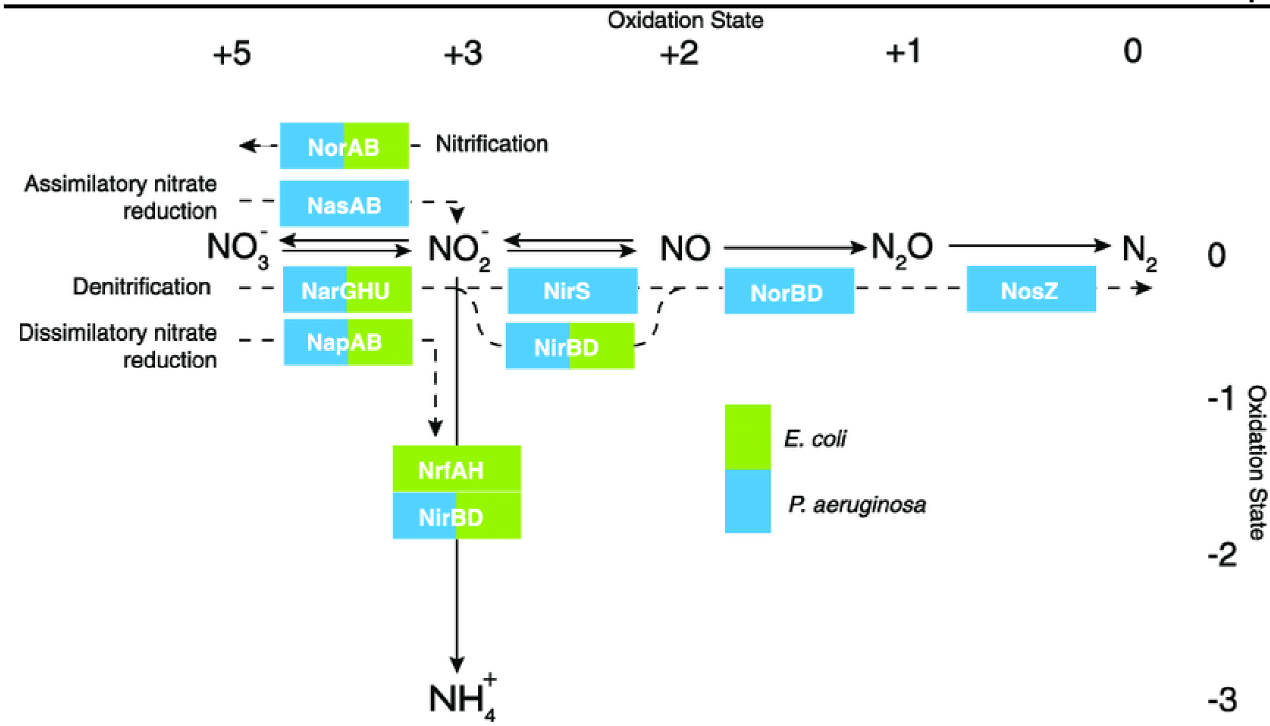

Figure 2: Nitrate conversion by microorganism with their oxidation state

2.4.2. *Pseudomonas fluorescence* and *Escherichia coli* bacteria for treatment of wastewater

Some bacteria able to reduce nitrate from ammonium can be used in their identification and isolation. For instant, *E. coli* can reduce nitrate only to nitrite from ammonium, P. fluorescence reduces it completely into molecular nitrogen, (Prescott, 2002). Different studies on the treatment of waste predict that aerobic soils are primary sources of NO and that N_20 is produced only when there is sufficient soil moisture to provide the anaerobic microsites necessary for denitrification by either denitrifiers or nitrifiers (Iris Cofman Anderson and Joel S. Levine, 1986). Nitrous oxide production was highest in the denitrifier *Pseudomonas fluorescence*, but only under anaerobic treatment situation; While seeing the molar ratio of NO/N_20 produced was usually greater than unity for nitrifies and much less (Iris Cofman Anderson and Joel S. Levine, 1986).

On some studies, the presence of 5mM nitrate provided a growth benefit and induced both nitrite and ammonia generation in *E. coli* and *L. plantarum* bacteria grown at oxygen concentrations compatible with the content in the gastrointestinal tract. While reducing the nitrate then nitrite and ammonia accumulated in the growth medium when at least 2.5 mM nitrate was present (Mauro Tiso and Alan N. Schechter, 2015). On the other hand, enzymes responsible for the conversion of ammonium in nitrification and denitrification are as follows: two key enzymes are necessary for energy conservation during the oxidation process, ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO) (Hermann Bothe et al., 2000).

2.5. Mixed culture

Biological wastewater treatment technologies can greatly reduce operation costs by wastewater bioremediation (Olga N. Tsolcha et al., 2018). On the other hand, mixed culture or microbial consortia has different advantages compared to pure culture especially for biodegradation for the treatment of wastewater and sewerage. Optimal product formations using mixed culture are still under development. In order to select among mixed and pure culture processes depend on the complexity of the bioprocess involved. However, mixed culture fermentation gives several benefits than the conventional pure culture fermentations, the predominating bio production processes are still based on pure culture (Wael Sabra and An-Ping Zeng, 2016).

The coexistence of microorganisms in wastewater treatment systems has been widely investigated in an attempt to simulate natural processes (Olga N. Tsolcha et al., 2018). Besides pure culture has different impediment on the microorganism metabolic activity. The overall product yield or production rate of a pure culture normally decrease with time due to accumulation and inhibition of intermediates or the final product of the desired bioconversion process and quite often also byproducts. This can be avoided through the use of a mixed culture in which the inhibiting intermediates or byproducts may be degraded by one of the species of the mixed culture (Wael Sabra and An-Ping Zeng, 2016).

However, studying on mixed culture has different challenges to proceed. On study Mixed-Culture Interactions; Studies of mixed batch cultures yield data that are often difficult to interpret, because many factors are changing simultaneously. On the other issue continuous cultures permit control of nutritional factors, physiological age, and physiochemical conditions by adjustment of flow rates and concentrations of nutrients and additives (Henry R Bungay et al., 1965).

2.6. Gypsum as wastewater treatments and its use

Gypsum is used in a wide variety of applications: studies shows that gypsum can be used to remove pollutants such as lead or arsenic from contaminated waters (wikipedia, 2018). Consequently, gypsum serves as a skeleton builder, forming a permeable and rigid lattice structure that can remain porous under high positive pressure. During the compression step after the cake growth of the filtration, thereby maintaining the size of the micro-passages through which water is expressed by making dewatering by removing sludge (Zhao, 2006).

On the other hand, the effect of gypsum on agriculture on the research made other author discussed on some water bodies suggest in Europe. This paper examines the motivations of participants in a large-scale pilot project that develops a new agriculture and environmental measure, gypsum treatment of arable fields, to reduce phosphorus loads to the Baltic Sea. The general model of crop production that allows for three motivations; they are profit maximization, utility from agricultural innovation, and stewardship towards the nature. These models are present in the sample, proved by farmer survey and confirmatory factor analysis. Strong profit motivation relates to large gypsum treated area and perceived easiness of gypsum as a water protection measure, and strong environmental motivation to environmentally friendly cultivation technologies (A.K. Kosenius and M. Ollikainen, 2018).

On the study, using a high pressure cell apparatus showed that a further decrease of two to seven percent of the equilibrium moisture content of the sludge cake was achieved, for sludge thicknesses for dewatering of 1 to 10 cm, by the addition of gypsum with 60% of the original sludge solids when compared to the single polymer conditioning. Thus, the benefit of adding of gypsum in alum sludge dewatering is not only the improvement in the extent of dewatering, but also the potential application of transforming dewatered alum sludge from ‘waste’ for landfill to useful ‘fertilizer’ or to be used as filter medium/adsorbent for wastewater treatment engineering (Zhao, 2006). However, the recovery of gypsum after treatment would be developed by using alternative method of disposal that involves drying and granulating the sludge, followed by high temperature calcination in a fluidized bed reactor to recover usable sulfur dioxide and lime (Smith L.L. et al., 1984).

2.7. Material balances

In dealing with food and agro-processing industry effluent's, whether formulating treatment and utilization strategy or planning the initial stages of a comprehensive management project, a basic understanding of the effects of mass flow rate or loading factors on process designs are essential. Stoichiometry is the material accounting for a chemical reaction. Given enough information, one can use stoichiometry to calculate masses, moles, and percent within a chemical equation that is an expression of a chemical process. Consider a simple reaction where a reactant A converts into resultant B.

During the growth of cells, substrates that provide energy and raw materials are needed for the synthesis of additional cell mass. Generally, in a biochemical process, the cell environment should contain elements required in order to form additional cell mass and the free energy from the substrate consumed should exceed the free energy of cells and metabolic products formed. The Figure 3 shows the simplified mass balance (Dutta, 2008).

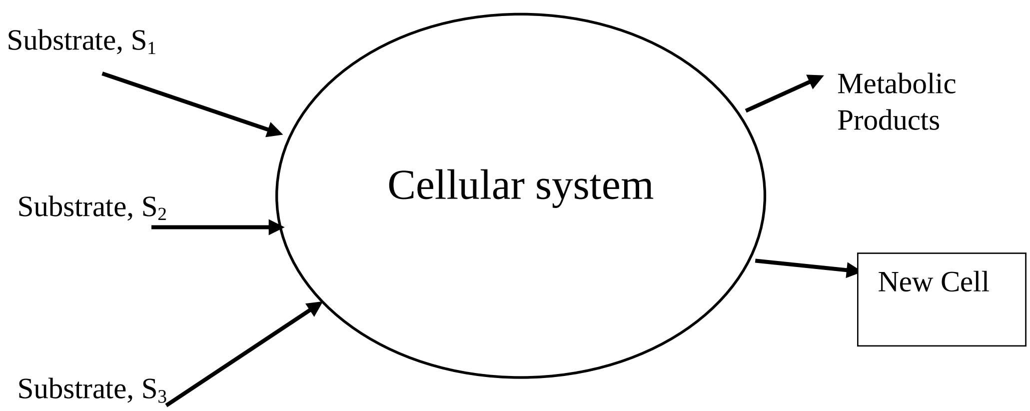

Figure 3: Simplified diagram for metabolism in microorganisms

CHAPTER THREE

MATERIALS AND METHODS

3.1. Description of study area

Dilla town is located in SNNPR, Gedeo zone and it has a total area of 2,141.21 ha and 365 km from the capital city of Ethiopia, Addis Ababa. Moreover, it is situated in the eastern escarpment of rift valley and within altitude of 1527 m.a.s.l., the town bordered with Bule woreda in the east, Guji zone in the west, Gungua town in the south, and Mechisho town in the north. The municipality is divided into 3 sub city and 10 Kebeles. Of ten kebeles, Dilla zuria kebele is the most coffee production site. The main economic activities of the town are cash crop mainly coffee, agro processing industry (Dry and wet coffee processing industry) and small and medium trade as well as commercial activities (University, 2012). Geographically, Dilla town is located at $6^{0}25'48.77''$N and $38^{0}17'47.65''$E (Figure 4).

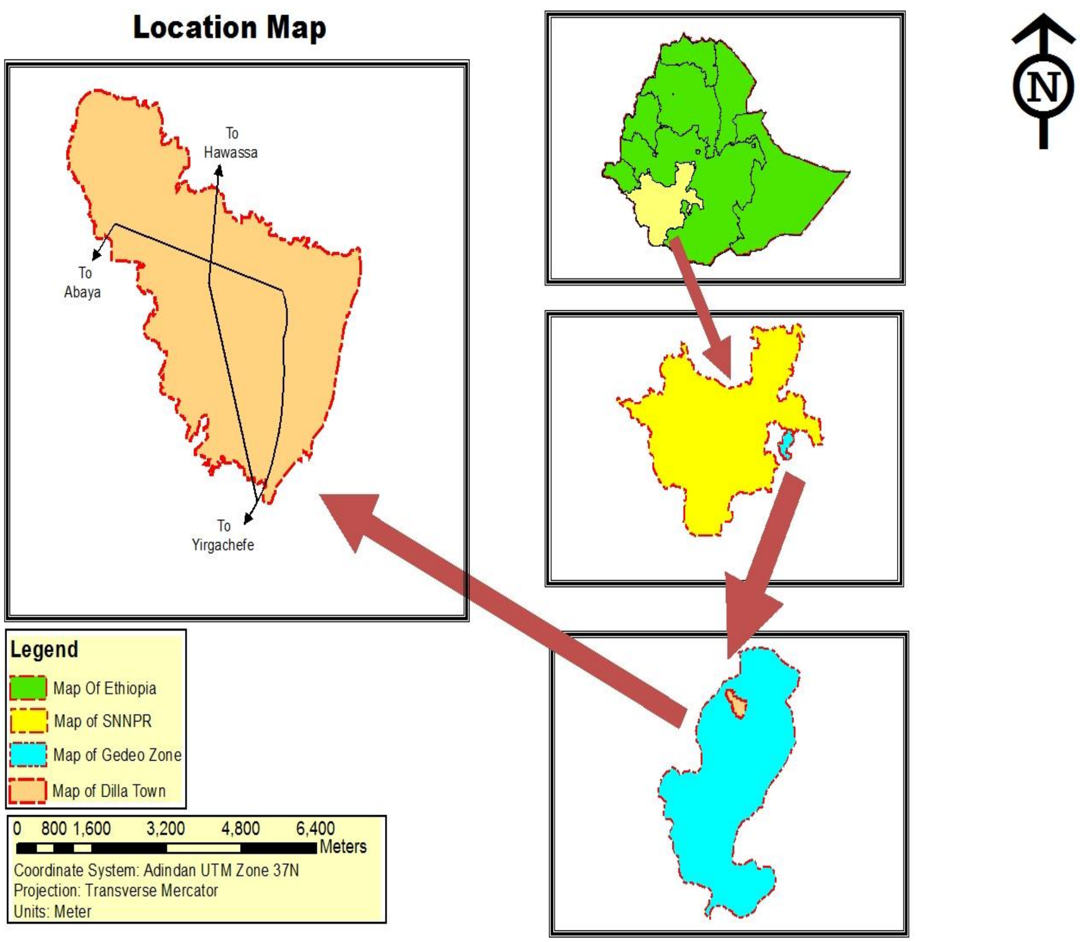

Figure 4: Location of the study area

3.2. Sample collection

The samples collected from private owned coffee processing industry situated near “Lagadara” River in Dilla, Ethiopia. Sample sites were selected based on some stated criteria, such as, the size of discharge to the rivers, the amount of water used for the process, the capacity of the processing industry and the location of coffee processing industry. The sample were collected equally from the top surface, bottom and medium part of the pond of total volume of 20L of wastewater was collected using polyethylene bags from. The sample was kept in ice box (cold storage) and transported to Addis Ababa Institute of Technology and kept at 4°C in refrigerator Bioengineering Lab until characterization process and analysis was conducted. Then, the sample was subjected for various analyses such as PH, BOD5, COD, TS, TDS, TSS, NO_2^- and NH_4^+. The physicochemical parameters were estimated using standard methods and the sample collection was shown in (Figure 5).

Figure 5: Sample collection from the coffee effluent

3.3. Chemicals and equipment

3.3.1. Chemicals and reagents

Pure and analytical grade chemicals were used in experiment including media preparation for the growth of microorganism. Nutrient agar and nutrient broth media mark in MacConkey Company were used for media preparation.

For BOD test reagents: Phosphate buffer solution, Magnesium sulfate solution ($MgSO_4$ $7H_2O$), Calcium chloride solution ($CaCl_2$), Ferric chloride solution ($FeCl_3$), Sodium hydroxide

(NaOH), 1 N, Sulfuric acid (H_2SO_4), 1 N, Sodium sulfite (Na_2SO_3), 0.025 N, Potassium iodide solution (KI), 10%, Acetic acid solution (CH_3CO_2H)(1+1), Sulfuric acid solution (H_2SO_4), (1+50), Starch indicator solution, Glucose-glutamic acid solution, nitrification inhibitor (2-chloro-6-(trichloromethyl) pyridine) and distilled water.

For COD test reagents: the following reagents were used such as standard potassium dichromate solution, (0.25 N): dissolve 12.2588 g of $K_2Cr_2O_7$; sulfuric acid reagent: Conc. H_2SO_4 containing 23.5 g silver sulfate, Ag_2SO_4, per bottle and Standard ferrous ammonium sulfate, 0.250 N: dissolve 98 g of Fe $(NH_4)_2(SO_4)_2\,6H_2O$ in distilled water.

3.3.2. Equipment and material used

All glass wares (conical flasks, measuring cylinders, Round bottom flask, Erlenmeyer flask, cylinder jar, dilution tube, beakers, petri dish and test tubes, burette, pippetes, crucible etc.) are purchased from M/s SAN Medico Ltd (Rourkela, India) under the name Borosil. Other materials used are spoon, loop spider, aluminum foil, cotton, filter paper, guans, gloves, rack, Bunsen burner, BOD bottles, and etc. The instruments and apparatus used throughout the experiment were listed in Table 3.

Table 3: List of instruments and apparatus

Equipment	Model	Use
Test tube shaker	IKA MS3 digital	Mixing
Microbial incubator	Memment	Preservation of culture
Incubator shaker	GFL-3017	Incubation and shaking of cultures
Laminar airflow hood	FlowFastV	Aseptic Environment
Electrical heated Vertical Autoclave	Model- LX-B5OL(Digital)	Sterilization
Electronic analytic balance		Weight Measurement
Automated BOD analyzer		BOD test
COD test kits		COD test
PH meter	Jenway 3505 PH meter	Measurement of PH
Electron microscope	B-350 OPIKA	Analyzing microbes
Colony counter	FUNKE GERBER	Cell count
Stop watch		Time measurement
Refrigerator (Deep)	LG	Preservation of culture medium
Oven dryer		Drying material
Micro- pipettes		Measuring small liquid volume

3.4. Experimental methods

3.4.1. Pre-experimental setup

Before the actual experiments were started pre-experimental setup was organized. These experimental setups were microorganism collection, inoculum preparation, characterization and analysis of *P. fluorescence* and *E. coli* bacteria were conducted.

A. Microorganism collection and preparation

Pseudomonas fluorescence and *Escherichia Coli* bacteria species in the form of slant of 1.5 ml each bacterium in nutrient broth medium was collected by test tube. The microbes were brought from Ethiopian Biodiversity Institute (EBI) inserting in the test tube in ice box to AAiT, Biochemical laboratory. After transported to laboratory, these nutrient broth medium was maintained at 4°C in deep freeze until further experimentation and was sub cultured from time to time for its maintenance.

The microorganisms were maintained on the medium containing: nutrient broth medium (where glucose and peptone were used as primary carbon and nitrogen sources) at PH 7. The medium was autoclaved at 121^{o}C and 15 lb pressure for 15 min. The media was then poured in the Petri-dish and allowed the agar to solidify. A loop full of microbial colonies were taken from the master slant and inoculated in the petri dishes. The petri dishes were then kept in the incubator. The growth of the bacterial colonies was observed after 24 hrs. of incubation at 30^{0}C. Then the slants were stored at 4°c till further use.

B. Inoculum preparation

<u>Procedures</u>

The experimental studies were carried out in the shake flask as lab scale. Measuring two 6.5 gm nutrient broth medium (where glucose and peptone were used as primary carbon and nitrogen sources) using electrical analytic balance and measured two 50ml distilled water in measuring cylinder and added to two 100ml conical flask. Adding nutrient broth media and distilled water then covering the top of the conical flask by cotton and aluminum foil. Using 1KA MS3 digital shaker well mixed. Then sterilized using electrical heated vertical steam sterilizer (LX-B50L (Digital) at 121.1^{o}c and 15psi for 15 minutes as shown in Figure 6: b) below. In laminar flow hood (FlowFastV) dispersed each 1ml *P. fluorescence* and *E. coli* bacteria from the master slant added to each conical flask using micropipette and again coved using cotton and aluminum foil not being contaminated further. The prepared medium incubated to incubator (memmet) at 30^{o}c for 72 hrs. Finally, the incubated microbial broth was kept in refrigerator at 4^{o}c until further use (Figure 6).

a) b) c)

Figure 6: a) Collected sample b) sterilization c) incubation in shaker

C. Characterizing and growth analysis for *Pseudomonas fluorescence* and *Escherichia Coli* bacteria

After collecting microbes characterization tests of microorganisms were done. Some of the tests were morphological, viability, growth in medium agar and growth of both microbes in one medium. This was done to check whether the microbes were able to grow in media and how much microbial concentration would be available in the broth nutrient; so these microbes could have been used in the study for treatment the effluent (Figure 7(b)). Characterization of microbes was carried at nutrient media PH 7 which it gives not only a high biomass growth of *E. coli* but also biodegradation pollutants (Debadatta Das and SUSMITA MISHRA, 2010).

(a) (b)

Figure 7: (a) Microbial analysis (b) Microbial growth in petri dish

1. Procedure for characterization (for morphology and viability test) of *Pseudomonas fluoresce* and *Escherichia Coli* (in different nutrient agar medium)

Using electrical analytic balance was measured two nutrient agar medium of 6.5 gm. and measured two distilled water in measuring cylinder of 50ml. Both nutrient agar and water was added in to 100 ml of conical flask. Nutrient agar media and distilled water was added and covered the conical flask by cotton and aluminum foil. Agitated the conical flask using 1KA MS3 digital shaker was done until mixed. Then, sterilized was done with electrical heated vertical steam sterilizer (LX-B50L) at 121.1°c and 15psi for 15 minutes. In laminar flow hood (Flow Fast V) was made a serial dilution 10^{-3}, 10^{-6}, 10^{-9}, 10^{-12} and a control by using each 1ml for 9 ml of nutrient agar *P. fluorescence* and *E. coli* added to each conical flask using micropipette and again coved using cotton and aluminum foil. Full loop drop speeded in to Petri dish. The prepared medium in the petri dish only dilution of 10^{-3}, 10^{-9}, 10^{-12} and a control incubated to incubator (memmet) at 30°c for 72 hrs. Using colony counter count every colony for both microorganisms. Using sterile loop picking and spreading one colonies from both Petri dish microbes put in to electron microscope using B-350 OPIKA taken image and seen the morphology and viability of both microorganisms (Figure 8).

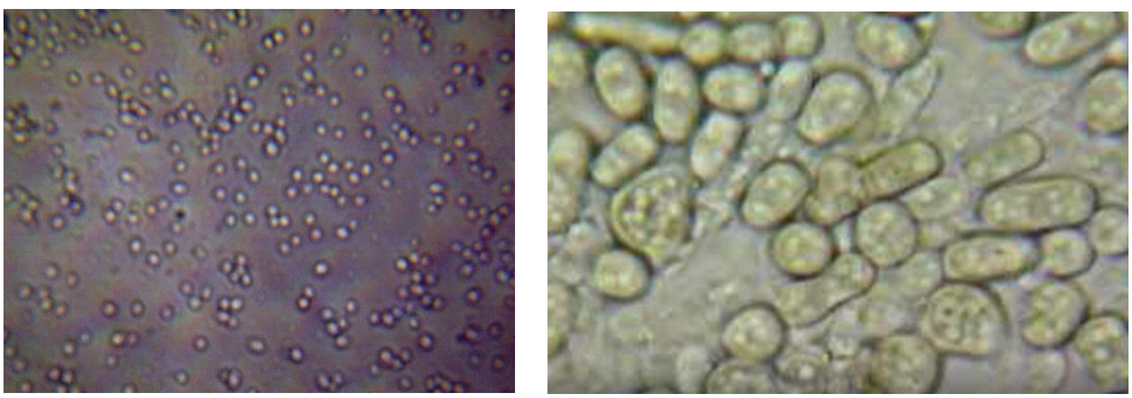

Figure 8: *Pseudomonas fluorescence* and *Escherichia coli* bacteria under electrical microscope

2. **Procedure for characterization (for morphology and viability test) of in mixed culture of *Pseudomonas fluoresce* and *E. Coli* in one/same nutrient medium**

Measured 6.5 gm. of nutrient agar medium using analytic balance and measured 50ml of distilled water in measuring cylinder. Both nutrient agar and water was added in to 100 ml of conical flask. Added nutrient agar media and distilled water then covering the top of the conical flask by cotton and aluminum foil. Using 1KA MS3 digital shaker well mixed. Then, sterilized was done using electrical heated vertical steam sterilizer (LX-B50L (Digital) at 121.1°c and 15psi for 15 minutes. In laminar flow hood (FlowFastV) was made a serial dilution 10^{-3},10^{-6},10^{-9} 10^{-12} and a control by using from each *P. fluorescence* and *E. coli* of 1ml for 9 ml of nutrient agar added to each conical flask using micropipette and again coved using cotton and aluminum foil not been contaminated. Full loop drop speeded in to Petri dish. The prepared medium in the petri dish only dilution of 10^{-3}, 10^{-9} ,10^{-12} and a control incubated to incubator (memmet) at 30^{o}c for 72 hrs. as shown in Figure 9 below. Using colony counter count every colony of microorganisms. Using sterile loop picking and spreading one colonies from petri dish microbes put in to electron microscope using B-350 OPIKA taken image and seen the morphology and viability of both microorganisms.

Figure 9: Poring nutrient agar for microbial growth

3.4.2. Characterization of the wastewater

The sample was kept in ice box (cold storage) and transported to Addis Ababa Institute of Technology and kept at 4°C in refrigerator bioengineering laboratory until characterization process and analysis was conducted. Then, the sample was subjected for various analyses such as PH, BOD5, COD, TS, TDS, TSS, NO_2^- and NH_4^+. Characteristics and compositions of contaminants present in the coffee industry effluent were studied.

3.4.3. Procedure for treatment effect mixed culture *Pseudomonas fluorescence* and *Escherichia coli* on the wastewater

Measuring 1000 ml wet processing of wastewater sample in one measuring cylinder and added 1500ml conical flask. The sample wastewater was covered the top of the conical flask by cotton and aluminum foil. Then, sterilized was done using electrical heated vertical steam sterilizer (LX-B50L (Digital) at 121.1^{o}c and 15psi for 15 minutes to reduce biasness in the treatment. Using conical flask 5ml from each for mixed culture *P. fluorescence* and *E. coli* bacteria were added using micropipette and again coved the conical flask using cotton and aluminum foil to kept in anaerobic treatment. Freshly prepared overnight culture of *P. fluorescence* and *E. coli bacterium* was inoculated into wastewater 5ml which have $3.215X10^{10}$cfu/ml. Using 1KA MS3 digital shaker well mixed and agitation speed was maintained at 200 rpm. The prepared medium incubated to incubator (memmet) at 30^{o}c for two different level of experimental run that is 72 hours (3 days) and 144 hours (6 days). After treatment of anaerobic mixed culture, the wastewater sample checked for physicochemical characteristics of wastewater sample.

3.4.4. Procedure for treatment effect of the wastewater using gypsum ($CaSO_4.2H_2O$) after microbial treatment

After treatment of anaerobic mixed culture, the wastewater sample checked for physicochemical characteristics of wastewater sample. Then, 20gm of Gypsum ($CaSO_4.2H_2O$) powder measured using electrical analytic balance and were added to fermentation conical flask after incubation and checked for physicochemical test finished and covered the measuring cylinder in aluminum foil. Then incubated in oven by putting in room temperature 25^{o}c with no vibration place for two different level of experimental run that was 24 hours (1 day) and 72 hours (3 days) as shown in Figure 10 below. Then the treated wastewater sample in the run slowly was poured the water in to jar from the top of measuring cylinder. Finally, physicochemical characteristics of the treated wastewater in every experimental runs were tested and the result was recorded immediately after incubation time finished.

3.4.5. Procedure for combined treatment effect of the wastewater using *Pseudomonas fluorescence* and *Escherichia coli* and with gypsum ($CaSO_4.2H_2O$)

Measuring 1000 ml wet processing of wastewater sample in one measuring cylinder and added 1500ml conical flask. The sample wastewater was covered the top of the conical flask by cotton and aluminum foil. Then, sterilized was done using electrical heated vertical steam sterilizer (LX-B50L (Digital) at 121.1^{o}c and 15psi for 15 minutes to reduce biasness in the treatment. Using conical flask 5ml from each for mixed culture *P. fluorescence* and *E. coli* bacteria were added using micropipette and again coved the conical flask using cotton and aluminum foil to kept in anaerobic treatment. Freshly prepared overnight culture of *P. fluorescence* and *E. coli* *bacterium* was inoculated into wastewater 5ml which have $3.215X10^{10}$cfu/ml. Using 1KA MS3 digital shaker well mixed and agitation speed was maintained at 200 rpm. The prepared medium incubated to incubator (memmet) at 30^oc for two different level of experimental run that is 72 hours (3 days) and 144 hours (6 days). Then, 20gm of gypsum ($CaSO_4.2H_2O$) powder measured using electrical analytic balance and were added to fermentation conical flask after incubation and checked for physicochemical test finished and covered the measuring cylinder in aluminum foil. Then incubated in oven by putting in room temperature 25^oc with no vibration place for two different level of experimental run that was 24 hours (1 day) and 72 hours (3 days) as shown in Figure 10 below. Then the treated wastewater sample in the run slowly was poured the water in to jar from the top of measuring cylinder. Finally, physicochemical characteristics of the treated wastewater in every experimental runs were tested and the result was recorded immediately after incubation time finished.

N.B: physicochemical characteristics were PH, TSS, TDS, TS, BOD5, COD, NH_4^+ and $NO2^-$

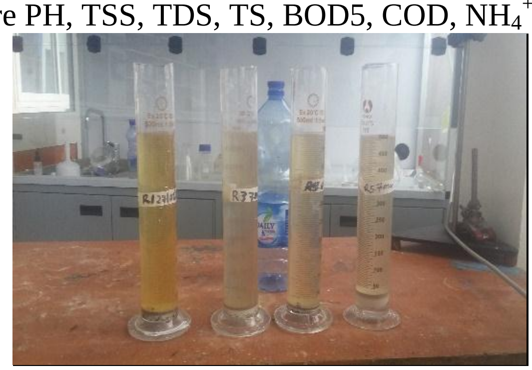

Figure 10: Treating the wastewater with gypsum

3.5. Microbial cell count by Colony Forming Units (CFU)

Plating techniques are simple, sensitive, and widely used for viable counts of bacteria and other microorganisms in samples of food, water, and soil. Several problems, however, could lead to inaccurate counts. Low counts would have resulted if clumps of cells were not broken up and the microorganisms well dispersed. Because it would not possible to be absolutely certain that each colony arose from an individual cell, in the study the results were expressed in terms of colony forming units (CFU) rather than the number of microorganisms. The samples used yield between 30 and 300 colonies for best results.

Procedure for CFU

1. Measured 3.5 gm nutrient agar and added to 50 ml distilled water
2. Prepared and mixed nutrient agar with 3.5gm for 50ml distilled water with conical flask
3. Heated the prepared nutrient agar until it mixed well
4. Washed all necessary equipment and sterilized the prepared nutrient agar at 121^{o}c for 15 minute at 15psi.
5. Labelled the petri dish and dilution tube i.e. the dilution concentration and the type of microbes' *P. fluorescence* and *E. coli* bacteria and also the controlled.
6. Added the sterile nutrient agar to the petri dish starting from controlled, dilution10^{-6}, 10^{-9}, and 10^{-12} for both *P. fluorescence* and *E. coli* bacteria respectively.
7. Prepared a serial dilution from 10^{-1} up to 10^{-12} in the laminar hood using micropipette from 9ml of sterilized distilled water and added 1ml the *P. fluorescence* and *E. coli* bacteria for the first and shack well and proceeded adding the sample from the previous dilution which already added the serial dilution and shacked well continue until 10^{-12} dilution as shown in Figure 11 below.
8. Waiting the nutrient agar until cooled in the petri dish; the serial dilution sample from the dilution tube was spread using sterile glass spreader in to already added agar in the petri dish.

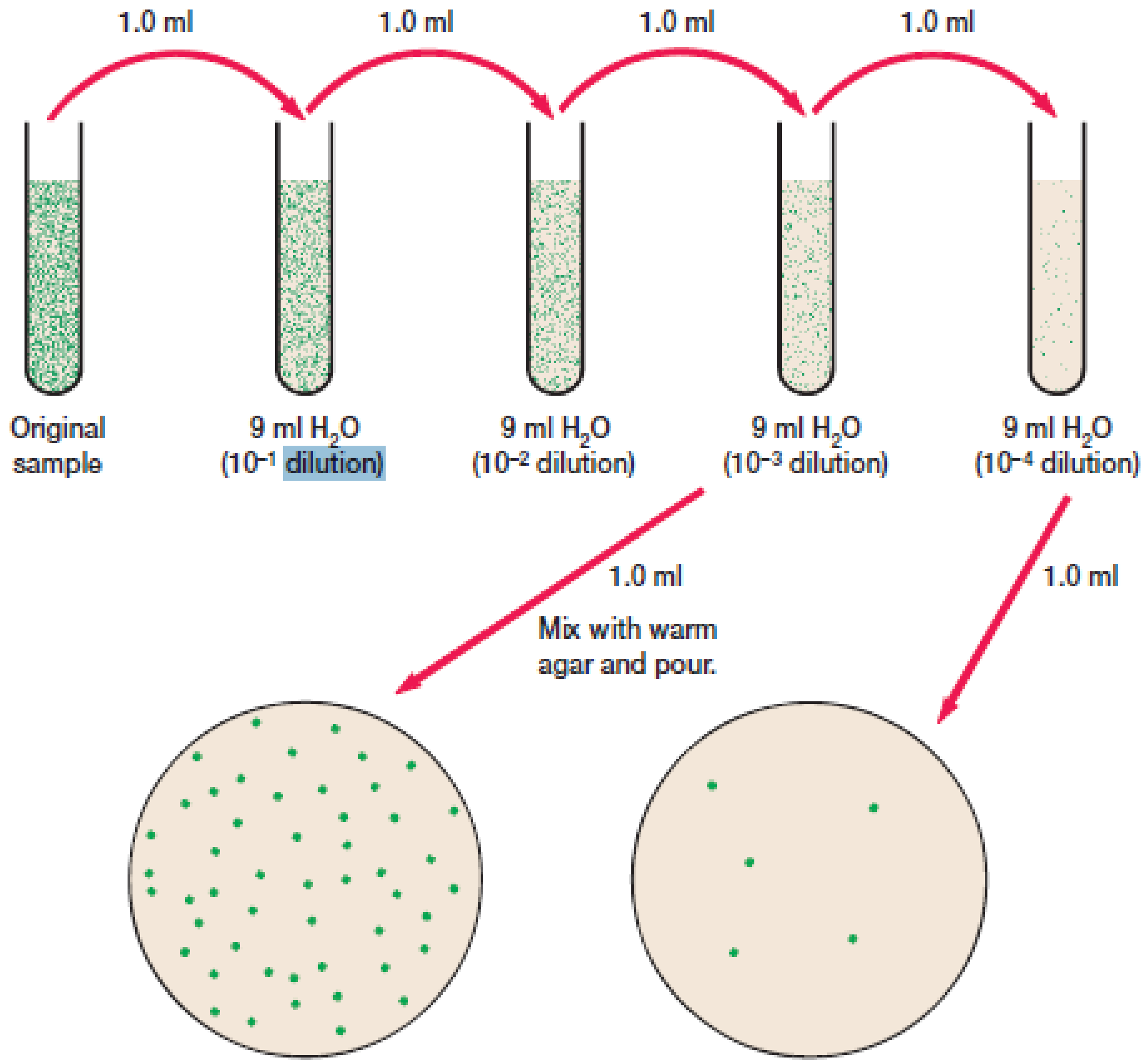

Figure 11: Technique of serial dilution and spread into petri dish

9. Incubated the sample for 25^{o}c until 72 hrs.
10. Counted the colony of the microorganism using colony counter
11. Colony forming unit is a unit used to estimate the number of viable bacteria cells in the sample was given by the following formula in equation below.

CFU= $\frac{n}{Sxd}$**…………………………………………………………..………………..Equation 1**

n= Number of colony

d= dilution blank factor

S= volume of transferred to culture plate

3.6. Procedures of physicochemical analysis for the treatment

3.6.1. BOD test procedure

BOD test was used to measure wastewater loads on the treatment sample, determine efficiency (in terms of BOD removal), and control processes. When a measurement was made of all oxygen consuming materials in a sample, the result was termed “Total Biochemical Oxygen Demand” (TBOD), or often just simply “Biochemical Oxygen Demand” (BOD). Because the test was performed over a five-day period, it was often referred to as a “Five Day BOD”, or a BOD_5.

In many biological treatments, the facility effluent contains large numbers of nitrifying organisms which was developed during the treatment process. These organisms could exert an oxygen demand as they convert nitrogenous compounds (ammonia and organic nitrogen) to more stable forms (nitrites and nitrates). At least part of this oxygen demand was normally measured in a five-day BOD.

Sometimes it was advantageous to measure just the oxygen demand exerted by organic (carbonaceous) compounds, excluding the oxygen demand exerted by the nitrogenous compounds. To accomplish this, the nitrifying organisms could have been inhibited from using oxygen by the addition of a nitrification inhibitor to the samples. The result was termed “Carbonaceous Biochemical Oxygen Demand”, or CBOD.

EQUIPMENT

BOD meter with probe for measurement of dissolved oxygen in 300 ml BOD bottles, 300 ml, Incubator, capable of maintaining 20 +/- 1°C, 250 ml graduated cylinders, 100 ml graduated cylinders, 25 ml measuring pipettes (wide-mouth), 10 ml measuring pipettes (wide-mouth), 100 ml beaker, 1000 ml beaker, 250 ml Erlenmeyer flask, Burette graduated to 0.1 Ml, Dilution water bottle of suitable volume for the number of tests to be performed, Pipette bulb, equipment for PH measurements **, Magnetic stirrer and stirring bars **,

N. B. ** Optional equipment

Procedure for BOD test

1. Completely filled two BOD bottles with sample water.

2. Into additional BOD bottles, partially filled with sample water, carefully measure out the proper volume of sample. Added sample water until the bottles were completely filled.
3. Stopper each bottle taking care to avoid trapping air bubbles inside the bottles as the bottle stoppers were inserted.
4. Filled the top of each bottle neck around the stopper with sample water.
5. Determined the initial DO content on one of each set of duplicate bottles, including the sample water blank by one of the approved methods and record data on the lab sheet.
6. Place the remaining bottles in the incubator at 20°C and incubated for five days.
7. At the end of exactly five days (+/-3 hours), test the DO content of the incubated bottles.
8. Calculate the BOD for each dilution. The most accurate BOD would have been obtained from those dilutions that had a depletion of at least 2 mg/L DO and at least 1.0 mg/L DO residual. If there was more than one dilution that meets these criteria, the BOD results should have been averaged to obtain a final BOD value.
9. The sample water blanks were used only to check the quality of the sample water.
10. If nitrification inhibition was used, the BOD test must also have been performed on a series of sample dilutions which have not been inhibited.
11. Report the results of the nitrification inhibited samples as $CBOD_5$ and uninhibited samples as BOD_5.
12. CALCULATIONS

Determined the value of the BOD5 in mg/L, used the following formula:

BOD5, mg/L = [(Initial DO - Final DO) x 300]/ml sample………………………Equation 2

3.6.2. COD test and procedures

When the chloride level exceeds 1000 mg/L the minimum accepted value for the COD was 250 mg/L. COD levels which fall below this value were highly questionable because of the high chloride correction which must be made.

Organic and oxidizable inorganic substances in an aqueous sample were oxidized by potassium dichromate solution in 50 percent (by volume) sulfuric acid solutions. The excess dichromate was titrated with standard ferrous ammonium sulfate using orthophenanthroline ferrous complex (ferroin) as an indicator.

Sample handling and preservation

The samples were collected in glass bottles. Used of plastic containers was permissible while it was known that no organic contaminants were present in the containers. Biologically active samples were tested as soon as possible. A sample containing settleable material was well mixed, preferably homogenized, to permit removal of representative aliquots. Samples was preserved with sulfuric acid to a PH < 2 and maintained at 4°C until analysis.

Standardization: Dilute 25.0 ml of standard dichromate solution to about 250 ml with distilled water. Added 20 ml concentrated sulfuric acid. Cool, and then titrate with ferrous ammonium sulfate titrant, using 10 drops of ferroin indicator. Normality = [(ml $K_2Cr_2O_7$) (0.25)] / [ml Fe $(NH_4)_2(SO_4)_2$], Mercuric sulfate: Powdered $HgSO_4$, phenanthroline ferrous sulfate (ferroin) indicator solution: Dissolve 1.48 g of 1-10-(ortho) phenanthroline monohydrate, together with 0.70 g of $FeSO_4$ $7H_2O$ in 100 ml of water. This indicator was purchased already prepared, Silver sulfate: Powdered Ag_2SO_4, Sulfuric acid (sp. gr. 1.84): Concentrated H_2SO_4.

Procedure for COD test

1. Pipetted a 50.0 ml aliquot of sample not to exceed 800 mg/L of COD into a 500 ml, flat bottom, Erlenmeyer flask. Added $HgSO_4$in the ratio of 10 mg to 1 mg chloride, based upon the mg of chloride in the sample aliquot and 5 ml of sulfuric acid. Swirl until all the mercuric sulfate had dissolved. Added 25.0 ml of 0.25N $K_2Cr_2O_7$ (6.1). Carefully added 70 ml of sulfuric acid-silver sulfate solution

2. Gently swirled until the solution was thoroughly mixed. A Glass bead was added to the reflux mixture to prevent bumping, which could be severe and dangerous.

The reflux mixture was thoroughly mixed before heat was applied. If this was not done, local heating occurs in the bottom of the flask, and the mixture might be blown out of the condenser.

3. While volatile organics were present in the sample, used an allihn condenser and added the sulfuric acid-silver sulfate solution through the condenser, while cooling the flask, reduced loss by volatilization.

4. Attached the flask to the condenser and refluxed the mixture for two hours.

5. Cooled, and washed down the interior of the condenser with 25 ml of distilled water. Disconnected the condenser and washed the flask and condenser joint with 25 ml of distilled water so that the total volume was 350 ml. Cooled to room temperature.

6. Titrated with standard ferrous ammonium sulfate using 10 drops of ferroin indicator. The color change was sharp, going from blue-green to reddish-brown and was taken as the end point.

7. Run a blank, had been used 50 ml of distilled water in place of the sample together with all reagents and subsequent treatment.

8. For COD values greater than 800 mg/L, a smaller aliquot of sample was taken; however, the volume was readjusted to 50 ml with distilled water having a chloride concentration equal to the sample.

9. Chloride correction (1): Prepared a standard curve of COD versus mg/L of chloride, using sodium chloride solutions of varying concentrations following exactly the procedure outlined. The chloride interval, as a minimum was 4000 mg/L up to 20,000 mg/L chloride. Lesser intervals of greater concentrations were run as per the requirements of the data, but at any case extrapolation had used.

3.6.3. PH value test and procedures

This test method was sets out the procedure for determining the PH (hydrogen ion concentration) of water using an electronic PH meter.

<u>Apparatus</u>

An electronic PH meter, Laboratory glassware including volumetric flasks etc., A wash bottle filled with distilled water, Buffer tablets of PH 7

Procedure for PH test

Filled the 50ml beaker in buffer solution of PH 7.0 and PH meter (Jenway 3505 PH meter) electrode was immersed in to the buffer solution. Adjusted using PH meter (Jenway 3505 PH meter) until it reached PH 7.0. The tip of the PH meter electrode was washed using distilled water and dried using cotton. The PH meter electrode was immersed in to experimental wastewater sample that was going to be tested.

3.6.4. Procedures to measure dissolved, suspended & total solids

A. Total Filterable Solids = Total Dissolved Solids (TDS)

Procedure

Filter paper was washed using distilled water. Dried the filter paper and dish oven on a tray with open lid and set for 1 hrs. at ~105 °C until the moisture was removed. Using electrical analytic beam balance weighed (A). The water sample was pipetted 50ml while stirring and three times filtered and washed. Using oven drier was transferred the filtrate to evaporating dish and dried. After allowed to cool in a desiccator for 30 minutes it was measured using electrical analytic beam balance (B). Then, calculated total dissolved solids (TDS) by using equation below.

Calculating total dissolved solids concentration:

$$\text{mg dissolved solids/L} = \frac{(A-B)X\ 1000}{ml\ sample} \quad \text{.......Equation 3}$$

Where:
A = weight of dried residue + dish, mg
B = weight of dish, mg.

B. Measurement of total suspended solids:

Procedure

Using analytical beam balance was weighted the 1.2 μm diameter dried filter paper and dish (A). Assemble filtration apparatus and 1.2μm filter paper was attached inside the apparatus. Added 100 ml experiment run test sample in to the assembled filtration apparatus and switch on the suction pump. After finished all 100ml in the sample finished switch off the suction pump and disassemble the apparatus. Dried the filter paper and dish oven on a tray with opened lid and set for 1 hrs. at ~105 °C until the moisture was removed. Using electrical analytic beam balance weighed. After allowed to cool in a desiccator for 30 minutes it was measured using electrical analytic beam balance (B). Then, calculated total suspended solids (TSS) by equation 4 below.

Calculating total suspended solids concentration:

mg dissolved solids/L=$\frac{(A-B)X\ 1000}{ml\ sample}$………………………………………………..Equation 4

Where:

A = weight of filter + dried residue, mg
B = weight of filter, mg

C. Measurement of total solids:

Procedure

Using electrical analytic beam balance was weighted the crucible (A). Added 20 ml in experiment run test sample to the crucible. On standard digital oven drier set for 1 hrs. at ~105 °C until the moisture was removed. After allowed to cool in a desiccator for 30 minutes it was measured the crucible using electrical analytic beam balance (B). Then, calculated total solids (TS) by using equation 5 below.

Calculating total solids concentration:

mg dissolved solids/L=$\frac{(A-B)X\ 1000}{ml\ sample}$………………………………………………..Equation 5

Where:
A = weight of dish + residue, mg
B = weight of dish, mg

Figure 12: Satiable wastewater debris in the gypsum

3.7. Analysis and optimization of experiment using Design Expert 11.0

Data analysis was carried out by Design Expert version 11.0 software evaluate (using general factorial) the treatment effects of the wastewater variables; Anaerobic Mixed culture bacterias fermentation time (72hrs.. and 144 hrs..) and gypsum hydraulic retention time (24 hrs. and72 hrs.). A 12 full factorial experimental design was employed and the experiment was performed with three replications. The response variable was removal percentage of BOD, COD and TS in both microbial and gypsum treatment and other responses was TSS, TDS, NH_4^+, NO_2 and PH. This design of the experiment helps us to optimize and set combination of treatment parameters. Significance of the result was set from analysis of variance (ANOVA).

Table 4: Selected values of parameters

Factors	**Lower level**	**Higher level**
Anaerobic Mixed culture *P. fluorescence* and *E. coli* bacteria fermentation time	72hrs.	144 hrs.
Gypsum hydraulic retention time as a factor	24 hrs.	72 hrs.

CHAPTER FOUR

RESULTS AND DISCUSSIONS

4.1. Characterization the raw wastewater sample

The coffee waste effluent collected from local coffee wet processing industry were assessed the physiochemical parameters using standard procedures. The result of physicochemical parameters of industrial coffee effluent was show in Table 5.

Table 5: Physiochemical characterization of wet processing coffee industry effluent in Dilla

Parameter	**Effluent (Untreated)**
BOD5 (mg/l)	320.26
COD (mg/l)	1261
TS (mg/l)	3545
PH	5.32
TSS (mg/l)	1475
TDS (mg/l)	2070
NH_4^+ (mg/l)	85
NO_2^- (mg/l)	0.229

Result in the study shows that the PH of coffee effluent was 5.32. Study showed by (Asrat et al., 2014) was PH of 3.90 - 4.36 due to organic and acetic acids from the fermentation of the sugars in the mucilage make the wastewater very acid. The standard discharge in Ethiopia by the study showed by (Tsigereda et al., 2013) as referred in (Asrat et al., 2014) showed PH value of 6-9. Nevertheless, the result in the study indicated the coffee effluent made from the coffee processing industry is slightly acidic. In the same study by (Asrat et al., 2014) under these acid conditions, higher plants and animals will hardly survive. As a result, the water was contaminated and the survivals of aquatic organisms were under questions.

Analysis of the solids particles is also an important parameter in the biological wastewater treatment process. The results of Total Solid (TS), Total Dissolved solids (TDS) and Total suspended solids (TSS) in coffee effluent were 3545 mg/l, 2070 mg/l and 1475 mg/l respectively. Similar reports showed by (Asrat et al., 2015) were TSS and TDS was 1975 + 322 mg/l and was 1801 + 245 mg/l respectively. Similar results were also reported by (A. Beyene et al., 2013) on treatment of wet processing coffee effluent industry. Though, the standard discharge in Ethiopia by the study showed by (Tsigereda et al., 2013) as referred in (Asrat et al., 2014) showed TSS was 100 mg/l and TDS was 3000 mg/l. Thus, when compared the result in the study with the standard discharge level of Ethiopia was higher TSS was recorded. Therefore, discharging the waste directly to the water causes pollution by increasing COD and BOD (Asrat et al., 2014).

COD and BOD are a measure of the strength of effluent and its pollution potential. They make up around 80% of the pollution load and are reaching 40g/l and more (Asrat et al., 2014). Luckily most of this material can be taken out of the water stream as precipitated mucilage solids and made into compost. In the study, results show that COD and BOD5 value of 1261 mg/l and 320.26 mg/l respectively. On the other this shows that the waste is high oxygen-demanding. In similar study showed COD and BOD5 were 5683± 304 mg/l and 1697 ± 391 mg/l respectively (Asrat et al., 2015). Though, the standard discharge in Ethiopia by the study showed by (Tsigereda et al., 2013) as referred in (Asrat et al., 2014) COD and BOD5 were 250mg/l and 80mg/l respectively; yet the result in this study shows much higher with the standard set value. The COD: BOD5 ratio is frequently used as an indicator of biological degradability in which ratios was showed less than 5:1 indicate (Asrat et al., 2014). Thus, the result shows that the ratio of COD: BOD5 was less than 5:1, which indicates the biological degradability of the waste. Therefore, the high COD and BOD value causing bad smells and speed up the death of aquatic life due to the quick use up of oxygen dissolved in the water and bacteria living in anaerobic conditions can also cause health problems for humans when found in drinking water.

The effluent shows of NO_2^- and NH_4^+ were 0.229 mg/l and 85 mg/l respectively. The effluent shows higher amount of nitrogen compound compared to the standard value. Yet nitrification is by no means limited to chemoautotrophs; numerous heterotrophic bacteria and fungi have the ability to oxidize a variety of nitrogenous compounds (Domenic Castignet and Thomas C. Hollocher, 1983). The high amount of nitrogen compound in the effluent might be due to anaerobic fermentation (Debadatta Das and SUSMITA MISHRA, 2010). For instant, *E. coli* can reduce nitrate only to nitrite from ammonium, P. fluorescence reduces it completely into molecular nitrogen, (Prescott, 2002). Therefore, bacteria able to reduce nitrate from ammonium can be used in their identification and isolation. Therefore, the effluent physicochemical characteristics have high degree of contaminants in the water generally induces unfavorable reactions to plants when it is released.

Thus, the physicochemical analysis of a typical coffee industry wastewater indicated that it contained other major contaminants, including sulphates and heavy metal ions and organic pollutants like phenol. Similar pollutants were reported by researchers [(Asrat et al., 2015), (A. Beyene et al., 2013)].

4.2. The effect of treatment by anaerobic mixed culture *Pseudomonas fluorescence* and *Escherichia coli*

Anaerobic treatment of coffee wastewater using mixed culture of *P. fluorescence* and *E. coli* response was showed in Table 6. Total Solid (TS) response starting initial value 3545 mg/l was reduced from lower level of fermentation time 72 hr. to higher level of fermentation time 144 hr. was 2844 mg/l, 3298 mg/l, and 3135 mg/l with three repetition of experimental value to 2347 mg/l, 2373 mg/l and 2428 mg/l respectively. Similarly, NH_4^+ response the starting initial value of 85 mg/l was reduced from lower level of fermentation time 72 hr.to higher level of fermentation time of 144 hr.

Table 6: Physiochemical characterization of the wastewater by the microbial treatment

Level of treatment		BOD5 (mg/l)		COD (mg/l)		TS (mg/l)		PH		NH_4^+ (mg/l)	
		Δ BOD5	Final BOD5	ΔCOD	Final COD	ΔTS	Final TS	ΔPH	Final PH	ΔNH_4^+	Final NH_4^+
Higher Level	R1	262.348	57.912	1115	146	1198	2347	1.33	7.65	61	24
	R2	261.04	59.22	1104	157	1172	2373	1.4	7.72	55	30
	R3	261.89	58.37	1109	152	1117	2428	1.3	7.62	68	17
Lower Level	R4	204.14	116.12	824	437	701	2844	1.5	7.82	18	67
	R5	202.91	117.35	828	433	247	3298	2.25	8.57	13	72
	R6	205.04	115.22	864	397	410	3135	1.92	8.24	19	66

In this study the microbial treatment of the BOD5 response from initial value of 320.26 mg/l was reduced at fermentation time of 144hrs. from mg/l to 57.91 mg/l, 59.22 mg/l and 58.37 mg/l value with three repetitions of experimental run respectively. Whereas, at fermentation time of 72 hrs. with the same experimental run on BOD5 response showed 116.12 mg/l, 117.35 mg/l and

115.22 mg/l respectively. This treatment had shown reduction in the BOD5 value and when fermentation time increases from lower level (72hrs.) to higher level (144 hrs.).

Besides, when COD: BOD5 ratio is less than 5:1 it indicates the biological degradability of the waste (Asrat et al., 2015). The wastewater was biodegradable substance it can degrade by microorganisms (Table 6). This is because of wastewater contains pulp and mucilage substances such as proteins, sugars and pectin's (Jan C. von Enden and Ken C. Calvert, 2010). Thus, *Pseudomonas florecence* and *Escherchia coli* bacteria are responsible for degerading polysaccharide carbohydrates molecules present in wet processing coffee wastewater in to more stable compound/ molecules. Therefore, these microbes would reduced organic load in the wet processing coffee wastewater hence the BOD5 the effluent would reduced.

Resistant organic materials broken down by Chemical Oxygen Demand (COD) make up 80% of pollution load (Jan C. von Enden and Ken C. Calvert, 2010). The run of value of COD in experiment showed 146 mg/l, 157 mg/l and 152 mg/l at higher level of fermentations time (144 hrs.). Whereas, the fermentation time of 72 hrs. with the same experimental run on COD response was showed 437 mg/l, 433 mg/l and 397 mg/l. Thus, reduction in the COD value was achieved while increased fermentation time from lower level (72hrs.) to higher level (144 hrs.).

The material making up the high COD can be taken out of the water as precipitated mucilage solids. This is achieved during a sufficient time of acidification of sugars present in the wastewater during which solids get out of solution. After full acidification, the clear, acid wastewater is treated by natural limestone to lift the PH to change from around PH 4 to a PH to around 6 (Jules B. Van Lier et al., 2008). Only at this PH levels, UASB digestion and constructed wetland would achieve optimal result (Jan C. von Enden and Ken C. Calvert, 2010). Acidification in acidogenesis process would have been achieved by anaerobic fermentation process so that it had reduced COD load.

The result showed that the effect of PH by microbial treatment at different fermentation hours were changed the PH value (referred and shown in Table 6 above). For example the effect of PH by microbial treatment at fermentation time of 144hrs. were showed that the initial PH value of 5.32 were increased to 7.65, 7.72 and 7.62 but, at fermentation time of 72hrs. the initial PH value were increased to 7.82, 8.57 and 8.24 with three repetition of experiment. These results showed the PH increase to slightly acidic to basic PH when fermentation time increases from lower level (72hrs.) to higher level (144 hrs.). The reason behind is anaerobic fermentation process after acidogenesis to acetogenesis produces Volatile Fatty Acids (VFAs) and H_2; in which both produce NH_3 leads to PH increase (Jules B. Van Lier et al., 2008). Similar findings by (Jules B. Van Lier et al., 2008), subsequently, once increased PH by production of NH_3 there is no net production of proton, which leads no changes in PH value further. Therefore, anaerobic treatment of coffee wastewater using mixed culture of *P. fluorescence* and *E. coli* were changed in to slightly acidic PH in to slightly basic PH.

4.3. The effect treatment by gypsum after anaerobic treatment

As it showed in Table 6, anaerobic treated coffee waste was slightly basic (PH = 7.62 to 8.57), therefore, discharging the waste directly to the water causes pollution. However, gypsum ($CaSO_4{\cdot}2H_2O$) treatment on the wastewater showed the PH value was reduce to slightly basic PH to neutral PH, which ranges between 7.69 to 7.43. These finding concurs with (Maria C Vazquez-Almazan, 2012) who reported gypsum ($CaSO_4{\cdot}2H_2O$) treated wastewater by neutral PH which benefit for reuse and water showed fluctuations between PH value 6.96 and 7.51.

Table 7: The characteristics of wet coffee waste by gypsum treatment after anaerobic treatment

Level of treatment		BOD5 (mg/l)		COD (mg/l)		TS (mg/l)		PH		NH_4^+ (mg/l)	
		Δ BOD5	Final BOD5	ΔCOD	Final COD	ΔTS	Final TS	ΔPH	Final PH	ΔNH_4^+	Final NH_4^+
Higher Level	R1	22.252	35.66	44	102	2272	75	-0.18	7.47	14.5	9.5
	R2	16.8	42.42	60	97	2304	69	-0.03	7.69	19.68	10.32
	R3	18.65	39.72	49	103	2360	68	-0.16	7.46	8.5	8.5
Lower Level	R4	13.748	102.372	99	338	2217	627	-0.34	7.48	0.8	66.2
	R5	22.162	95.188	93	340	2658	640	-0.95	7.62	5	67
	R6	17.92	97.3	62	335	2500	635	-0.81	7.43	1.75	64.25

Gypsum was agglomerate and flocculates the solid particles in the wet processing coffee wastewater effluent in to settable solids. The turbidity reported for the same doses was 74.05, 80.5 and 74.5 NTU, respectively (Maria C Vazquez-Almazan, 2012). Slowly increasing from lower level to higher level in gypsum retention time would had higher increasing effet on the TS response.

Gypsum would reduce BOD5, COD, TS and NH_4^+ by reducing solid sludge unacceptable to microorganisms by forming coagulation and forming tougher solid settlable at the bottom of the measuring cylinder. More effective treatment showed on TS than other responses. On the study (Zhao, 2006) using gypsum treatment, an organic polymer is often used as a chemical conditioner to improve sludge filterability by flocculating small gel-like sludge particles into large aggregates with less affinity for water. Theoretically, the flocs are porous, permeable to water flow and incompressible, so that the pores will not be blocked under pressure during filtration and dewatering (Zhao, 2006). Once the sum of suspended solid and dissolved solid are removed in the form of solid sludge the microbial load would decrease since the biodegradable substance is present in wet processing coffee wastewater effluent would reduce; thus TS inter decreased.

4.4. Combined effect of treatment by anaerobic mixed culture *Pseudomonas fluorescence* and *Escherichia coli* bacteria and gypsum

The treatment effect of anaerobic mixed culture *Pseudomonas fluorescence* and *Escherichia coli* bacteria with gypsum had able to treat the coffee processing effluent. The effect of the treatment by the responses BOD5, COD, TS, NO_2^- and NH_4^+ had reduced from the initial raw wastewater characteristics and neutralized the PH (Table 8).

Table 8: Physicochemical characteristics of effluent after microbial and gypsum treatment

Std	RuN	Factor 1 A:MFT	Factor 2 B:GRT	Response 1 BOD 5 mg/l	Response 2 COD mg/l	Response 3 PH	Response 4 TS mg/l	Response 5 TDS mg/l	Response 6 TSS mg/l	Response 7 NO_2^- mg/l	Response 8 NH_4^+ mg/l
Raw sample				**320.26**	**1261**	**6.32**	**3545**	**2070**	**1475**	**0.229**	**85**
6	1	1	-1	49.32	133	7	215	135	80	0.037	22.5
2	2	-1	-1	102.37	338	7.48	627	523	104	0.06	67
10	3	1	1	35.66	102	7.47	75	54	21	0.023	9.5
4	4	1	-1	48.37	125	7.66	207	132	75	0.035	20.66
12	5	1	1	42.42	97	7.69	69	46	23	0.025	10.32
1	6	-1	-1	95.188	340	7.62	640	490	150	0.067	66.2
5	7	1	-1	49.35	126	7.39	209	116	93	0.038	21
3	8	-1	-1	97.3	335	7.43	635	515	120	0.061	64.25
9	9	-1	1	60.69	215	7.42	90	54	36	0.046	24.5
8	10	-1	1	66.25	208	7.47	95	55	40	0.048	25
7	11	-1	1	64.52	210	7.5	87	44	43	0.043	26.5
11	12	1	1	39.72	103	7.46	68	35	33	0.021	8.5

Note: Factor A = MFT (Microbial Fermentation Time)

Factor B = GRT (Gypsum Retention Time)

As it was indicated in (Table 8) both gypsum and anaerobic mixed culture fermentation time of *P. florecence and E. coli* bacteria treatment on wet processing coffee wastewater effluent had reduced BOD5 response. Yet, when campared reduction rate BOD5 response showed microbial treatement were more effective than gypsum treatment (in Table 6 and 7). In the meantime, *P. florescence* and *E. coli* bacteria in mixed culture had consumed on biodegradable substance and gypsum would unviable the solid particles in the wet coffee processing wastewater effluent. Consequently, when COD: BOD5 ratio is less than 5:1 the wastewater is more of biodegradable (Asrat et al., 2014). Likewise, the wastewater by this study was in general organic matter that shows the COD: BOD5 ratio was less than 5:1; in which, microorganisms like *P. florescence and E. coli* can be able to decompose in the wastewater. Far ahead, BOD is a measure of the rate at which micro-organisms use dissolved oxygen in the bacterial breakdown of organic matter (food) under aerobic conditions (Gerry Carty G. O., 1997). In the same book by (Gerry Carty G. O., 1997) under anaerobic conditions, readily biodegradable organic matter (i.e. BOD) becomes fermented to short chain fatty acids (SCFA). Thus, microbial decomposition of organic wastewater had reduced BOD5 by these microorganisms.

Gypsum would reduced BOD5 by sinking solid sludge in which creates unacceble to micrrorganism by coagulation and forming more tough solid settlable at the bottom of the measuring cylinder. This is articulated in the study by (Zhao, 2006), gypsum the flocs are porous, permeable to water flow and incompressible, so that the pores will not be blocked under pressure during filtration and dewatering. Thus, once the some of suspnded solid and dissolved solid are removed in the form of solid sludge the microbial load would decrease since the biodigradable substance was present in wet processing coffee wastewater effluent would reduced; thus BOD5 inter decreased.

Anaerobic mixed culture *Pseudomonas florescence and Escherichia Coli* bacteria fermentation time had reduced (Total Solid) TS with smaller that the change effect of gypsum treatment. Yet, anaerobic bacteria in the wastewater treatment forms conglomerates and become matured due to time forms a round shape granular sludge (Jules B. Van Lier et al., 2008). In the same study by (Jules B. Van Lier et al., 2008) the treatment removes the sludge by a mechanism of sedimentation, flocculation and entrapment in a sludge blanket. Microorganisms especially bacteria are the most populous of the microorganisms used in wastewater treatment; these single-celled organisms directly break down the polluting matter in wastewaters (Gerry Carty G. O., 1997). *P. florescence and E. coli* bacteria were using biodegradable material as their food.

Effect of Gypsum on removal of TS was bigger than the effect of mixed culture *Pseudomonas florescence* and *E. coli* bacteria when we see in different studies. Gypsum in the study by (Maria C Vazquez-Almazan, 2012) showed with respect to turbidity removal were high reduction due to coagulation and flocculation mechanism of sludge present in the effluent. Whereas, the reduction NO_2^- and NH_4^+ where also showed the treatment had reduced. Gypsum by itself would be able to coagulate and flocculate the solid particles found in the waste. Thus, in different techniques from microbial treatment above seen gypsum addition to the wastewater develops solid slug at the bottom of the measuring cylinder (Jules B. Van Lier et al., 2008).

On the other hand, *E. coli* can reduce nitrate only to nitrite from ammonium, *P. fluorescence* reduces it completely into molecular nitrogen, (Prescott, 2002). Different studies on the treatment of waste predict that aerobic soils are primary sources of NO and that N_20 is produced only when there is sufficient soil moisture to provide the anaerobic microsites necessary for denitrification by either denitrifiers or nitrifiers (Iris Cofman Anderson and Joel S. Levine, 1986). However, the mechanism of removal of ammonia from wet processing of coffee wastewater was different but the result both treatments help to reduce ammonia from effluent.

Result after the treatment on PH response showed that neutral ranging from PH value 7-7.69. This is because anaerobic fermentation process after acidogenesis to acetogenesis produces Volatile Fatty Acids (VFAs) and H_2; which both produce NH_3 leads to PH increase (Jules B. Van Lier et al., 2008). In the same study by (Jules B. Van Lier et al., 2008), subsequently, once increased PH by production of NH_3 there is no net production of proton, which leads no changes in PH value further. Hence, anaerobic treatment of coffee wastewater using mixed culture of *P. fluorescence* and *E. coli* bacteria leads to slightly basic PH of the effluent. Conversely, gypsum ($CaSO_4 \cdot 2H_2O$) treatment on the wastewater showed reduced the slightly basic PH to more neutral PH. These can be seen on the study by (Maria C Vazquez-Almazan, 2012), gypsum ($CaSO_4 \cdot 2H_2O$) resulting neutral PH of the treated water is an advantage for reuse and water showed fluctuations between PH value 6.96 and 7.51. Thus, the combined treatment of gypsum and microbial on the effluent in the coffee processing industry is effective to neutralization of PH value.

4.5. Microbial analysis

A. Characterization of microorganism

The result showed that both microorganisms in petri dish have different colony colors. For example the color of *P. fluorescence* was white and *E. coli* was brown color. When it was observed under microscope both colonies are dipoles. As it was showed in table 6 the results of cells of *P. fluorescence* and *E. coli* were grown in nutrient agar medium mark in MacConkey different lengths of time from which inoculums were collected and tested for growth microorganism.

B. Colony count result

The microbial colony counts in nutrient agar up to 3 days there are no colonies but in the third day both *P. fluorescence* and *E. coli* bacteria colony was grown (Table 9).

Table 9: Colony count result for *P. florescence* and *E. coli*

Microbes	Time of in days	Dilution			Average CFU(CFU/ml)
		10^{-6}	10^{-9}	10^{-12}	
		Nutrient agar	Nutrient agar	Nutrient agar	
Pseudomonas florescence	1-2 days	0	0	0	
	3 days	100	48	17	
	CFU(CFU/ml)	1x108	3.8x1010		$1.904X10^{10}$
***Escherichia coli* bacteria**	1-2 days	0	0	0	
	3 days	123	52	23	
	CFU(CFU/ml)	1.23x108	5.2x1010	-	$3.215X10^{10}$

This shows when the dilution increases the number of colonies decreases from higher values to lower values. *P. florescence* bacteria can grow in nutrient media at slower rate than *E. coli* bacteria.

4.6. Statistical analysis

4.6.1. BOD5 response on the treatments the wastewater

Table 10: Analysis of variance showing BOD5 response

Source	Sum of Squares	Df	Mean Square	F-value	p-value
Model	6012.21	3	2004.07	238.45	< 0.0001
A-A	4087.78	1	4087.78	486.39	< 0.0001
B-B	1466.11	1	1466.11	174.45	< 0.0001
AB	458.31	1	458.31	54.53	< 0.0001
Pure Error	67.24	8	8.40		
Cor Total	6079.44	11			

The "Lack of Fit F-value" of 0.76 implies the Lack of Fit is not significant relative to the pure error. There is a 54.55% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good we want the model to fit.

The normal probability plot in the experiment the experimental runs were done in the randomized and to reduce other effects it is repeated. Thus the influence/ leverages of all the runs in the design were identical. As we can see from the report in design expert 11 the standard errors of the residuals are different. Thus the normal probability plot the response follows a normal distribution follows straight line. That means they are following not exactly on the straight line but it is near to the straight line.

Design Expert analyzed data on BOD5 response shows the following result:

Table 11: Fit Statistics for BOD5 response

Std. Dev.	2.90	**R^2**	0.9889
Mean	62.60	**Adjusted R^2**	0.9848
C.V. %	4.63	**Predicted R^2**	0.9751
		Adeq Precision	35.2619

The predicted R- squared of 97.51% is reasonable agreement with adjusted R- squared of 98.48%; i.e. the difference was less than 0.2%. When we see the adequacy precision it was 35.262 which is greater than 4, then the model had strong enough signal to be used optimization.

Residual Vs Predicted: It tests constant variance and the plot shows constant range of residuals a cross the graph which also intern random scatter. If it was out of the range values, the experiments should be needed to be repeated again but in this case the BOD5 response was within the constant range.

Residuals Vs Run: Experimental run order on the independent variable and residual on the dependent variable shows that the lurking (in line with) variables. That may have influenced the response (BOD5) during the experimental values. Response (BOD5) in the experimental runs was different in their values.

Predicted Vs Actual: The predicted value and the actual value are in straight line or near to each other. This means that the experiment can be modeled. The increase in the fermentation time mixed culture would have had more BOD5 reduction effect than factor B (gypsum treatment by retention time) in wet processing coffee wastewater effleunt teatment effect. Slowly increasing from lower level to higher level in MO treatment would had higher increasing effet on the BOD5 response than increasing from lower level to higher level in gypsum treatment by using retention time as factor treatment and vise versa.

MODEL GRAPH

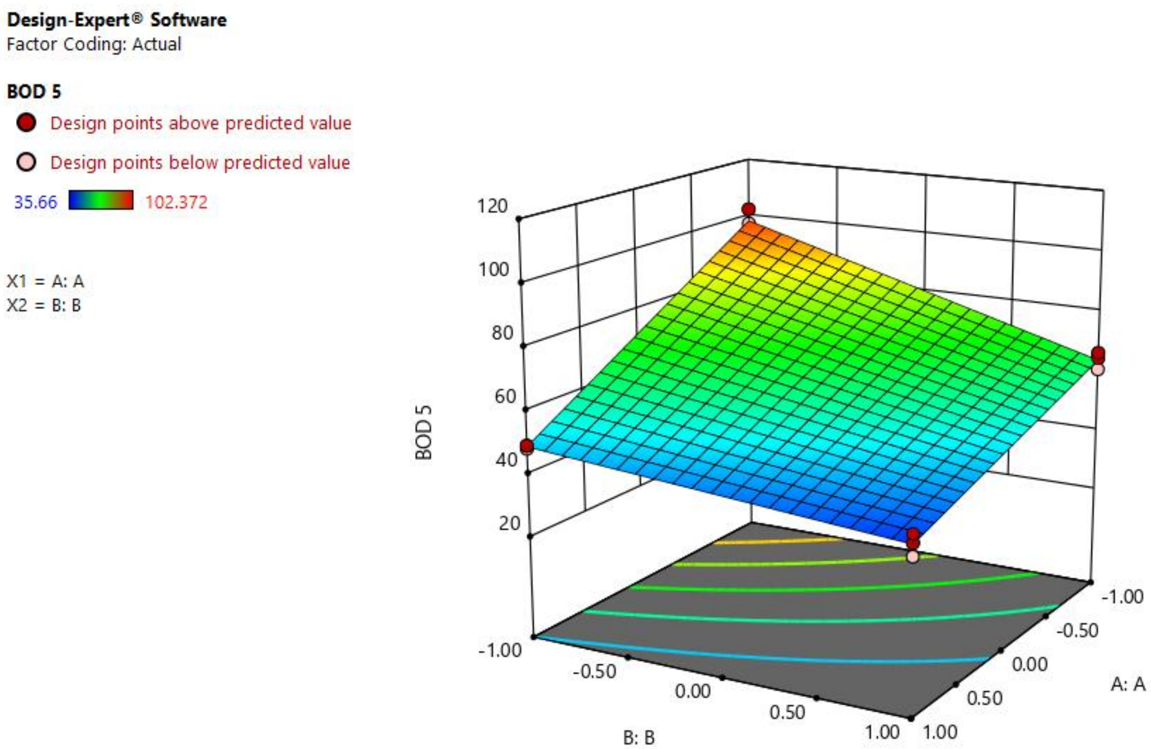

Figure 13: Model graph for BOD5 response by the fermentation time and retention time factors

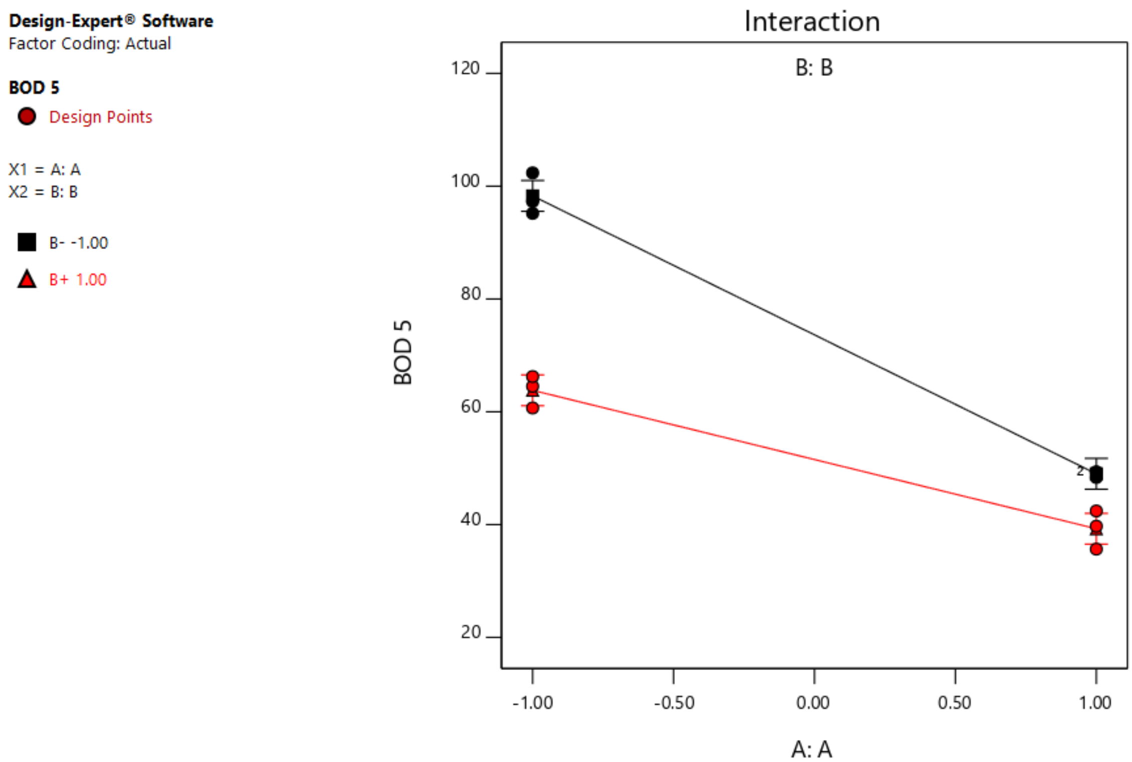

Figure 14: Interaction effect for the treatment mixed culture *P. fluorescence* and *E. coli* and gypsum on BOD5

Model equation for BOD5 as two model equation the coded model equation and actual model

<u>Coded model equation</u>

BOD5= 62.6 – 18.46*A – 11.05*B+ 6.18*A*B……………………………………..Equation 6

<u>Actual model equation</u>

BOD5= 62.59667 – 18.45667*A – 11.0533*B+ 6.1800*A*B……….……………..Equation 7

We can see that the BOD5 had more effect *Pseudomonas florescence* and *E. coli* mixed fermentation time than gypsum retention time. This is because when we can see the coefficient of factor A (*P. florescence and E. coli* mixed fermentation time) was 18.46 and that of coefficient factor B (gypsum retention time) was 11.05 which is 18.46 is greater than 11.05. so the effect of *P. florescence* and *E. coli* mixed fermentation time on the wet processing of coffee wastewater treatment in BOD5 response had more significance that of gypsum retention time.

4.6.2. COD **response on the treatments of the wastewater**

Table 12: ANOVA result on **COD** response on the wastewater treatment

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	1.020E+05	3	33995.78	2794.17	< 0.0001
A-MFT	76800.00	1	76800.00	6312.33	< 0.0001
B-GRT	17787.00	1	17787.00	1461.95	< 0.0001
AB	7400.33	1	7400.33	608.25	< 0.0001
Pure Error	97.33	8	12.17		
Cor Total	1.021E+05	11			

The **Model F-value** of 2794.17 implies the model was significant. There was only a 0.01% chance that an F-value this large could occur due to noise. **P-values** less than 0.0500 indicate model terms are significant. In this case A, B and AB were significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there were many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

Table 13: Fit Statistics of response COD in the treatment of the wastewater

Std. Dev.	3.49	**R^2**	0.9990
Mean	194.33	**Adjusted R^2**	0.9987
C.V. %	1.79	**Predicted R^2**	0.9979
		Adeq Precision	117.6856

The **Predicted R^2** of 99.79% was in reasonable agreement with the **Adjusted R^2** of 99.87%; i.e. the difference was less than 0.2%. **Adeq. Precision** measures the signal to noise ratio. A ratio greater than 4 was desirable. Your ratio of 117.686 indicates an adequate signal. This model can be used to navigate the design space.

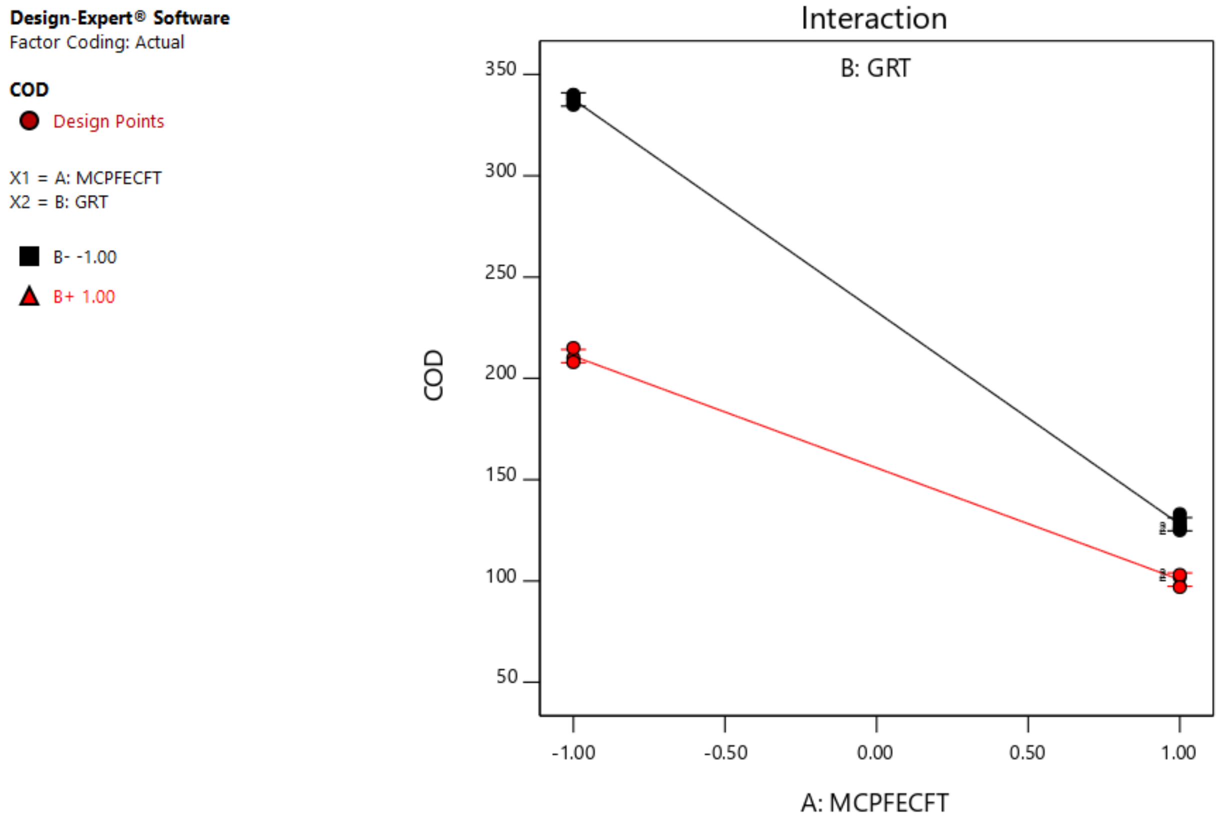

Figure 15: Interaction effect factors by microbial fermentation time and gypsum retention time as on COD as a response

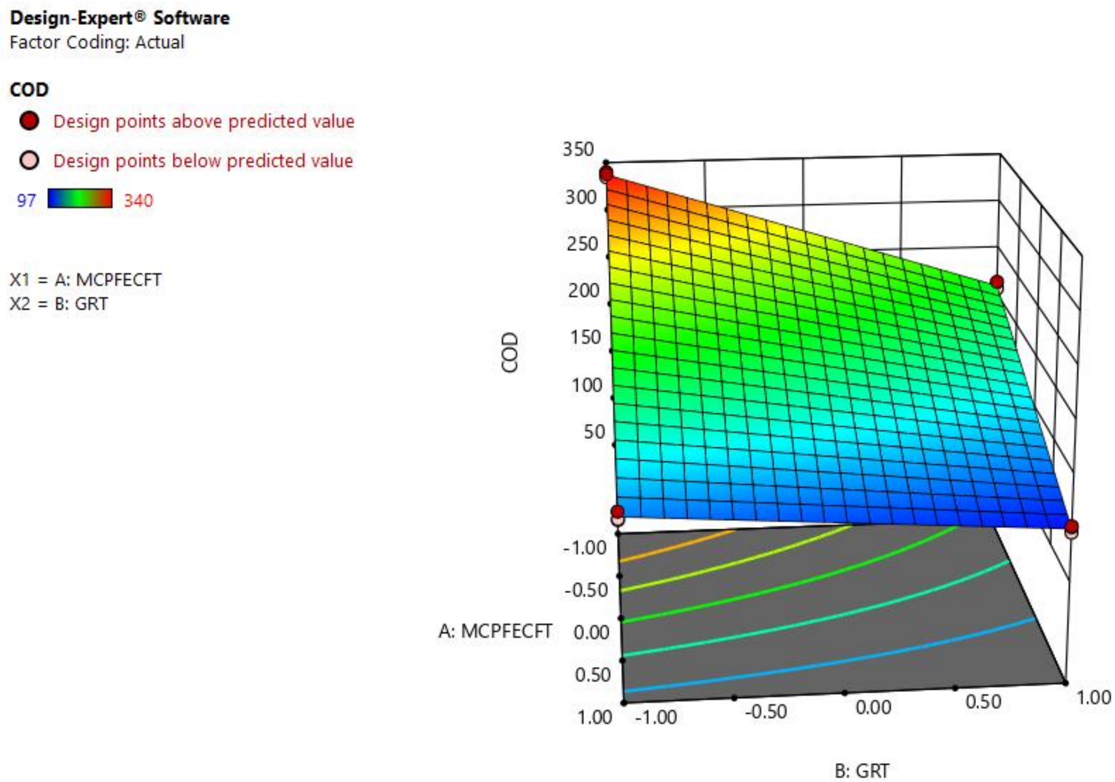

Figure 16: COD response by the fermentation time and retention time factors

Final equation in terms of coded factors

COD = 194.33 - 80.00*A -38.50*B + 24.83*A*B……………………………….Equation (8)

The equation in terms of coded factors can be used to make predictions about the response for given levels of each factor. By default, the high levels of the factors are coded as +1 and the low levels are coded as -1. The coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients.

Final equation in terms of actual factors

COD= 194.3333- 80*A - 38.50*B + 24.8333*A*B…………………………..Equation (9)

The equation in terms of actual factors can be used to make predictions about the response for given levels of each factor. Here, the levels should be specified in the original units for each factor. This equation should not be used to determine the relative impact of each factor because the coefficients are scaled to accommodate the units of each factor and the intercept is not at the center of the design space.

4.6.3. TS response on the treatments the wastewater

Table 14: ANOVA for Total solid response

Source	Sum of Squares	Df	Mean Square	F-value	p-value
Model	6.197E+05	3	2.066E+05	9079.95	< 0.0001
A-A	1.476E+05	1	1.476E+05	6489.23	< 0.0001
B-B	3.499E+05	1	3.499E+05	15378.76	< 0.0001
AB	1.222E+05	1	1.222E+05	5371.87	< 0.0001
Pure Error	182.00	8	22.75		
Cor Total	6.199E+05	11			

The Model F-value of 9079.95 implies the model was significant. There was only a 0.01% chance that an F-value this large could occur due to noise. P-values less than 0.0500 indicate model terms are significant. In this case A, B and AB were significant model terms. P-values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

Table 15: Fit Statistics for total solid response

Std. Dev.	4.77	**R^2**	0.9997
Mean	251.42	**Adjusted R^2**	0.9996
C.V. %	1.90	**Predicted R^2**	0.9993
		Adeq Precision	204.5669

The predicted R- squared of 99.93% was reasonable agreement with adjusted R- squared of 99.96%; i.e. the difference was less than 0.2 %. When we see the lack of fit was not significant that means the replicate in the experiment data was not exactly the same but it had little bit difference on TS response although the parameter was the same.

The adjusted R-squared and predicted R- squared 99.93% was near 100% that was theoretically 0.9% acceptable. If the experiment less than 0.9% the experiment should be repeated since the result was greater than 0.9% it would have not been repeated. When we subtract the predicted R-squared from adjusted R- squared the difference was 0.0003% which was less than 0.2 %. Thus the model was fitting the data and can reliably be used to interpolate.

When we see the adequacy precision it was 204.567 which was greater than 4, then the model had strong enough signal to be used optimization. When we see the report from design expert 11.1 the repeat experiment with the value of actual and predicted has large margin.

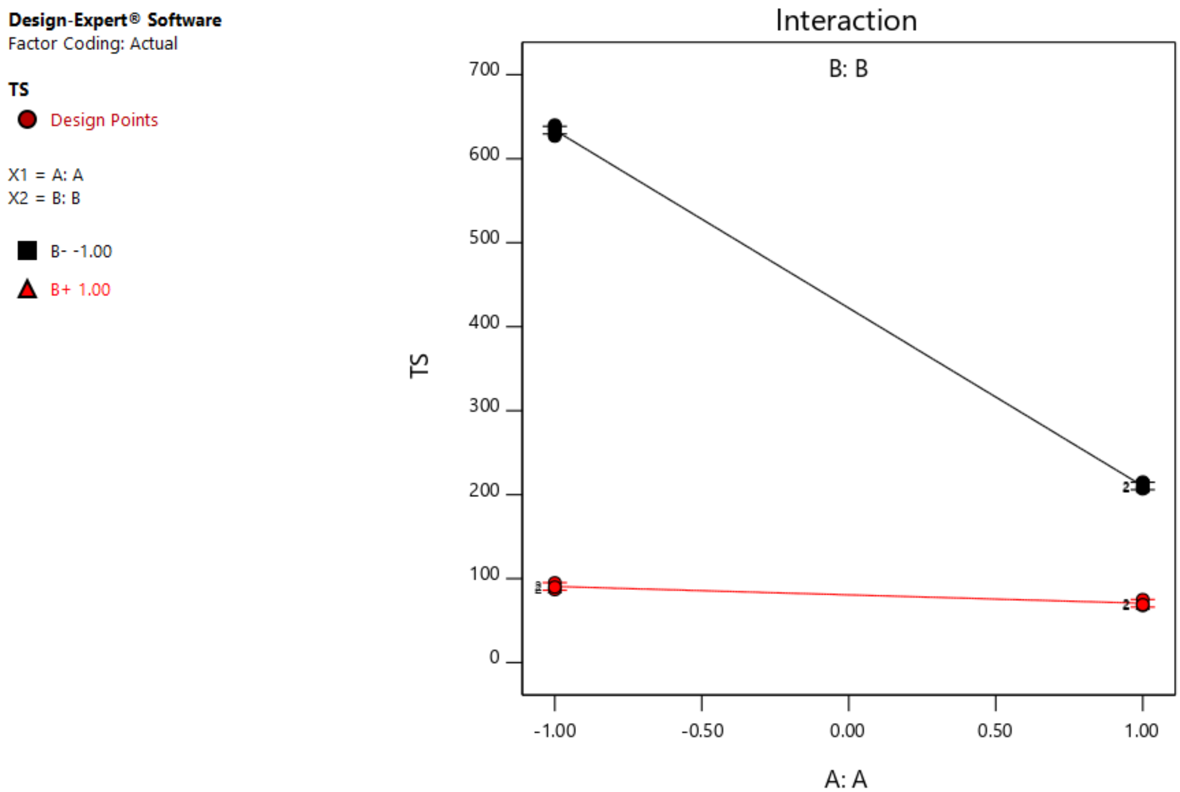

Figure 17: Interaction effect of the treatment using *Pseudomonas florescence* and *Escherichia coli* and gypsum on TS

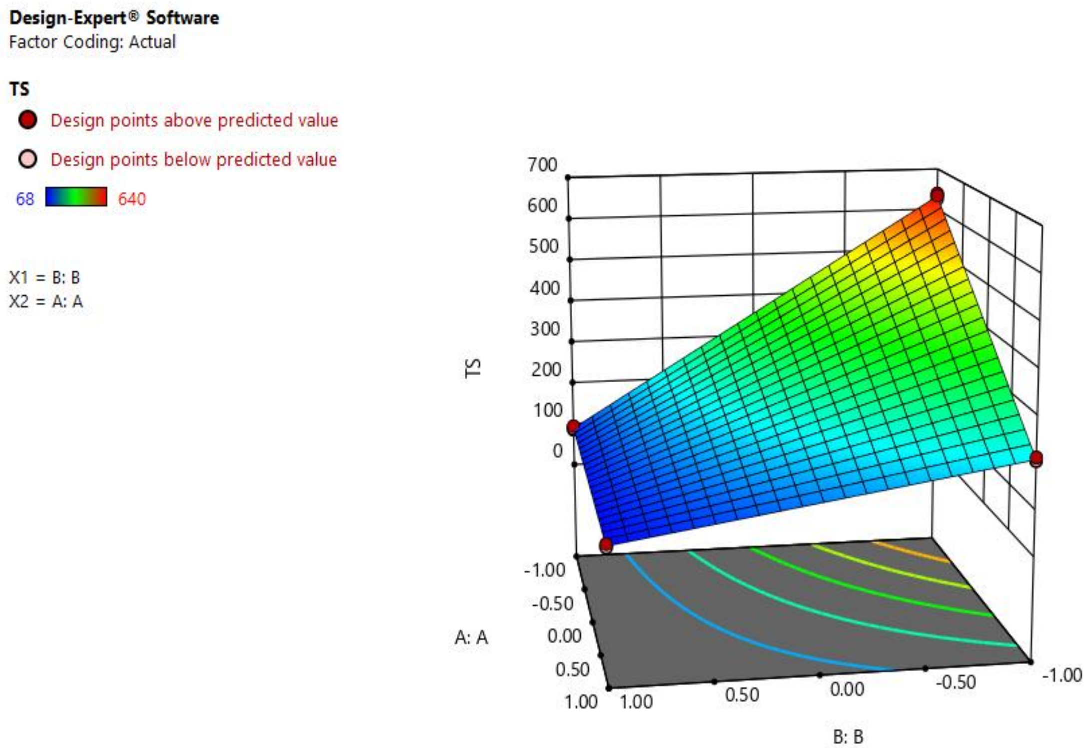

Figure 18: TS response by the fermentation time and retention time factors

Model equation for TS as two model equation the coded model equation and actual model

Final Equation in Terms of Coded Factors

TS= 251.42 – 110.92*A – 170.75*B+ 100.92*A*B………………………………..Equation 10

Final Equation in Terms of Actual Factors (TS)

TS= 251.41667 – 110.91667*A – 170.7500*B+ 100.91667*A*B…………………Equation 11

The equation in terms of actual factors can be used to make predictions about the response for given levels of each factor. Here, the levels should be specified in the original units for each factor. This equation should not be used to determine the relative impact of each factor because the coefficients are scaled to accommodate the units of each factor and the intercept is not at the center of the design space. The result of TS response had more effect by gypsum retention time than *Pseudomonas florescence* and *Escherichia coli* mixed fermentation time. Consequently, the coefficient of factor B (gypsum retention time) was 170.75 and that of coefficient factor A (*Pseudomonas florescence* and *E. coli* mixed fermentation time) was 110.92; i.e. gypsum treatment is greater effect than microbial treatment on treatment of coffee industry effluent.

4.7. Optimization of parameters for the effluent treatment

In order to develop a treatment on the effluent, a number of factors influencing the treatment like BOD5, COD, TS, PH, NH_4^+ and NO_2^- should be considered, but the important factors first three were studied. Yet, the studying of the each and every factor is quite tedious and time consuming. Thus, a factorial design could minimize the above difficulties by optimizing all the affecting parameters collectively at a time. Factorial design was employed to reduce the total number of experiments in order to achieve the best overall optimization of the process. The design determines the effect of each factor on response as well as how the effect of each factor varies with the change in level of the other factors. Interaction effects of different factors could be attained using design of experiments only. Factorial design comprises the greater precision in estimating the overall main factor effects and interactions of different factors. The optimization of treatment parameters for BOD5, COD and TS were summarized as follows (in Table 16 and Table 17).

Table 16: Goal of Parameters for the optimal removal efficiency of the treatment

Parameters	Goals	Minimum mg/l	Maximum mg/l	Optimized value(mg/l)	Initial value mg/l	Removal efficiency (%)
BOD5	Minimize	35.66	102.372	39.267	320.26	87.74
COD	Minimize	97	340	100.667	1261	92.02
TS	Minimize	68	640	70.667	3545	98.01

Table 17: Optimal possible solutions of factorial design

No.	Factor A = MTFT	Factor B = GRT	BOD5	COD	TS	Desirability	Selection
1.	**1.000**	**1.000**	**39.267**	**100.667**	**70.667**	**0.973**	**Selected**
2.	0.983	1.000	39.480	101.627	70.841	0.971	
3.	1.000	0.961	39.455	101.194	73.357	0.968	
4.	0.951	1.000	39.872	103.387	71.161	0.967	
5.	1.000	0.933	39.595	101.588	75.374	0.965	
6.	0.937	1.000	40.046	104.167	71.302	0.965	
7.	0.925	1.000	40.186	104.800	71.416	0.963	
8.	1.000	0.863	39.932	102.533	80.200	0.957	

Note: MTFT= Microbial fermentation time and GRT= Gypsum retention time

Based on the results the optimum possible solutions in treatment of coffee industry effluent were presented in Table 16 and Table 17 above. The predicted optimum BOD5 removal efficiency was 87.74%; which from raw wastewater of 320.26 mg/l amount to 39.267 mg /l was observed at possible factors parameters of 144 hrs. and 72 hrs., microbial fermentation time and gypsum retention time respectively. Under these conditions the COD removal efficiency was 92.02% and TS removal efficiency was 98.01% which was close to the general factorial design optimal result of 97.3 %, and this shows that the experimental values were found to be close to the predicted values and hence the model validation of the optimal solutions.

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1. Conclusions

The coffee wastewater effluent characterized had BOD5 value of 320.26mg/l, COD value of 1261mg/l and TS value of 3545mg/l and PH value of 5.32. These wastes had serious side effect on the surrounding environment. The effluent had many compounds and microorganism could be able to decompose organic matter inside the wastewater. Moreover, physicochemical characteristics the effluent like COD: BOD5 ratio is determinant factors to select among the treatment method; it had COD: BOD5 ratio of less than 5 which indicates more of organic matter. Thus biological treatment method could be able to treat coffee wastewater. Nevertheless, if it is not treated properly, the organic matter in the wastewater consumed by microbes the oxygen in water; resulting in the death of plants and animals due to the lack of oxygen or the increased acidity.

The treatment effect of anaerobic mixed culture of *P. fluorescence* and *E. coli* reduced BOD5, COD and TS while increasing fermentation time from 72 hr. to 144 hr. The method in the treatment had reduced BOD5 response from initial value of 320.26mg/l up to 57.91mg/l. Similarly, COD response was reduced up to 397mg/l from initial value of 1261mg/l; additionally, Total Solid (TS) response starting from initial value 3545mg/l was reduced up to 2373mg/l by the treatment. Therefore, the microbial treatment on the effluent in the coffee processing industry is effective to for reduction of value BOD5, COD and TS.

On the other hands, gypsum treatment reduced from the value of BOD5, COD and TS. Addition of gypsum powder after anaerobic treatment had reduced BOD5, COD and TS value further up to 35.66mg/l, 97mg/l and 69mg/l respectively. However, when campared reduction rate of COD and BOD5 unlike TS response showed microbial treatement were more effective than gypsum treatment. Therefore, gypsum had able to reduce BOD5, COD and TS by reducing solid sludge unacceptable to microorganisms by forming coagulation and forming tougher solid settle at the bottom of the measuring cylinder.

When we see the combined effect of anaerobic mixed culture *Pseudomonas fluorescence* and *Escherichia coli* bacteria with gypsum had able to treat the coffee processing effluent The treatment method reduced BOD5, COD and TS from its initial concentration of 320.26 mg/l, 1261 mg/l, and 3545 mg/l to 35.66 mg/l, 97 mg/l and 68 mg/l respectively as well as neutralize PH. The study showed 87.74% removal of BOD5 load, 92.02% removal of COD as well as 98.01% removal of TS from the initial load using optimization of the treatment method. Thus, a factorial design could minimize the above difficulties by optimizing all the affecting parameters collectively at a time. This shows that the experimental values were found to be close to the predicted values and hence the model validation of the optimal solutions.

5.2. Recommendations

Thus, based on this study I would like to forward that;

- The wastewater have reduce to standard value of BOD5, COD and TS by using combined treatment method of anaerobic mixed culture *Pseudomonas fluorescence* and *Escherichia coli* bacteria with gypsum. Thus, the treatment method in the study can be used for wastewater treatment.
- Mixed culture of microorganisms in the study is helps to treat coffee wastewater; since it have reduced BOD5, COD and TS.
- Mixed cultures are the rule in nature so the interaction between the microorganisms helps for easy to scale up the process. However, the characteristics of these microorganisms should be studied well before suggesting the microorganisms.
- *Pseudomonas florescence* and *Escherichia coli* bacteria best harmonized microbes that degrade both on organic and non-organic matter (ammonium) and less residual effect on the environment.
- Gypsum is needed to study well on the wastewater treatment in a better reduction in pollution in the wastewater; since it is cheap price and less residual effect.
- The treatment method in the study biological and gypsum is ecofriendly.
- Needed further study on design on the treatment plant in pilot scale for further scale up for industrial level.
- The experimental values were found to be close to the predicted values and hence the model validation of the optimal solutions.

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APPENDIX

Appendix I: Design Expert 11 and Design Expert 6.0.8 BOD5 response

BOD 5 response in Design Expert 6.0.8

Use your mouse to right click on individual cells for definitions.

Response: BOD5 .

ANOVA for Selected Factorial Model

Analysis of variance table

Source		**Sum of Square**	**Df**	**Mean square**	**F value**	Prob > F	
Model		0.000	0				significant
Residual		6079.44	11	552.68			
Lack of Fit		*1353.39*	*3*	*451.13*	*0.76*	*0.5455*	*not significant*
Pure Error		*4726.05*	*8*	*590.76*			
Cor Total		6079.44	11				

Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case there are no significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

The "Lack of Fit F-value" of 0.76 implies the Lack of Fit is not significant relative to the pure error. There is a 54.55% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good we want the model to fit.

Appendix II: Design Expert 11 TDS response

ANOVA for selected factorial model

Response 3: TDS

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	4.381E+05	3	1.460E+05	1104.84	< 0.0001	significant
A-MCPFECFT	1.127E+05	1	1.127E+05	852.82	< 0.0001	
B-GRT	2.195E+05	1	2.195E+05	1660.86	< 0.0001	
AB	1.058E+05	1	1.058E+05	800.84	< 0.0001	
Pure Error	1057.33	8	132.17			
Cor Total	4.391E+05	11				

The Model F-value of 1104.84 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. P-values less than 0.0500 indicate model terms are significant. In this case A, B, AB are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

Fit Statistics

Std. Dev.	11.50		**R^2**	0.9976
Mean	183.25		**Adjusted R^2**	0.9967
C.V. %	6.27		**Predicted R^2**	0.9946
			Adeq Precision	69.9567

The **Predicted R^2** of 0.9946 is in reasonable agreement with the **Adjusted R^2** of 0.9967; i.e. the difference is less than 0.2. **Adeq Precision** measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 69.957 indicates an adequate signal. This model can be used to navigate the design space.

Appendix III: Design Expert 11 TSS response

ANOVA for selected factorial model

Response 4: SS

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	18063.00	3	6021.00	35.14	< 0.0001	significant
A-MCPFECFT	2352.00	1	2352.00	13.73	0.0060	
B-GRT	15123.00	1	15123.00	88.27	< 0.0001	
AB	588.00	1	588.00	3.43	0.1011	
Pure Error	1370.67	8	171.33			
Cor Total	19433.67	11				

The **Model F-value** of 35.14 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. **P-values** less than 0.0500 indicate model terms are significant. In this case A, B are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

Fit Statistics

Std. Dev.	13.09		**R^2**	0.9295
Mean	68.17		**Adjusted R^2**	0.9030
C.V. %	19.20		**Predicted R^2**	0.8413
			Adeq Precision	13.1001

The **Predicted R^2** of 0.8413 is in reasonable agreement with the **Adjusted R^2** of 0.9030; i.e. the difference is less than 0.2. **Adeq Precision** measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 13.100 indicates an adequate signal. This model can be used to navigate the design space.

Appendix IV: Design Expert 11 NO_2^- response

ANOVA for selected factorial model

Response 5: NO_2^-

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	0.0025	3	0.0008	122.96	< 0.0001	significant
A-MCPFECFT	0.0018	1	0.0018	263.16	< 0.0001	
B-GRT	0.0007	1	0.0007	104.49	< 0.0001	
AB	8.333E-06	1	8.333E-06	1.23	0.2988	
Pure Error	0.0001	8	6.750E-06			
Cor Total	0.0025	11				

The **Model F-value** of 122.96 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. **P-values** less than 0.0500 indicate model terms are significant. In this case A, B are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

Fit Statistics

Std. Dev.	0.0026		**R^2**	0.9788
Mean	0.0420		**Adjusted R^2**	0.9708
C.V. %	6.19		**Predicted R^2**	0.9522
			Adeq Precision	26.4444

The **Predicted R^2** of 0.9522 is in reasonable agreement with the **Adjusted R^2** of 0.9708; i.e. the difference is less than 0.2. **Adeq Precision** measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 26.444 indicates an adequate signal. This model can be used to navigate the design space.

Appendix V: Design Expert 11 PH response

ANOVA for selected factorial model

Response 3: PH

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	0.0626	3	0.0209	0.6036	0.6308	not significant
A-MCPFECFT	0.0052	1	0.0052	0.1506	0.7081	
B-GRT	0.0154	1	0.0154	0.4455	0.5232	
AB	0.0420	1	0.0420	1.21	0.3025	
Pure Error	0.2767	8	0.0346			
Cor Total	0.3393	11				

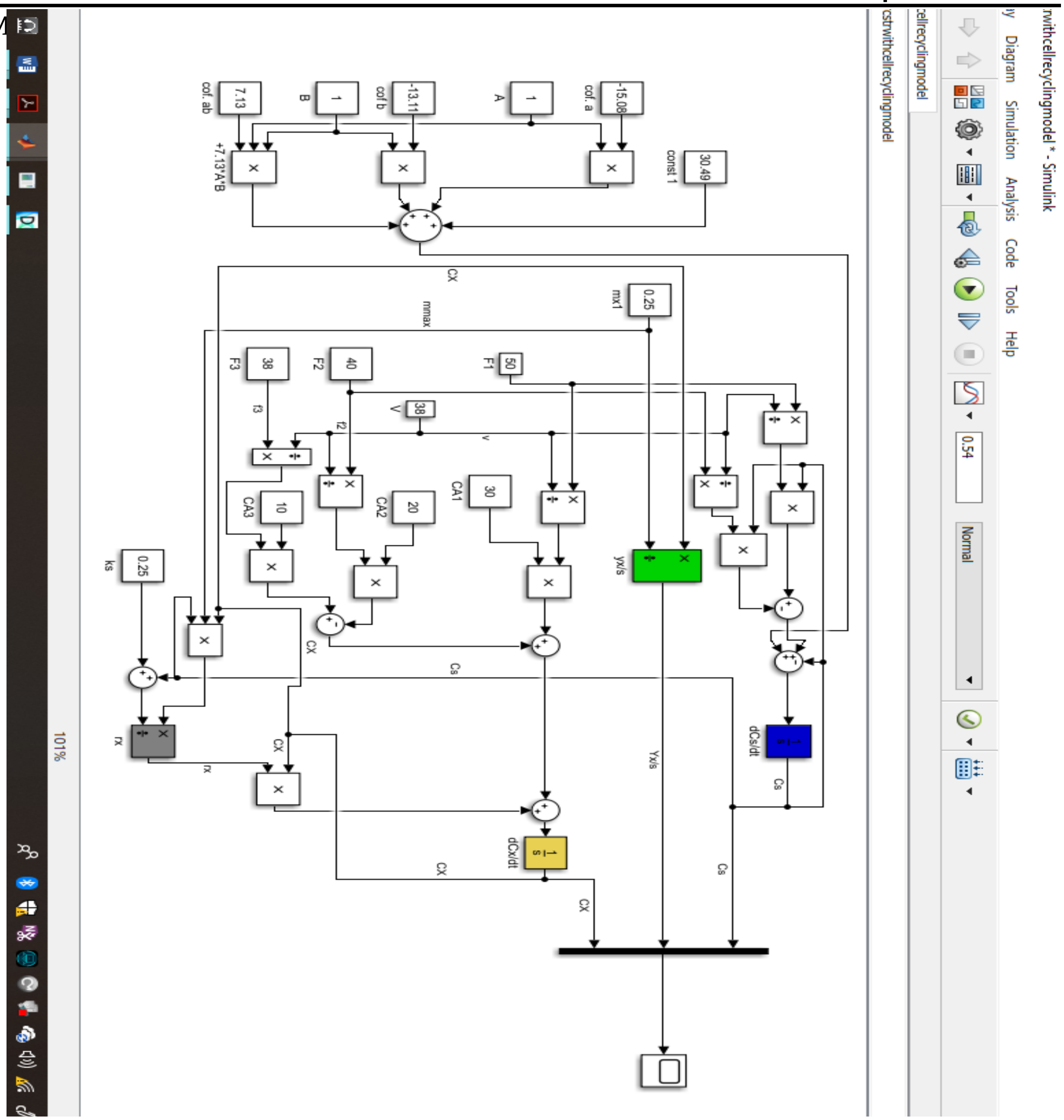
The **Model F-value** of 0.60 implies the model is not significant relative to the noise. There is a 63.08% chance that an F-value this large could occur due to noise. **P-values** less than 0.0500 indicate model terms are significant. In this case there are no significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

Fit Statistics

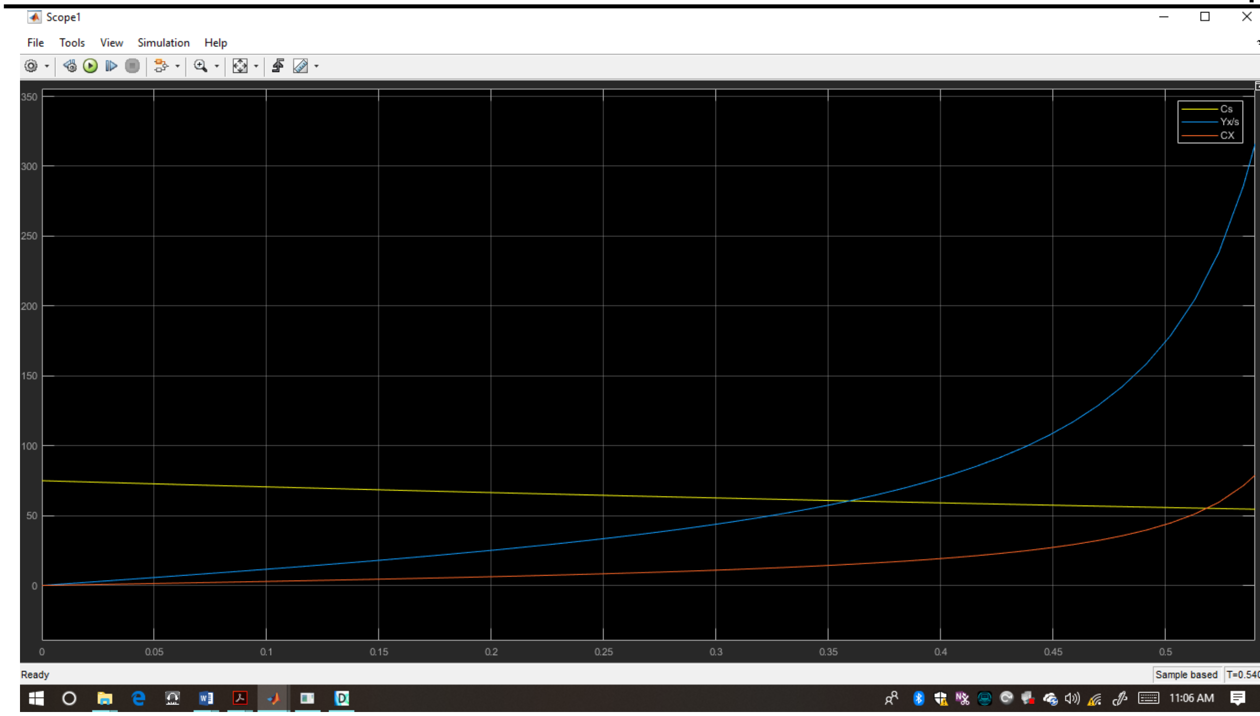
Std. Dev.	0.1860		**R^2**	0.1846
Mean	7.47		**Adjusted R^2**	-0.1212
C.V. %	2.49		**Predicted R^2**	-0.8347
			Adeq Precision	1.7696

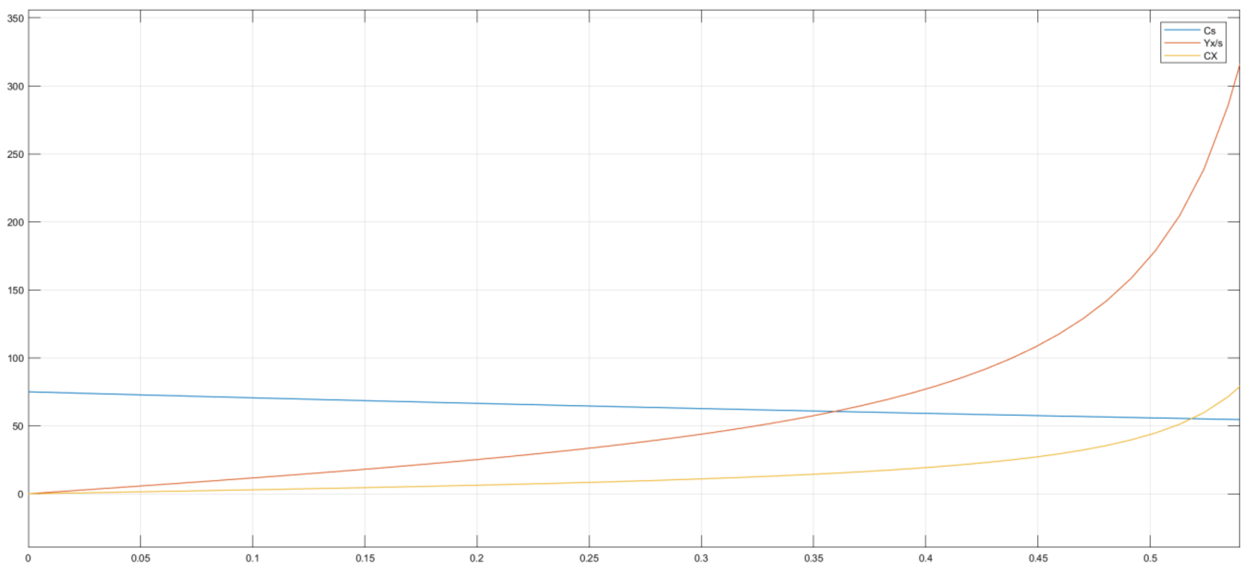
A negative **Predicted R^2** implies that the overall mean may be a better predictor of your response than the current model. In some cases, a higher order model may also predict better. **Adeq Precision** measures the signal to noise ratio. A ratio of 1.77 indicates an inadequate signal and you should not use this model to navigate the design space.

Appendix VI: M

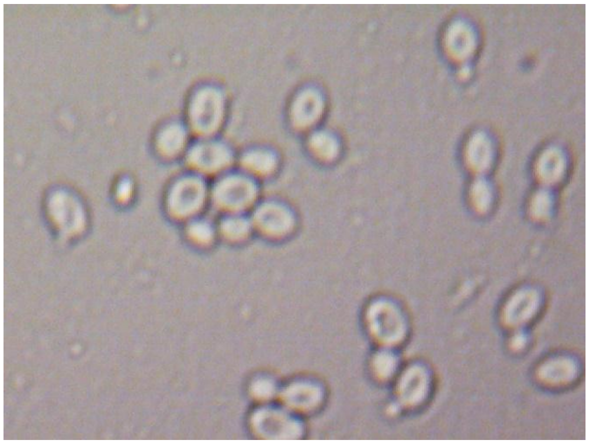


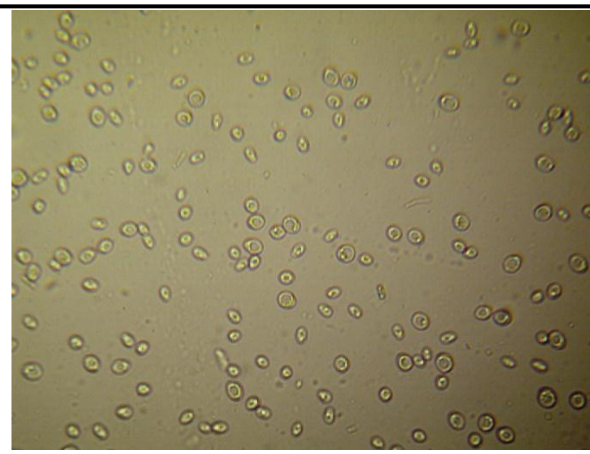
Appendix VII: Mat lab Result for CSTR Model

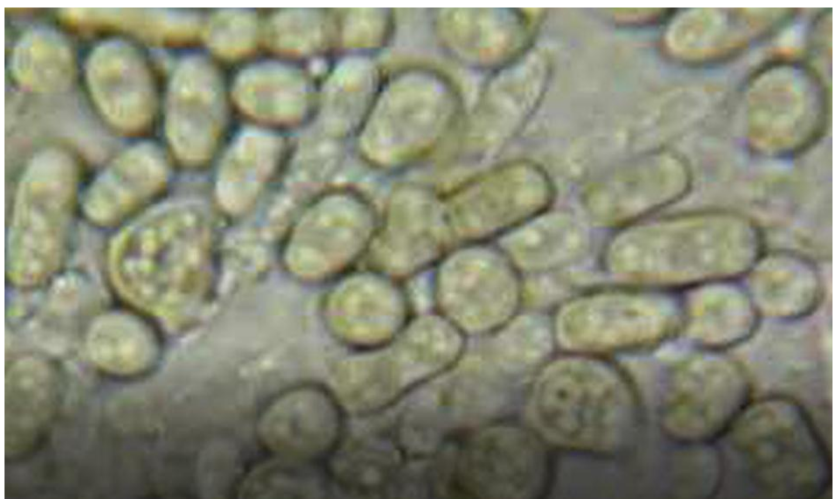




Appendix VIII: Electronic microscope image of *Pseudomonas fluorescence* and *Escherichia coli* Bacteria







Appendix IX: Preparation of nutrient media and colony count

Appendix X: Treatment of the wastewater at different level


Hiwot
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