IMPROVEMENT OF METHYLENE BLUE DECOLOURIZATION USING TEA WASTE AND *Phanerochaete chrysosporium* FOR ENHANCING DYE DECOLOURIZATION PROCESS

PROJECT REPORT

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M.Sc. Microbiology (Honours)

TO

Department of Microbiology

School of Bio Engineering and Bio Sciences.

In partial fulfilment of the requirement for the award of the degree of Master of Science in Microbiology (Honours)

Under the guidance of

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Declaration

I, hereby declare that the project report entitled "**Improvement of Methylene Blue decolourization using modified Tea Waste and *Phanerochaete chrysosporium* for decolourization process**" carried out under the supervision of Dr. Shalini Singh, Associate professor, Department of School of Bio Engineering and Bio Sciences, for partial fulfilment of degree of M.Sc. Microbiology (Honours), Lovely Professional University, Punjab, is entirely my original work and all the ideas and references have been acknowledge.

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Certificate

This is to certify that **Sarvjeet Singh** bearing registration number **11512294** is undergoing his M.Sc. Microbiology (Honours) project work titled "**Improvement of Methylene Blue decolourization using tea waste and *Phanerochaete chrysosporium* for enhancing dye decolourization process**" under my guidance and supervision. To the best of my knowledge, the present work is the result of his original investigation and study. No part of this study has ever been submitted for any other degree award or diploma. This project work is fit for the award for submission as part of the requirement necessary for the award of the degree of M.Sc. Microbiology (Honours.).

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Acknowledgement

First of all, I express my sincere gratitude to Dr. Shalini Singh, Associate Professor, Microbiology Department, Lovely Professional University, Punjab (India), for her suggestions to do this innovative work. In fact she is a great visionary and researcher who had contributed immensely towards this project work. Without her constructive criticism and great efforts I was unable to do this work accurately.

I would like to express my extreme sense of gratitude to Dr. Ashish Vyas, (H.O.D.) and all faculty members of Department of Bioengineering and Biosciences, Lovely Professional University, (Phagwara, Punjab) for giving me permission and supporting to do this project work. I am very much thankful to Mr. Gajender Singh, who helped me and necessary material required during my project work. Without his help I would not have completed my project successfully.

I heartily thanks to my lab-mates and all my friends who helped me in each and every way to complete this thesis successfully.

I bow my head before my beloved Parents who grew me up mentally and spiritually with prayers and Devine love.

Last but not the least, I would like to thank Lovely Professional University Punjab, for giving me the opportunity to get this relevant exposure in the biological world.

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TERMINOLOGY

Terminology	Term
MEA	Malt Extract Agar
TWA	Tea Waste Agar
TG	Tea Granules
TL	Tea Leaves
MTL	Modified Tea Leaves
MTG	Modified Tea Granules

Chapter 1

Introduction

For every living organism in this earth the crucial factor is the requirement of water. But unfortunately, today throughout the world, a huge number of persons are suffering with the current problem and that is shortage of potable water. Today the potable water sources are continuously getting polluted due to increasing the number of people, industrial growth, very poor conditions of urban management. And the factors which are doing this are mainly the harmful waste products of industries, unmanaged disposal of harmful and toxic waste products of industries, sanitary waste discharge and harmful and toxic agricultural products and their mixing with potable water. It is found that in developing countries, due to consumption or use of contaminated water about the overall illness percentage is about 80%. And if we talk about the most susceptible sex or age group is children and with them the women are also most affected. Today the harmful and toxic waste products, which is directly going to the water bodies or water sources have the ability to be prove as fatal and harmful to the life residing in the water bodies (WHO, 2000, Bhatnagar and Sillanpaa, 2010).

In the various areas of the industries for example textile production, paper, leather industries, pharmaceutical industries, cosmetic products, and food related, dyes are regularly and heavily used. According to the report of a literature, the annual production of dying stuffs and synthetic dyes are generally exceeding 700,000 tones (Gupta and Suhas, 2009), (McMullan *et al.,* 2001). The total number of dyes production known is about more than 100,000 types of commercial and about greater 700,00 metric tons per year, out of more than 70,000 dyes are used to release in water bodies or water sources (Tunc *et al.*,2009). The dyes which are used in the industries related with textile are generally classified as: (a) anionic dyes (direct, acid, reactive dyes), (b) cationic dyes (all basic dyes) and (c) non-ionic (dispersed dyes).Out of these, industries related to textile commonly use anionic and cationic more due to choice of their preferred colour water solubility, low cost production and easy to use (Karadag *et al*.,2007).The procedure of dying is generally done at high temperature under the alkaline conditions due to which covalent bonds which are stable in nature are formed in the fibre with the respect to the dye. When we give treatment to the dye, hydrolysis process with the fixation reaction occurs and resulting loss of dye which is hydrolysed in the waste. It is estimated that about amount of dye which is gone from starting is about 50-90 %.

Long lasting colour and brightness to the product is obtained when dye molecules containing reactive group make the formation of covalent bonding to the fibres (Jonstrup *et al*., 2011).

When dyes released in the environment, they may cause various types of harmful and fatal results to the surrounding plants and animals. It may cause the less availability of oxygen and amount of sunlight in water, which can prove as fatal to the lives living in water and also it can badly effect the human beings also. Due to these reasons, dye contamination in the water source is considered as a major environmental problem. Coloured wastes disposed in water can cause complications like lowering the amount of light entering in water, kills the beautiful lives residing in surface of the water and less amount of photosynthesis etc. (Saha *et al.*, 2010). And we talk about the harmful effects on human then it includes kidney problem, brain problems, allergic conditions, liver complications, irritation on skin, dermatitis, problems in reproductive system, central nervous system problems etc. (Khan *et al.*, 2011).

And if we talk about the methylene blue dye then it is very frequently used dye by a number of companies like rubber related, cosmetic production, and industries related with food, textile based, plastic based, pharmaceutical oriented, paper production leather based companies etc. And when during and after the production work the waste product generated usually having the dye components in it. Due to this a less amount present in waste is also observable in the waste (Alkan 2004**,** Turhan and Ozturkcan, 2012).

Silk products, cotton products and wood products are also used to dye with the methylene blue. But the person who are working or animals who are exposed to this can easily get infected with the problem of eye burns and if prolonged exposure is there then it can easily lead to the stable damage to eyes. For certain medical applications it is also used in a big scale. For colouring cotton, in paper industries, colouring of wool, etc. it is also used. Basically if we talk about methylene blue, generally not harmful in nature it is able to produce certain complications. For example, for a less interaction it can cause vomiting, due to liver damage; jaundice, necrosis formation of tissues, elevated rate of heart beat, condition like; shock, formation of Heinz bodies, quadriplegia, cyanosis (Rafatullah, 2010).

For the purpose of elimination and clarification of waste water generated from dyes, several industries follow different kinds of treatment procedures and different techniques like treatment by biological way, process of oxidation, method of coagulation, technique of Nano filtration, method of ozonisation and process of floatation etc. If we talk about physical treatment then the process of adsorption, method of reverse osmosis, procedure of membrane filtration, technique of electrolysis etc. plays their role. According to the reports, for the treatment of waste water, adsorption method is the best way from all physical methods. Due

to the reason of having cheap in nature and easy procedure the adsorption method is most beneficial for the purpose of the decolouration. From the water bodies; dyes, various types of inorganic with organic polluting compounds are removed by well-known and very much efficient adsorption technique. For the formation of target adsorbent against determined pollutant this method provides higher flexibility by its easy operating nature and procedure. Further adding, good nature of treated water determines the power and effectiveness of this technique. Low cost and quality makes the effectiveness of the adsorptive separation technique. If we take the examples of natural minerals, activated form of carbon, many lignocellulose, lot of chitosan, with functionalized polymers, zeolites, are used as an adsorbents and regular efforts towards their development were seen in recent years (Rafatullah, 2010).

But there are some reasons like certain limit in diffusion or non-availability of active sites on the surface which results in the non-effectiveness and due to secondary effluent production and expensive nature or some kind of complications in separation of waste water produces some problems. Due to these problems now the work towards finding the option for the removal of dye through the adsorption which is more cost effective, an effective, compounds in on progress. In the last few years, a number of investigations have done on the adsorption of anionic dyes with the help of certain economical adsorbents and waste of agriculture. And there are many benefits of using waste produced by agricultural source like simple to regenerate, requires very common procedure, better property of adsorption, cheap in cost, very less requirement of processing, and easily availability. With all of these generation of steam can also get by the burning and treating the waste adsorbents with heat (Gaballah *et al*., 1997).

There are also a number of complications if we use the unprocessed plant, agricultural material in the form of adsorbent like more need of COD (Chemical Oxygen Demand) and BOD (Biological Oxygen Demand), less property of adsorption and also a high value of TOC (Total Organic Carbon) as a result of presence of certain organic compounds of the waste of plant (Crini and Badot, 2008). Due to this the modification of treatment of agriculture waste is necessary if we want to use them as affective adsorbent (Hameed, 2009). In our regular life, and in industries like pulp industry, textile related, paper industries, surfactant molecules are used. Hydrophobic moieties and hydrophilic moieties are found in the surfactant molecules. Different types of dyeing processes like photography purpose, in pharmaceutical methods and in textile industries, association of dye and surfactant plays its role (García *et al.*, 2007). Because of its nature of better dispersion, property of levelling and ability to wet

the agent surfactant imparts their vital importance in the dying process in textile industries. The property of fast and wetting homogenously the surface of the fibre, makes the surfactants preferred and standard part of the textile industries. By the ability of adsorption in the fibre and development of complicated formations to the ionic dyes, surfactants work (Simoncic and Kert, 2002).

For the removal of toxic materials and ion of the metal from the waste water and water by treatment, the application of surfactant is used in the last few years. Physical activation involves the activation of the char under the following conditions like CO2, steam and the air. The environment of the carbonization is an inert condition. And if we talk about the chemically activation then it means the treatment of carbon with some chemicals which may be in the solid form or in the liquid form prior the heating treatment in the presence of an inert environment. It results as the surface area increases and formation of pores in the carbon material which aids in the formation of an ideal adsorbent. For the process of activation here is the list of some used chemicals, which are as follows: (Tseng, 2005), (Deng, 2010), sodium hydroxide; (Aksu and Akin, 2010, Guo, 2002), zinc chloride; (Mohanty, 2005, Dolas, 2011), phosphoric acid; (Yagmur, 2008), sulphuric acid; (Duran, 2011), hydrochloric acid; (Dolas, 2011), hydrogen peroxide; (Zhao, 2005) etc.

For performing the activation of any adsorbent whatever the chemical we decide, determines the kind of the method or treatment should be followed with respect to the precursor type. Having the ability to form big surface area and big sizes pores of biomass, potassium hydroxide is preferred as a choice of activation agent. Potassium acetate is compound of potassium and whenever we used to dissolve it in the water, it gets ionize and further gives acetate ions and potassium. To make the molarities equal the ionization of the pure water depends on the temperature to the same value of H3O+ and OH- ions. In the solution of the water and potassium acetate the attraction of the two opposite charges under the ideal conditions results in the development of KOH due to the hydrolysis of the metal ion and results in the formation of pores (Deng, 2010).

Tea is taken by huge number of people throughout the world. On the basis of the consumption tea is having the second place all over the world. Water is the only one having the first place more than tea. According to one study it was assessed that about 18 to 20 billion of tea cups are consumed every day. For providing that huge amount of tea, industries are producing it on a large scale by making the process of extraction of tea leaves by hot water resulting the bottled packed and cans of tea production. But the problem they face after

that is where and how to dispose the tea waste. So the use of that waste should be done in any beneficial process. According to the investigations of the recent years, it is found that tea waste is excellent agent for the elimination of the pollutants from the different contaminated water bodies, also containing methylene dye. If we see then we will find that most of the Asian countries mostly dominantly consume tea and due to which a huge amount of tea waste is generated. For using that for useful purpose, many researchers studied the action of tea waste on the removal of different types of dyes. Methylene blue removal by the use of the tea waste is the best example of that. From the solution or water, the methylene blue dye can be easily removed by using the tea leaves or tea waste, this was analysed by the results of the Hameed study (Hameed and Ahmad, 2009).

The frequently used dye in industries is methylene blue (MB). There are a huge number of industrial waste water treatment methodologies and technologies which are used for the elimination and purification of water but most of them are very complex in procedure and costly in application. So we are heading towards the investigation of some type of substitute, which will prove as cost-effective with similarly as potential for the elimination of methylene blue. From all types of methods the best method which has proved as the best is the adsorption. If we talk about the recent years it has confirmed that spent tea leaves and tea waste has the ability to elimination of diverse kinds of dyes, pollutants etc., and comprising methylene blue. For increasing the affectivity of the tea waste and to utilize tea waste as an adsorbent, it is better to treat it or modify it prior using. For the elimination purpose of toxic substances and dyes from the industrial waste or water, use of surfactants is observed in previous years. So in our study, we will use some modification methods on tea waste and or by treating it with diverse types of parameters we will observed the efficiency of the tea waste (Owamah, 2013).

Chapter 2

Review of Literature

Innovation of the Synthetic dyes was the real change, if we talk about the change from the old time to the present time from where everything start to change. In the present day, the new dyes which are available have a numerous properties like more bright, colour variations, cheap in cost, their simple way of application and quick action to the fabrics. Different researchers have made various beautiful colours variations and due to this factor synthetic dyes are the thing of high demand in various sectors. We admit this thing that beautiful coloured dyes have made our world beautiful and brought a change but we cannot ignores the carcinogenicity, harmful explosive nature and toxicity present in the chemicals which are used to make dyes (Sharma, 2011).

Today we use different products which usually comes in different colours which is being produced by the use of different synthetic dyes in the plastic industries, textile works, paper industries, leather industries, cosmetics and printing industries etc. And when these industries after production, releases waste water then it contains different types of dyestuffs in it. For example if we talk about the dyes which are commonly used in the textile are differentiated into these classes: (1) Cationic (basic) (2) Anionic and (3) reactive dyes. Out of these due to the ability of solubility in water, simple in use, providing desired bright colour and cheap nature reactive and basic dyes are mostly preferred (Karadag, 2007, Karcher, 2002, Purkait, 2005).

In the paper industries, rubber industries, paper industries, cosmetics related, pharmaceutical sectors, leather industries, food sectors commonly used dye is methylene blue. The waste water discharged after processing usually contains the fraction of dyes. Even the very amount in waste water is visible (Alkan *et al.,* 2012). If the waste water produced in companies are not pre-treated before releasing into the water bodies or environment then it can easily cause a number of complication to the lives residing in water bodies as well as to the animals, plants and human beings also due to the toxic components present and high chemical oxygen demand. According to one study, around 10,000 varieties of pigments and various dyes are available today and around 7×105 tons of dyes which are synthetic in nature are regularly being produced throughout the world (Owamah, 2013).

At the time of the processing of the dyes it is calculated that about 10 to 15 percent of dye components are being lost. The harmful effect caused by the coloured waste in the water is that it reduces photosynthesis rate, decreasing the ability of light to enter in water, killing

the living world inside the water (Soni, 2012). The waste generated in the process of dying in the different industries is harmful due to toxic materials present in it and carcinogenic nature of dyes so that is why it must be pre-treated before being disposed into the water bodies to make the safety. What the dyes do is that when sunlight comes to the water bodies then the deposition of dyes on the surface of water bodies take it and cause the reflection of the light which hinders the sunlight to enter in the water. Due to which the bacteria which help in the removal of impurities in water get affected and it causes the hinder the process of photosynthesis which harm aquatic plants. According to the amount and the time for which any one is getting exposed to these dyes determines the degree of severity i.e. normal or severe. Even the low amount like 0.005 ppm can affect the complications at a big level (Couto, 2006). There are many problems like; liver damage, skin problems, central nervous system problems, allergic conditions, kidney complication etc. can be done by dyes (Khan, 2011).

Also there is a danger of this incorrectly disposed dye containing waters entering the water bodies used for drinking by both cattle and humans. Benzidine along with certain aromatic substances which are carcinogenic in nature are used to make dyes. The main source of the presence of the carcinogens in the water bodies is the dyes which are metal consisting like chromium which ends up to the production of chromium i.e. carcinogen. In the fungi and algae it has been observed that a huge amount of waste products containing the ions of the heavy metal and the deposition of dispersed dyes are there due to the textile waste. We have different ways to treat textile waste for the purpose of clearing of the dye. It includes three types which are; physico-chemically processes, advanced oxidation processes and biological processes (Wesenberg, 2003).

Organic dyes must be removed colour from the waste water because they are injurious to us. And if think in the point of view of environment then it is also it is good. Synthetic dyes which are commonly used are basically of their aromatic nature, and it is hard to remove that from dyes because they are non-degradable in nature (Aksu *et al*., 2010). Now, the topic of interest in the field of the research is how fungi can help in the removal of the harmful dye waste. And it was found that the fungus suits in the work of removal of the dye wastes. Over the property of having single cell, mycelia of the fungus have the ability to produce certain enzymes which solubilize the impurities. By having the property of higher cell to surface value, to the environment fungi contain a very high enzymatic and physical contact. Due to the extra-cellular property present in the fungal enzymes they are best for bearing an elevated concentrations of the toxins (Walker and Weatherley, 2000).

For the process of decolourization of the dyes many types of fungi are used both in the dead and living condition. These methods are divided into two types on the mechanism it work, these are bio sorption and other one is biodegradation process. In the water sources, the coloured components and products are usually act as the unwanted thing. So that is the reason that all the coloured materials present in the waste water should be considered as the pollutant and must be removed by different methods and treatments before discharging it to the water sources. For the industries, dealing with the paper work, textile industries, pulp industries etc., it is the big difficulty that how to treat the waste water most efficiently. All these industries requires a lot of water and after processing it creates a large amount of waste water. So it is very necessary to remove or reduce these harmful dye components and for doing so it is necessary to get the knowledge of that dye as a result we can apply the best methods (Robinson *et al.,* 2001).

Before discharging the harmful dye waste water in the water sources it is very compulsory to treat it with suitable methods, due to the fatal natures of dye components. There are a number of dyes which have the ability of causing cancer, able to act as toxin, etc. which causes the harmful effects to the organisms that lives in the water (O'Neill, 1999, Vandevivere, 1998). But the problem is that a number of dyes shows the resistant towards the digestion by aerobic condition, agents that is oxidizing in nature, heat, light treatment, etc. (Sun and Yang, 2003).

Today we have a number of processes and methods by which we can remove the waste water harmfulness, and few of those are: process of electrolysis; (Jin, 2003), method of electro-coagulation and photo catalysis (Malekbala, 2012), process of adsorption, foam flotation method and process of biodegradation, (Yue, 2008). The basic methods under which all the treatments comes are divided into: (1) Chemical treatment, (2) Physical treatment and (3) Biological treatments.

Chemical treatment**s** are the chemical methods, flocculants and coagulants are used as a big agents for the removal of waste water (Rauf, 2009). The process of the flocculation is started by the addition of aluminium, calcium, ferric ions, etc. (Zhou, 2008). According to the (Shi *et al.,* 2005), we can also increase the effectiveness of the procedure of removal of dyes by combination or using two methods together. The chemical method is generally effective in the removal of dyes but the limitation is due to the reason that there is a huge demand of these chemicals in the industries and the process of the production, in the market these chemicals are pretty expensive and cost of them are also different due to their much demand (Shi, 2007).

Physical methods are the type of dye removal method, techniques like electrolysis process, membrane filtration technique, adsorption, reverse osmosis process etc. From all of these methods, the adsorption techniques is more effective and beneficial, because of the comfort of the procedure with having the cheap cost, adsorption method is recognized as a best and more reliable method for the decolourization of the dyes. In the work of the purification of the polluted waste produced by the industries and water bodies, usage of the commercial available activated carbon as an adsorbent is done. But the activated carbon is available in the very high price for its regeneration process and for its production. Natural adsorbents like; Chitosan, fly ash paper mill sludge, various clay minerals Coal, and zeolites, should be used for the elimination of undesirable dangerous components present in the waste water of the industries (Rafatullah, 2010, Unuabonah, 2009).

Biological methods are the methods of the treatment of the harmful components from the waste water are far better than the chemical and the physical methods due to the cheap cost nature of this method. It makes it the better substitute for the dye removal processes. In this type of the treatment bio degradable materials like; living or non-living microbial biomass, fungus, degradation by microbial agents, bioremediation etc. are used for the removal of harmful waste water products. The ability to attach and damage the various polluting elements makes the bacteria, algae, fungi and yeast make them better option but there are some limits in the use of them which makes the use of them restricted (Banat, 2003).In the process of dying of the textile products, surfactants imparts their important action because of having the property of diffusing, levelling and ability to make the surface wet. All the processes followed in the industries which requires the liquid quickly and consistently the wetting of fibre surface, makes the surfactants as standard and appropriate constituent. The mechanism of the action of the surfactants is by the creation of the complicated species with the respect to the adsorption or ionic dyes to the fibre.

Chapter 3

Scope of the study

Dyes are the major chemicals contributing towards the environmental pollution. Today the entire world is facing a very big and increasing problem of decreasing level of potable water and its sources. With other factors, the industries are making a great contribution in making potable water contaminated and polluted. And the main reason is population of the human beings are increasing at an alarming rate. For the fulfilment of the diverse requirement of the human beings, industries are working continuously and in doing so they are causing harm to the nature and producing toxic and harmful waste products. The waste material produced after processing is being dumped into water sources and water bodies directly or indirectly. As a result, the life of the water bodies and surrounding areas is getting harmed and killed. The animals, water lives, plants, birds, human beings, etc. is facing fatal results.

So, in order to save the fresh water, life present in the water bodies and the environment, we are working on the methods which are naturally available as a bio sorbents which may be considered as an attractive option for the elimination of the dyes from the water and waste water generated in industries. Though different kinds of bio sorbents has not investigated as well as proposed for the dye adsorption yet, tea waste has been rarely studied for this purpose .A few reports suggest the use of natural/ untreated tea waste as biosorbent followed by its use for growth of *Phanerochaete chrysosporium*. A variety of substrate modifications have been proposed by researchers, which potentially can improve ability of tea waste as a bio sorbent as well as substrate for *Phanerochaete chrysosporium* growth. Thus, present work will explore the adsorptive properties of tea waste and also the use of dye adsorbed tea waste as a substrate for fungal growth and dye degradation.

Chapter 4

Objectives of the study

Keeping in view the above mentioned considerations, the present study is carried out to further study the use of modified tea waste as an adsorbent for the tested dye methylene blue which is the common dye used in the industries for dying purposes The work on the tea waste usage as an adsorbent for dye decolourization by using *Phanerochaete chrysosporium* was previously done by (Sharma, 2016). According to their results, laccase activity under the optimized conditions was found out be 89.20 U/ml by spent tea granules and 180.1 U/ml by the spent tea leaves. And on the reutilization of dye adsorbed substrates again the percentage of the dye decolourized was found to be 70% in sent tea granules and 91.4% in the sent tea leaves. Now we are taking their results as a reference and following few modification of tea leaves and granules for the removal of methylene blue by using *Phanerochaete chrysosporium.*

The objectives for the current study are as follows:

1. To study the effect of the different modifications on tea waste as dye absorbent.

2. To optimize the modified tea waste as dye absorbent for Methylene blue.

3. To evaluate the use of modified methylene blue dye absorbed tea waste as a substrate for the growth and laccase production of *Phanerochaete chrysosporium*.

Chapter 5

Materials and Research Methodology

Materials

All the glass wares used during the work were made up of Borosil/Rankem. All the materials and chemicals (except tea leaves and the tea granules) which we have used were of the company named Loba-Chemie, were of analytical grade nature and obtained from Mumbai, India.

5.0 Proposed Research Methodology

5.1 Revival of fungal culture

White rot fungi *Phanerochaete chrysosporium* was revived from the already available plates of the *Phanerochaete chrysosporium* by preparing fresh plates of MEA (Malt Extract Agar). MEA plates and slants were inoculated with the culture and were kept inside the incubator at the temperature of 28°C for the time period of 4-5 days and finally kept at the temperature of 4°C inside the refrigerator for the future use. Sub-culturing on MEA was done at regularly (Richter *et al*., 2010).

5.2 Preparation of adsorbent

Tea leaves and tea granules were purchased from local market Phagwara (Punjab). Then after collection, the collected tea leaves and tea granules were boiled initially with the water and then with the distilled water in order to wash out the colour and impurities of the tea leaves and tea granules. After that the washed and boiled tea leaves and tea granules were dried with the help of the hot air oven at the temperature of 60°C and for the time period of 48-60 hours and finally with the help of the steel sieve, sieved tea leaves and tea granules to a size range of 100 to 500 µm to obtain the uniform size (Pirbazari *et al*., 2014). At the end the adsorbents were stored in zip lock bags till further use.

5.3 Preparation of Dye solution

The tested dye we used in our work is methylene blue which was obtained from Merck and having the No.115943 and used for the checking of the tea leaves and tea granules ability on its removal. A stock solution for the use in the experiment made by adding 1.0gm methylene blue in 1 litre of the distilled water, and the concentrations of the methylene blue prepared by

diluting of the stock solution. The pH of the different dye samples was adjusted by adding a little drops of 0.01HCL / 0.01M Sodium hydroxide (Pirbazariet *et al*., 2015).

5.4 Thermal activation of tea leaves and tea granules

For performing the thermal activation of the tea leaves and tea granules, both of them were subjected to heat in muffle furnace at 200-400°C for 0.5 to 2 hours. Thermally activated material collected and stored in zip lock bags for future use (Zuorro *et al*., 2013).

5.5. Preparation of Potassium acetate modified tea leaves and tea granules

Tea leaves and granules were collected, properly washed with distilled water. After that they were boiled and dried at 60˚C for 48 hours. Then they were sieved at a size of 500µm and stored in zip-lock bag. For the modification of tea waste with potassium acetate, tea waste and potassium acetate were impregnated at a ratio of 2:1 g/g. Then they were incubated for overnight at room temperature. Then they were treated at the temperature of 80 ˚C for 8 hours. Then the modified sample is cooled to room temperature and followed with the washing step with distilled water. The pH was maintained to 6.8-7. Then modified sample was dried and packed in the air tight zip-lock bag. (Hameed and Auta, 2011)

5.6 Study on the comparative dye adsorptive ability of the potassium acetate modified and unmodified tea leaves and granules under different parameters

Different experiments were done by taking 250 ml Erlenmeyer flasks with 100 ml of dye solution in the concentration 10 mg/L. In the flasks, addition of 0.5 gm. of adsorbent were done and different experiments were done to check different parameters.

5.6.1 Effect of pH on the decolourization of dye by potassium acetate modified and unmodified tea leaves and granules

Dye solutions were made and different pH were set to check the effect of pH by the addition of either 0.1 N HCL or NaOH. Four different pH were taken 5.5, 6.5, 7.5, 8.5 and 0.5 gm. potassium acetate modified tea leaves and granules sample were added. In other set the same was done but the unmodified tea sample was taken. The flasks were kept at 60 ˚C (tea leaves) and 70˚C (tea granules) in the water bath for the time period of 2 hours. After that at 664 nm spectrophotometry was done and recorded the results (Hameed and Auta, 2011).

5.6.2 Effect of contact time on the decolourization of dye by potassium acetate modified and unmodified tea leaves and granules

Five different time values: 30 min, 60 min, 90 min, 120 min and 150 min were taken. The amount of the sample was 0.5 gm., potassium acetate modified tea leaves and granules sample were added. In other comparison set unmodified sample was taken. The flasks were kept at 70˚C in the water bath for the time period of 2 hours. After that at 664 nm spectrophotometry was done and recorded the results.

5.7 Preparation of H_2SO_4 modified tea leaves and tea granules

For the modification of tea leaves and granules with the H_2SO_4, H_2SO_4 and double distilled water were mixed with each other in the ratio of 1:1. After that 20 gm. of the tea leaves and granules were added into that, mix it well and kept for overnight. After that it was properly washed with distilled water to remove acid and until its pH became 7.0. Then it was dried in hot air oven at the temperature of 70˚C for 24 hours. After that it was crushed well in 300 µm sieve size (Sharma *et al*., 2015) and stored in zip lock bags.

5.8 Study on the comparative dye adsorptive ability of the H_2SO_4 modified and unmodified tea leaves and granules under different parameters

Stock solution preparation: By dissolving 5 mg dye in 1000 ml of distilled water a stock solution of methylene dye was prepared. By the addition of either 0.1 N HCL or NaOH different pH dilutions were performed.

A number of experiments were done by using 100 ml of dye solution in the 250 ml Erlenmeyer flask with the concentration 10 mg/L. The addition of 0.5 gm. of adsorbent was done and different experiments were done to check different parameters.

5.8.1. Effect of pH on the decolourization of dye by H_2SO_4 modified and unmodified tea leaves and granules

Dye solutions were made and different pH were set to check the effect of pH by the addition of either 0.1 N HCL or NaOH. Four different pH were taken 5.5, 6.5, 7.5, 8.5 and 0.5 gm. H_2SO_4 modified tea leaves and granules sample were added. The flasks were kept at 70˚C in the water bath for the time period of 2 hours. After that at 664 nm spectrophotometry was done and recorded the results (Foroughi-dahr *et al.,* 2015).

5.8.2. Effect of contact time on the decolourization of dye by H_2SO_4 modified and unmodified tea leaves and granules

Dye solutions were made and pH was set to 7.0 by the addition of either 0.1 N HCL or NaOH. Five different time values: 30 min, 60 min, 90 min, 120 min and 150 min were taken. 0.5 gm. H_2SO_4 modified tea leaves and granules sample were added. The flasks were kept at 70˚C in the water bath for the time period of 2 hours. After that at 664 nm spectrophotometry was done and recorded the results (Foroughi-dahr *et al*., 2015).

5.8.3. Effect of adsorbent dose on the decolourization of dye by H_2SO_4 modified and unmodified tea leaves and granules

The adsorbent dose was taken from 0.25 gm., 0.50 gm., 0.75 gm., 1.0 gm., 1.25 gm. and 1.50 gm. Then the pH was set to 7.0 by the addition of either 0.1 N HCL or NaOH. Amount of 0.5 gm. H_2SO_4 modified and unmodified tea leaves and granules sample were added. The flasks were kept at 70˚C in the water bath for the time period of 2 hours. After that at 664 nm spectrophotometry was done and recorded the results (Pirbazari, *et al*., 2014)

5.8.4. Effect of temperature on the decolourization of dye by H_2SO_4 modified and unmodified tea leaves and granules

Five different temperature values; 55˚ C, 65˚ C, 75 ˚ C, 85 ˚ C, and 95 ˚ C were taken with their controls. They were treated for 2 hours' time period. The pH was set to 7.0 by the addition of either 0.1 N HCL or NaOH. Amount of 0.5 gm. H2SO4 modified and unmodified tea leaves and granules sample were added. The flasks were kept at 70˚C in the water bath for the time period of 2 hours. After that at 664 nm spectrophotometry was done and note down the results.

5.9 Effect of the substrate or nutrient medium composition on the growth of *Phanerochaete chrysosporium.*

After the substrate had adsorbed the dye, it was dried and test for the ability to use it as a substrate for the production of laccase enzyme by the fungus *Phanerochaete chrysosporium.* Two types of the nutrient medium were made which were tea waste agar (TWA) and malt extract agar (MEA). Both of them were inoculated with the disc of fungi at the middle of the petri-plate. Incubation was done in incubator at 28 days for 3-5 days. Every day the diameter of the growth was noted and compared with each other.

5.10 Effect of incubation temperature on the laccase enzyme production by Phanerochaete chrysosporium under SSF

Moistening agent prepared with 6.0 pH, autoclaved and inoculation was done as done before. Here, the incubation temperature values are varied as 20˚ C, 25˚ C, 30˚ C and 35˚ C. Incubated at optimized time period and after that estimation of laccase was done (Muthezhilan *et al*., 2007).

Fermentation medium was prepared, consisting of the nutrient salt solution and dye adsorbed tea samples in a ratio of 1:3 in 250 ml flask. Composition was kept according to the standard procedure provided by Ivanka *et al*., (2010) with 6.0 pH. Autoclaving was done and after sterilization, the prepared media was inoculated with 2 discs of fungus into each flask. Incubation done at 37˚C to check the production of enzyme (Gupte *et al*., 2007). Every day the enzyme was harvested.

Every day one flask is removed and 15 ml of distilled water was added to the flask. Media was used to crush and mixed in a rotary shaking incubator for 10 minutes at the speed of 200 rpm. Then mixture is filtered by muslin cloth. Extract was centrifuged at the speed of 4000 rpm for time period of 15 minutes. After that supernatant was treated as crude enzyme (Muthezhilian *et al*., 2007).

Laccase production estimation was performed. Guaiacol was used as a substrate for laccase activity. In the 10 mM sodium acetate, 2mM Guiacol was prepared with 5.0 pH. Reaction mixture contains acetate buffer; 1.5 ml, guaiacol; 0.5 ml and 0.5 enzyme source. For the blank distilled water was used. Enzyme activity is expressed in International Units (IU), where 1IU means the amount of laccase enzyme required to oxidize 1 micromole of the guaiacol per minute.

Laccase activity can be calculated by:

E.A. = (A*V) / (t*e*v)

Where, E.A. =Enzyme activity (U/ml),

A=Absorbance of mixture at 450 nm,

V= Total volume of reaction mixture used (ml),

t= Incubation time (min), and

e=Extinction coefficient (M^{-1} cm^{-1}).

Chapter 6

Results and Discussion

6.1 Revival and sub-culturing of the tested Fungus: We have done the revival and sub-culturing of white rot fungi: *Phanerochaete chrysosporium* in the Malt Extract Agar (MEA). We prepared the MEA agar plates and with the disc method we inoculated the inoculum and incubated at the temperature of 25°C for 5 days. After getting the desired growth, we kept the plates at the refrigerator for our future use. We also preserved our culture in the MEA slants containing glycerol by streaking with the culture of *P. chrysosporium* and incubating it at the temperature of 25°C for 5 days and kept in the refrigerator at 4°C (Richter *et al*., 2010).

Growth conditions were provided as the temperature was maintained at 25°C to 30°C, atmospheric conditions were maintained **t**ypically aerobic. After the proper incubation colony morphology observed on the Malt extract agar media plates (MEA), at the temperature of 25°C after 5 days, (Picture 1a-b) colonies observed were creamy white, moist and cottony colonies. And on the Malt extract agar slants containing glycerol, which were kept at the temperature of 25°C, after 5 days colonies of the fungus *Phanerochaete chrysosporium* were observed creamy white, moist and cottony colonies. After getting growth on Malt extract agar slants, we store them in the refrigerator at the temperature of 4°C.

6.2 Collection and preparation of adsorbent (tea leaves and tea granules):

We have collected the tea leaves and tea granules from the Phagwara, local market of Punjab. We noted down the properties of that. After that we have done the processing of the tea waste: tea leaves and tea granules, by washing and boiling it with the water and distilled water. Then we dried it at the hot air oven at the temperature of 60°C for 24-48 hours. (Picture 2a-b) After that we stored it in the zip-locked bags for our future use (Pirbazari *et al*., 2015).

Tea leaves and tea granules

Tea leaves; Dry, flat, blackish in colour. After preparation of adsorbent: sieved at 500 µm size.

Tea granules; Dry, rough, brownish in colour and in granular form. After preparation of adsorbent: sieved at 500 µm size.

6.3 Preparation of heat activated tea leaves and tea granules

We have performed the modification of the tea leaves and tea granules by the heat activation of the tea leaves and granules. We have taken 25 grams tea leaves and tea granules separately in crucibles. We took them and placed in them inside the muffle furnace and exposed them in the muffle furnace at the temperature of 400°C for 2 hours. After that we kept the treated sample in the desiccator. After that we stored the thermally modified tea waste in the zip-lock bags for our future use (Zuorro *et al*., 2013**).**

6.4.1 Effect of pH on decolourization of dye by Potassium acetate modified and unmodified tea leaves and granules

Incubation conditions were maintained as dye concentration (mg/L): 10, time (Hours): 2, temperature (˚C): Tea Leaves: 60˚C, Tea Granules: 70˚C, pH: 5.5, 6.5, 7.5 & 8.5, adsorbent dose (gm.): 1.0. After incubation, (as shown in table 1) it was observed that in case of potassium modified tea leaves the maximum dye colourization was observed at 6.5 pH (38.56 %), where in case of unmodified tea leaves the maximum dye colourization was observed at 7.5 pH (75.96 %). Where in case of potassium modified tea granules the maximum dye decolourization was observed (47.28 %) at 7.5 pH where in case of unmodified tea granules the maximum dye decolourization was observed (79.24) at 6.5 pH. Results showed that the unmodified tea leaves were showing better dye decolourization percentage than the potassium acetate modified tea leaves.

6.4.2 Effect of contact time on the decolourization of dye by Potassium acetate modified and unmodified tea leaves and granules

Both the potassium acetate modified and unmodified tea samples were treated with five different contact time: 30, 60, 90, 120 and 150 minutes. The temperature maintained was 60˚ C and 70˚C and the quantity was 1.0 gm. After the incubation it was observed that, (as shown in table 2) in case of potassium modified tea leaves the maximum dye colourization was observed after 120 minutes (59.64 %) and minimum dye decolourization was observed after 30 minutes (33.58 %). Where in case of unmodified tea leaves the maximum dye colourization was observed after 90 minutes (73.63 %) and minimum dye decolourization was observed after 8.5 (62.35 %). Results showed that the unmodified tea leaves were showing better dye decolourization percentage than the potassium acetate modified tea leaves.

Where in case of in case of potassium modified tea granules the maximum dye colourization was observed after 120 minutes (54.56 %) and minimum dye decolourization was observed after 30 minutes (39.09 %). Where in case of unmodified tea granules the maximum dye colourization was observed after 120 minutes (71.35 %) and minimum dye decolourization was observed after 150 minutes (53.64 %). In this case also results showed that the unmodified tea leaves were showing better dye decolourization percentage than the potassium acetate modified tea leaves.

6.5.1 Effect of pH on the decolourization of dye by H_2SO_4 modified tea leaves and granules

The effect of the various pH on the decolourization of dye was observed (as shown as in table 3) which are shown in table as; the maximum dye decolourization (63.69 %) by H_2SO_4 modified tea leaves at pH 6.5 and minimum decolourization (56.00 %) at pH 8.5 was observed. Where in the unmodified tea leaves the maximum dye decolourization (11.94 %) by H_2SO_4 modified tea leaves at pH 7.5 and minimum decolourization (7.11 %) at pH 5.5was observed (Picture 3a-d). It showed that in the use of tea leaves for the dye decolourization the best pH range was about pH 6-7.

Where in case of H_2SO_4 modified tea granules; the maximum dye decolourization (77.02%) by H2SO4 modified tea granules at pH 5.5 and minimum decolourization (66.39 %) at pH 8.5 was observed. Where in the unmodified tea granules; the maximum dye decolourization (50.20%) by unmodified tea granules at pH 5.5 and minimum decolourization (34.95 %) at pH 6.5 was observed. It showed that in use of tea granules for the dye decolourization the effective pH value was around 5-6.

6.5.2. Effect of contact time on the decolourization of dye by H_2SO_4 modified and unmodified tea leaves and granules

After performing the test, the spectrophotometric results was observed. (As shown as in the table 4) The maximum dye decolourization (85.70 %) by H_2SO_4 modified tea leaves after 30 minutes and minimum decolourization (76.62 %) after 90 minutes was observed. And (Picture 4a-e) as in the case of the unmodified tea leaves as the time increase (30 min-120 min) the dye decolourization also increase maximum (71.25 %) after 120 minutes but after one point it start decreasing (67.45 %) after 150 minutes. The effective contact time in case of modified tea leaves was 30 minutes. Where in case of H_2SO_4 modified tea granules; the

maximum dye decolourization (96.90%) after 90 minutes and minimum decolourization (83.77 %) after 30 minutes was observed.

Where in the unmodified tea granules; the maximum dye decolourization (86.18 %) by after 90 minutes and minimum decolourization (81.73 %) after 150 minutes was observed. It was observed that in case of modified tea granules, the increased contact time increased the decolourization.

6.5.3. Effect of adsorbent dose on the decolourization of dye by H_2SO_4 modified and unmodified tea leaves and granules

Different adsorbent doses were taken and observed that (as shown in table 5) in case of H_2SO_4 modified tea leaves the maximum dye decolourization (99.06 %) by with the 0.25 gm. of dose and minimum decolourization (91.16 %) with 0.75 gm. dose was observed. It was observed that (Picture 5a-f) a low amount of modified tea leaves are more effective which is useful in the decolourization of the dye. As in the case of the unmodified tea leaves the maximum (84.76 %) with 1.25 gm. of dose and minimum dye decolourization (70.62 %) with 0.25 gm. dose.

Where in case of H_2SO_4 modified tea granules; the maximum dye decolourization (82.47 %) with 0.25 gm. dose and minimum decolourization (52.42 %) with 1.25 gm. dose was observed. Where in the unmodified tea granules; the maximum dye decolourization (69.01 %) with 0.25 gm. dose and minimum decolourization (30.73 %) with 1.0 gm. dose was observed. Also in the case of modified tea granules, the low quantity of modified tea proved as effective in the decolourization of the dye.

6.5.4 Effect of temperature on the decolourization of Methylene blue dye by H_2SO_4 modified and unmodified tea leaves and granules

The following incubation conditions were maintained during the test; dye concentration (mg/L): 10, time (hours): 2, temperature (˚C): 20, 25, 30, 40, pH: 7.0 Incubation conditions: dye concentration (mg/L): 10, time (hours): 2, temperature (˚C): 20, 25, 30, 40, pH: 7.0. After the incubation it was observed that (as shown as in table 6) in the case of H_2SO_4 modified tea leaves, the maximum dye decolourization was observed at the temperature of 25˚C (82.24 %) and the minimum was observed at the temperature of 20˚C (79.07 %). Where in the unmodified tea leaves the maximum dye decolourization was observed at the temperature of 35˚C (58.47 %) and the minimum was observed at the temperature of 20˚C (40.45%).

Where in case of H_2SO_4 modified tea granules; the maximum dye decolourization (86.62%) at the temperature of 30˚C and minimum decolourization (82.70 %) at the temperature of 20˚C was observed. Where in the unmodified tea granules; the maximum dye decolourization (69.66 %) at the temperature of 25˚C and minimum decolourization (66.95%) at the temperature of 35˚C was observed. So it was observed that the best temperature range for the maximum dye decolourization was about 25˚C - 30˚C.

6.6 Effect of the substrate or nutrient medium composition on the growth of *Phanerochaete chrysosporium*

On the malt extract agar and potato dextrose agar, the *Phanerochaete chrysosporium* inoculated and after the incubation at 28˚ C temperature, good growth was observed (as shown in table 7). In the prepared dye absorbed tea waste medium, the inoculation was done and the fair growth of *Phanerochaete chrysosporium* was observed (Picture 6) after proper incubation at 28˚ C temperature and in aerobic conditions. The good growth was not observed, may be due to the modification its functioning as a substrate reduced by H_2SO_4.

6.7 Laccase enzyme production by *P. chrysosporium* in the case of H_2SO_4 modified tea granules and tea leaves

After performing the inoculation of *Phanerochaete chrysosporium*, in the SSF conditions (Picture 8a-b), in the flasks, and by maintaining the temperature (˚C): 29, inoculum size (mm): 0.5, pH of the moistening agent: 6.0 and incubation duration (days): 7 (TG) and 8 (TL), It was observed that the (as shown in table 8) in the H_2SO_4 modified tea leaves the laccase production was more 145 ± 1.4 (U/ml) as compared to H_2SO_4 modified tea granules 74 ± 0.9 (U/ml). But overall the laccase production was less in both of cases may be due to the modification done to the tea waste, which resulted in the less laccase production.

6.8 Effect of incubation temperature on the production of laccase by *Phanerochaete chrysosporium*

By following the Incubation conditions: Inoculum size (mm): 0.5, pH of the moistening agent: 6.0, substrate: moistening agent: 1:3, Incubation duration (days): 7 (TG) and 8 (TL), the dye absorbed H_2SO_4 modified tea leaves and granules were treated with 20 ˚C, 25, 30 ˚C and 35˚C temperature. After incubation it was observed that (as shown as in table 7) in the H_2SO_4 modified tea leaves at the temperature of 30 ˚C laccase production 153 ± 1.9 (U/ml)

was maximum than in the H_2SO_4 modified tea granules at the same temperature of 30˚C the laccase production 118 ± 1.3 (U/ml) was maximum (Picture 7a-d).

As the temperature was increasing from 20˚C to above the enzyme production was increasing and was maximum at 30˚C in both H_2SO_4 modified tea leaves and granules but after that in decreased, showed 30˚C the best temperature range.

Table 1. Effect of pH on decolourization of dye by Potassium acetate modified and unmodified tea leaves and granules

S. No.	Parameter	Dye decolourized (%)			
	pH	Tea Leaves		Tea Granules	
		Potassium acetate modified	Unmodified	Potassium acetate modified	Unmodified
1	5.5	29.68	45.12	31.49	63.56
2	6.5	38.56	63.65	34.06	79.24
3	7.5	33.05	75.96	47.28	78.07
4	8.5	35.61	50.29	38.74	65.94
Incubation conditions: Dye concentration (mg/L): 10 Time (Hours): 2 Temperature (˚C): Tea Leaves: 60˚C, Tea Granules: 70˚C pH: 5.5, 6.5, 7.5 & 8.5 Adsorbent dose (gm.): 1.0					

Effect of contact time on decolourization of dye by Potassium acetate modified and unmodified tea leaves and granules

S. No.	Parameter	(%) Dye Decolourized			
	Contact Time (min.)	Tea Leaves		Tea Granules	
		Potassium acetate modified	Unmodified	Potassium acetate modified	Unmodified
1	30	33.58	62.35	39.09	59.06
2	60	39.16	68.36	42.19	65.86
3	90	49.49	73.63	48.18	56.96
4	120	59.64	71.61	54.56	71.35
5	150	53.18	65.37	46.80	53.64

Incubation conditions:

Dye concentration (mg/L): 10

Time (Hours): 2

Temperature (˚C): Tea Leaves: 60˚C, Tea Granules: 70˚C

Adsorbent dose (gm.): 1.0

Table 3 Effect of pH on decolourization of dye by H_2SO_4 modified and unmodified tea leaves and granules

S. No.	Parameter	Dye Decolourized (%)			
	pH	Tea Leaves		Tea Granules	
		H_2SO_4 Modified (%)	Unmodified (%)	H_2SO_4 Modified (%)	Unmodified (%)
1.	5.5	63.61	07.11	77.02	50.20
2.	6.5	63.69	09.68	70.02	34.95
3.	7.5	56.10	11.94	66.88	39.03
4.	8.5	56.00	11.54	66.39	40.41

Incubation conditions:

Dye concentration (mg/L): 10

Time (Hours): 2

Temperature (˚C): Tea Leaves: 60˚C, Tea Granules: 70˚C

pH: 5.5, 6.5, 7.5 & 8.5

Adsorbent dose (gm.): 1.0

Table 4. Effect of contact time on decolourization of dye by H_2SO_4 Modified and unmodified tea leaves and granules

S. No.	Parameter	Dye Decolourization (%)			
	Contact Time (min.)	Tea Leaves		Tea Granules	
		H_2SO_4 Modified (%)	Unmodified (%)	H_2SO_4 Modified (%)	Unmodified (%)
1.	30	83.31	68.16	83.77	83.31
2.	60	83.51	69.66	85.98	83.06
3.	90	76.62	69.73	96.90	86.18
4.	120	85.00	71.25	85.60	83.62
5.	150	85.68	67.45	86.62	81.73
Incubation conditions: Dye concentration (mg/L): 10 Time (Hours): 2 Temperature (˚C): Tea Leaves: 60˚C, Tea Granules: 70˚C Adsorbent dose (gm.): 1.0					

Table 5. Effect of adsorbent dose on decolourization of dye by H_2SO_4 modified and unmodified tea leaves and granules

S. No.	Parameter	Dye Decolourized (%)			
	Adsorbent Dose (gm.)	Tea Leaves		Tea Granules	
		H_2SO_4 Modified (%)	Unmodified (%)	H_2SO_4 Modified (%)	Unmodified (%)
1.	0.25	99.06	70.62	82.47	69.01
2.	0.50	95.81	74.13	69.42	54.55
3.	0.75	96.16	76.68	58.91	47.51
4.	1.0	91.79	79.05	61.19	30.73
5.	1.25	96.16	84.76	52.42	47.35
6.	1.50	91.22	74.40	60.55	53.80

Incubation conditions:

Dye concentration (mg/L): 10.

Time (hours.) : 2

Temperature (˚C): Tea Leaves: 60 Tea Granules: 70.

pH: 7.0

Table 6. Effect of temperature on the decolourization of Methylene blue dye by H_2SO_4 modified and unmodified tea leaves and granules

S. No.	Parameter	Dye Decolourization (%)			
	Temperature (˚C)	Tea Leaves		Tea Granules	
		H_2SO_4 Modified (%)	Unmodified (%)	H_2SO_4 Modified (%)	Unmodified (%)
1.	20	79.07	40.45	82.70	68.16
2.	25	82.24	47.30	85.51	69.66
3.	30	81.77	54.90	86.62	67.45
4.	35	79.85	58.47	83.62	66.95

Incubation conditions:

Dye concentration (mg/L): 10.

Time (hours) : 2

Temperature (˚C): 20, 25, 30, 40.

pH: 7.0

Table 7. Effect of modification of tea leaves and granules on the dye adsorption capability

S. No.	Modification method	Dye decolourized (%)
1.	Thermal activation (Tea Leaves)	89.7
	Thermal activation (Tea Granules)	84.3
2.	H_2SO_4 modified (Tea Leaves)	94.6
	H_2SO_4 modified (Tea Granules)	86.7
	Incubation Conditions Incubation temperature (˚C): 70 (TG) and 80 (TL) Incubation time (minutes): 120 Dye concentration (mg/L): 10 pH: 7 (TG) and 8(TL).	

Table 8. Effect of composition of nutrient medium on the growth of *Phanerochaete chrysosporium*

S. No.	Medium	Incubation period (days)	Fungal growth
1.	Potato Dextrose Agar	Day 1	+
		Day 2	+
		Day 3	++
		Day 4	+++
		Day 5	++++
2.	Malt Extract Agar	Day 1	-
		Day 2	+
		Day 3	+
		Day 4	++
		Day 5	+++
		Day 6	++++
3.	Tea Waste agar	Day 1	-
		Day 2	-
		Day 3	-
		Day 4	+
		Day 5	+
		Day 6	++

Incubation conditions:

Temperature (˚C) : 29

Result Interference:

No Growth: -

Poor: +

Fair: ++

Good: ++++

Table 9. Effect of incubation temperature on the production of laccase by *P.chrysosporium* under SSF

S. No.	Incubation Temperature (˚C)	Laccase Activity (U/ml)	
		H_2SO_4 Modified T.L.	H_2SO_4 Modified T.G.
1.	20	51.6 ± 1.2	39.6 ± 0.5
2.	25	110 ± 1.5	86 ± 0.8
3.	30	153 ± 1.9	118 ± 1.3
4.	35	147 ± 1.6	109 ± 0.9
Incubation conditions: Inoculum size (mm): 0.5 pH of the moistening agent: 6.0 Substrate: moistening agent: 1:3 Incubation duration (days): 7 (TG) and 8 (TL)			

Table 10. Laccase enzyme production by *P. chrysosporium* in the case of H_2SO_4 modified tea granules and tea leaves as a substrate

S. No.	Substrate Used	Laccase Production (U/ml.)
1.	H_2SO_4 modified tea granules	74.3 ± 0.9
2.	H_2SO_4 modified tea leaves	145.2 ± 1.4
	Incubation conditions: Temperature (˚C): 29 Inoculum size (mm): 0.5 pH of the moistening agent: 6.0 Incubation duration (days): 7 (TG) and 8 (TL).	

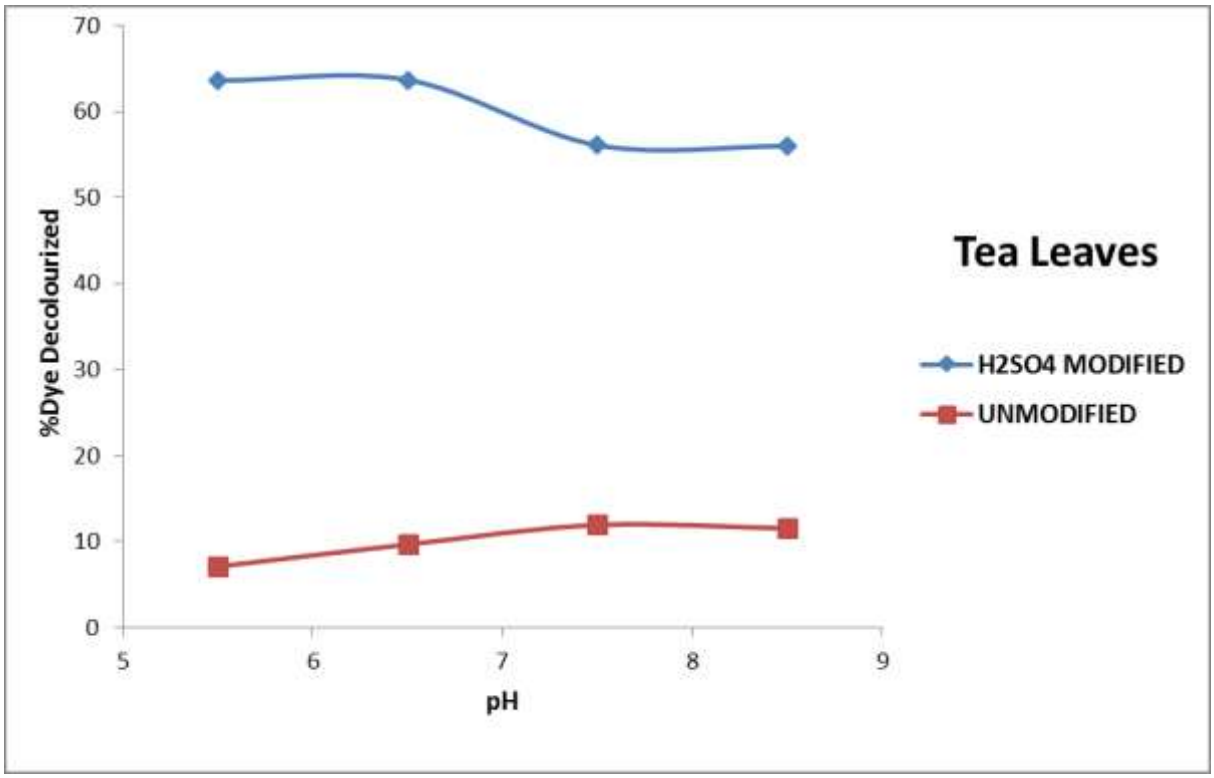


Figure 1. Effect of pH on decolourization of dye by H_2SO_4 modified and unmodified tea leaves

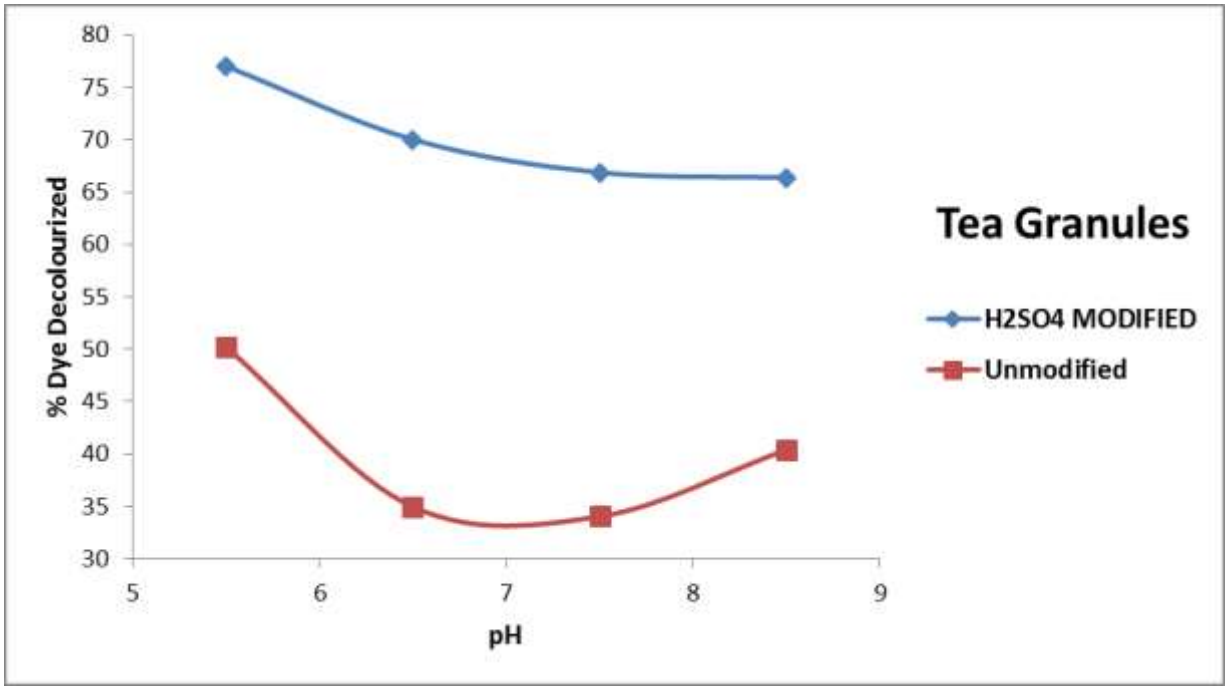


Figure 2. Effect of pH on decolourization of dye by H_2SO_4 modified and unmodified tea granules

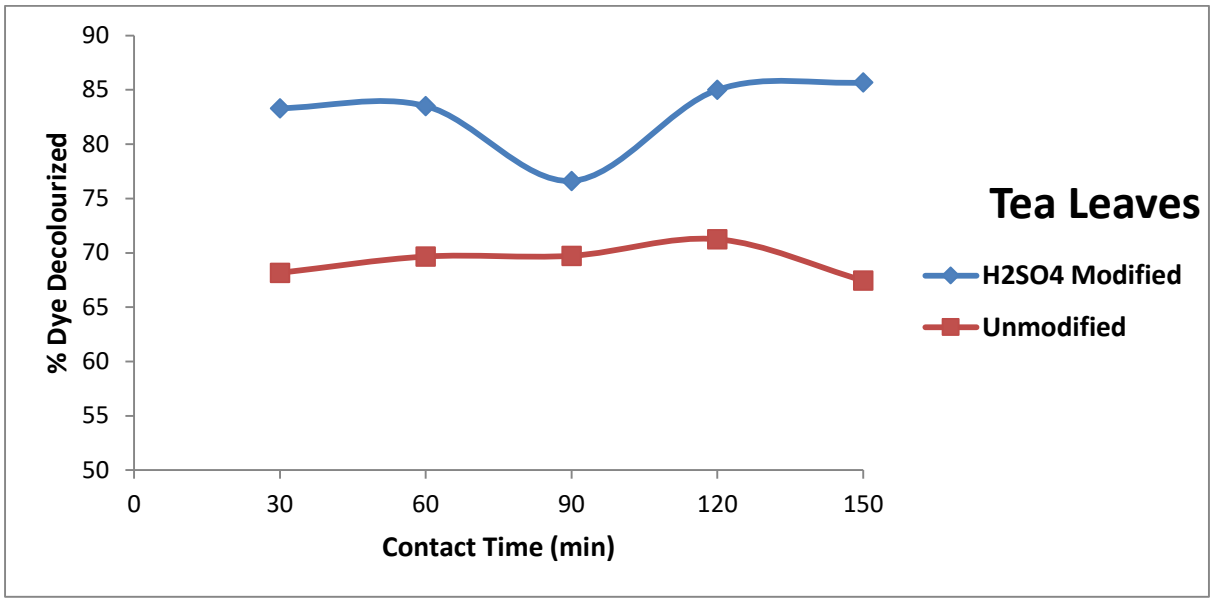


Figure 3. Effect of contact time on decolourization of dye by H_2SO_4 modified and unmodified tea leaves

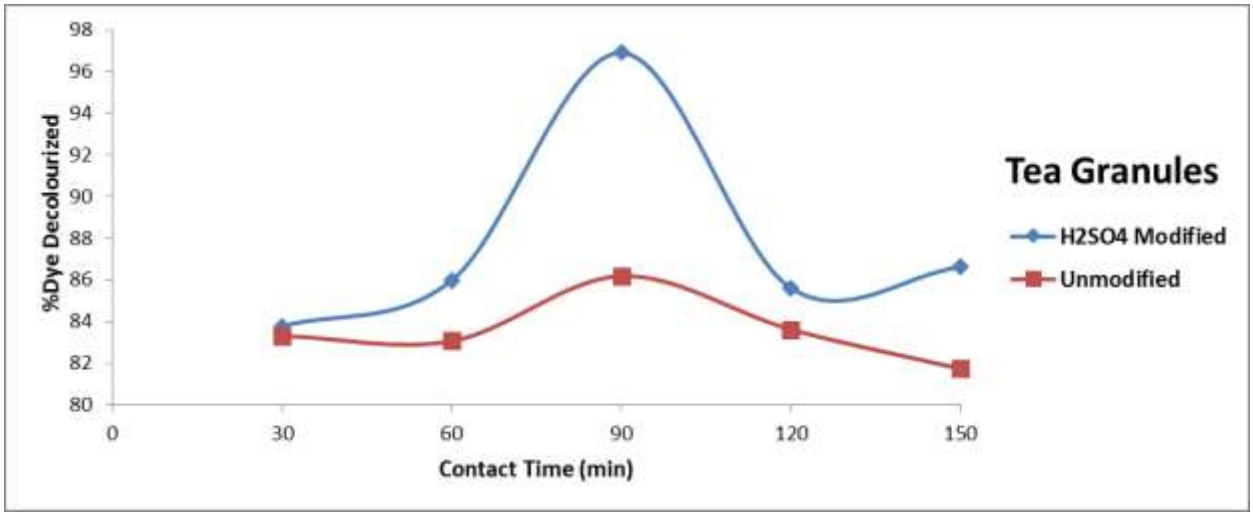


Figure 4. Effect of contact time on decolourization of dye by H_2SO_4 modified and unmodified tea granules

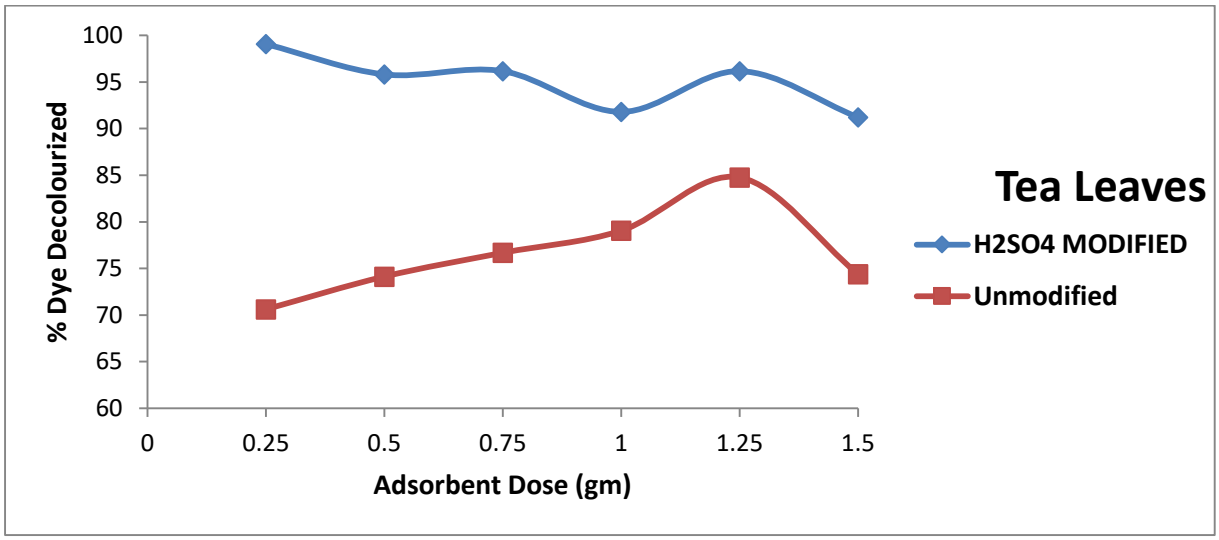


Figure 5. Effect of adsorbent dose on decolourization of dye by H_2SO_4 modified and unmodified tea leaves

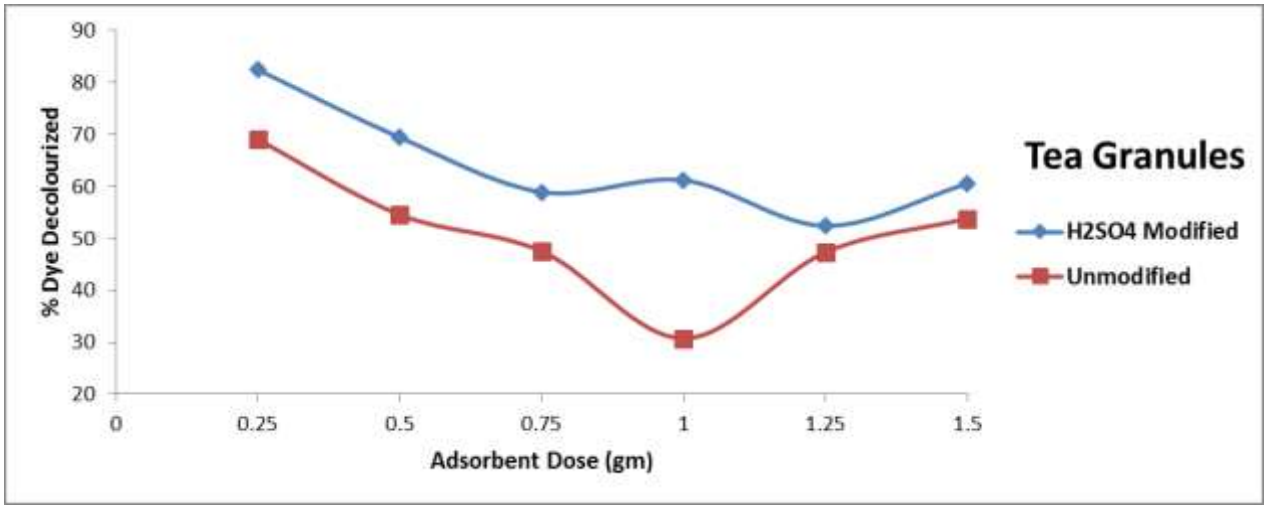


Figure 6. Effect of adsorbent dose on decolourization of dye by H_2SO_4 modified and unmodified tea granules

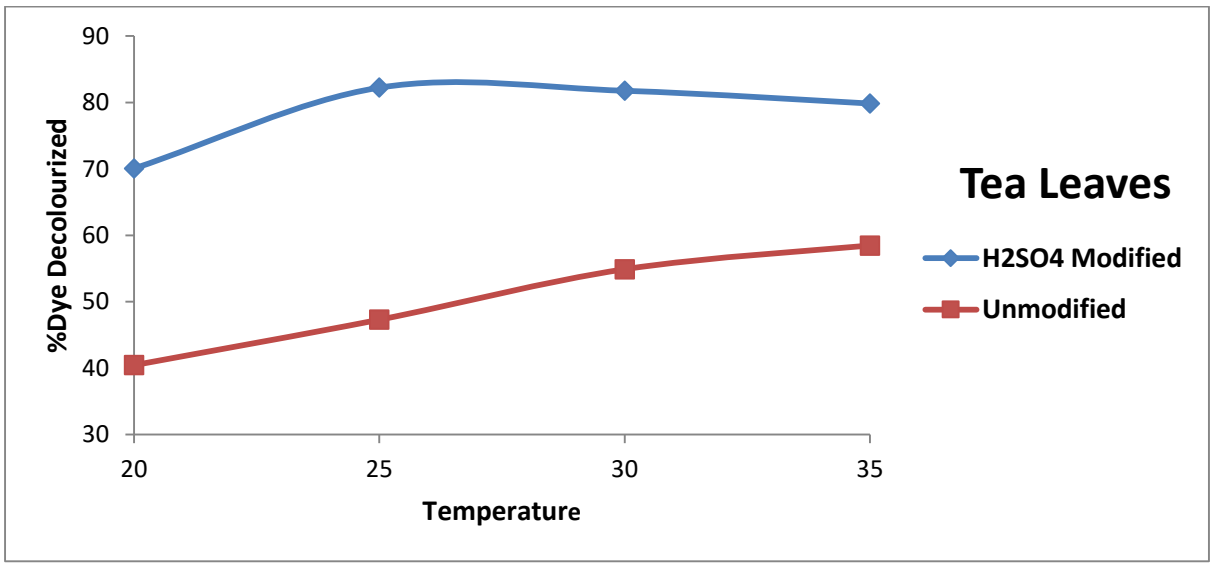


Figure 7. Effect of temperature on the decolourization of Methylene blue dye by the H_2SO_4 modified and unmodified tea leaves

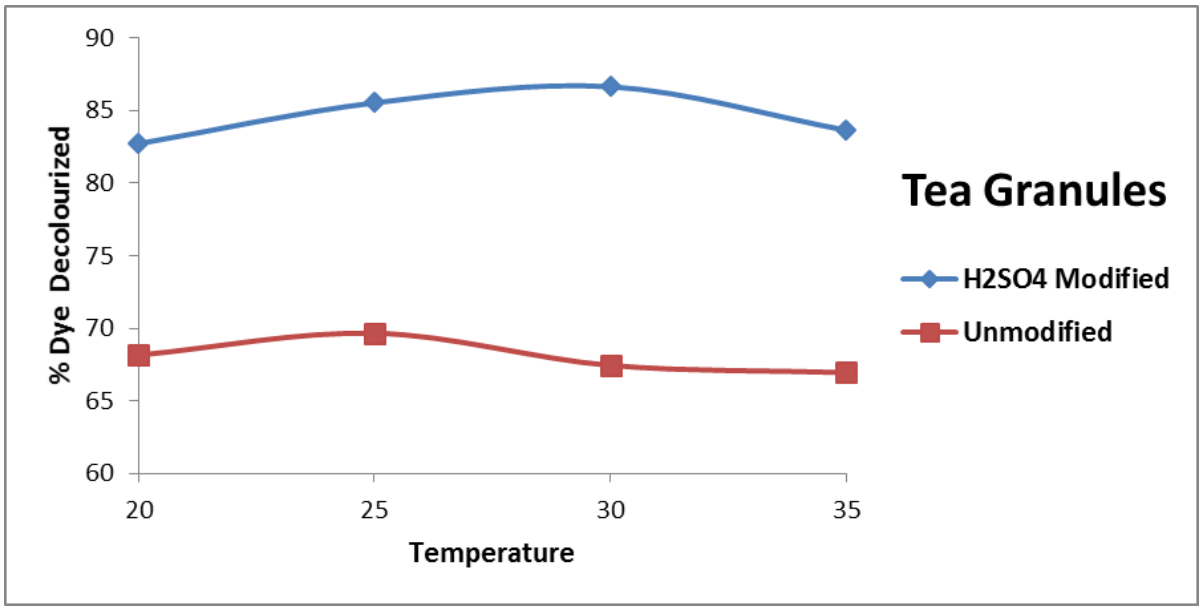


Figure 8. Effect of temperature on the decolourization of Methylene blue dye by the H_2SO_4 modified and unmodified tea granules

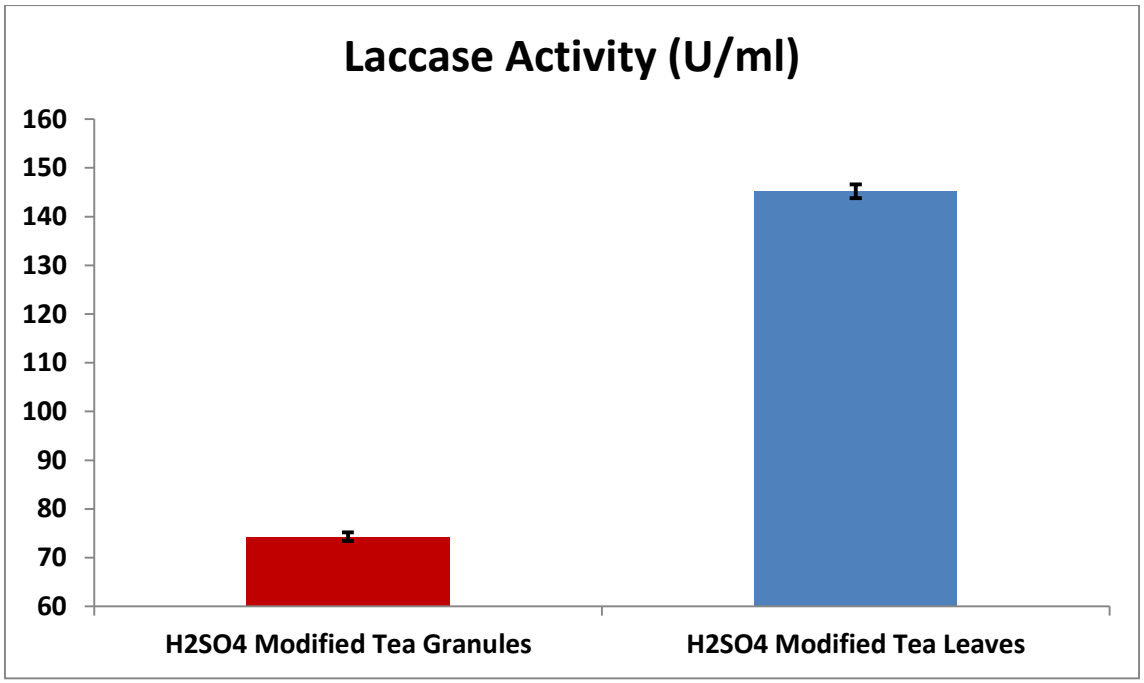


Figure 9. Laccase production by *Phanerochaete chrysosporium* in the H_2SO_4 modified tea leaves and granules SSF.

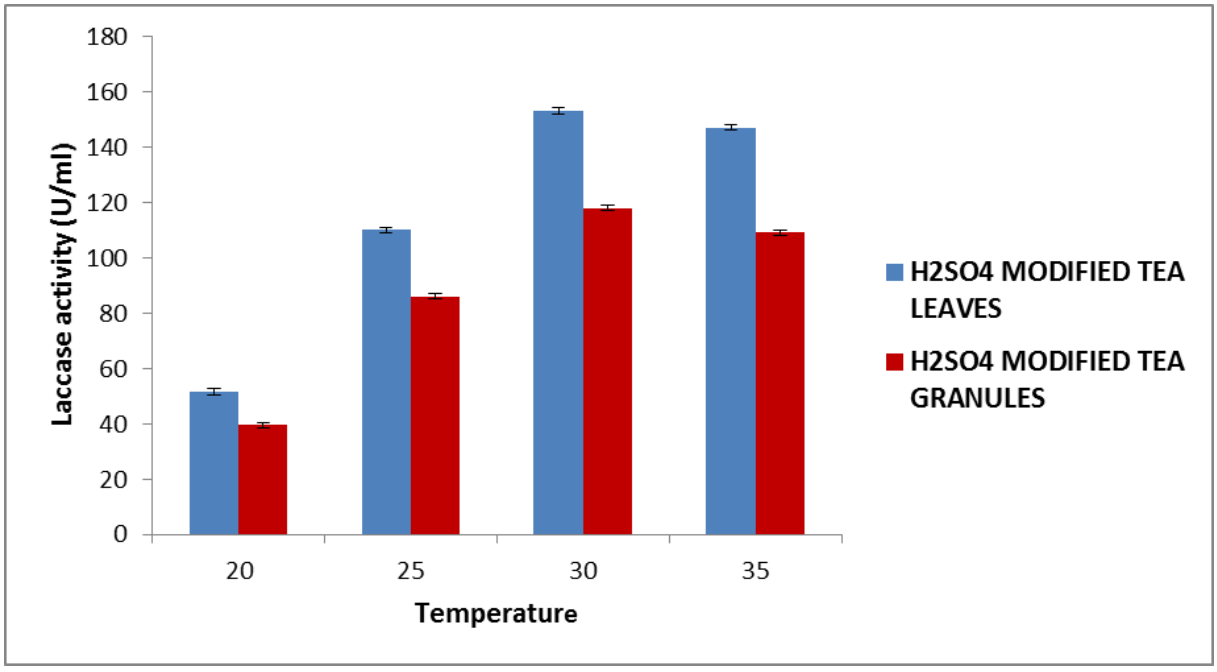


Figure 10. Effect of incubation temperature on the production of laccase by *Phanerochaete chrysosporium*

Picture 1(a) Growth of *Phanerochaete chrysosporium* on MEA plates.

Picture 1(b) Growth of *Phanerochaete chrysosporium* on MEA slants.

Picture 1. Growth of *Phanerochaete chrysosporium* on Malt Extract agar plates and slants (a-b)

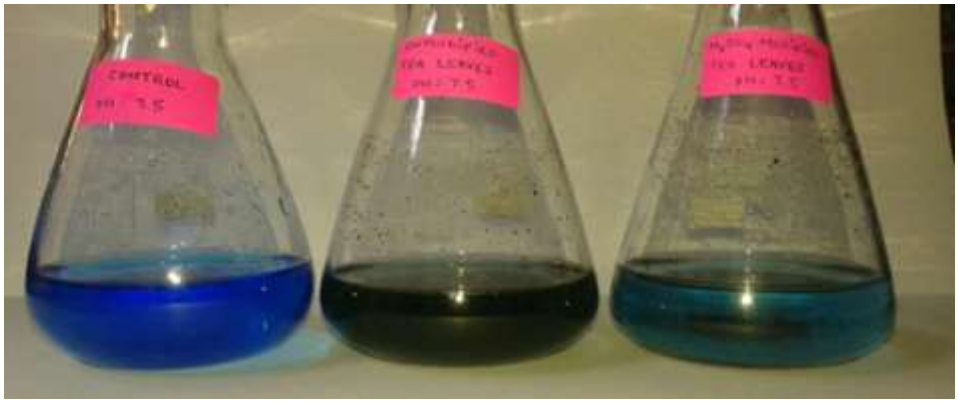
Picture 2(a) Tea Granules

Picture 2(b) Tea Leaves

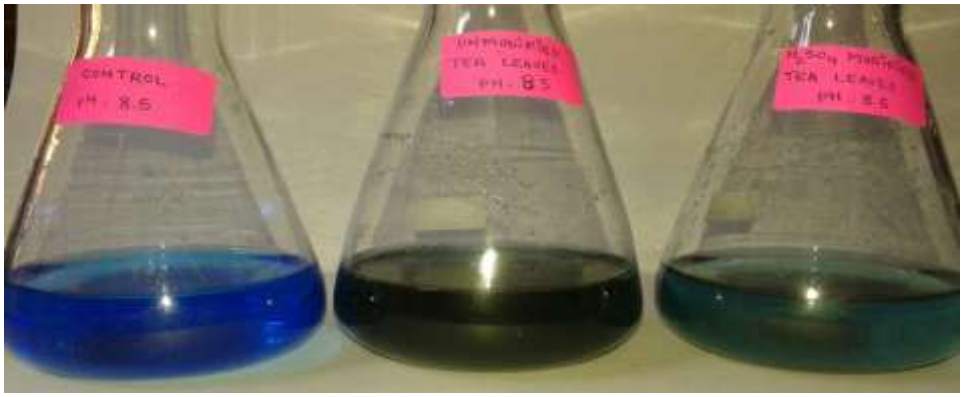
Picture 2. (a - b) Collection and preparation of tea leaves and granules samples

3 (a) Dye decolourization by H_2SO_4 modified and unmodified TG and TL at pH: 5.5

3(b) Dye decolourization by H_2SO_4 modified and unmodified TG and TL at pH: 6.5



3(c) Dye decolourization by H_2SO_4 modified and unmodified TG and TL at pH: 7.5



3(d) Dye decolourization by H_2SO_4 modified and unmodified TG and TL at pH: 8.5

Picture 3. Effect of pH on dye decolourization by H_2SO_4 modified tea leaves and granules (a-d)

4(a) Dye decolourization by H_2SO_4 modified and unmodified TG and TL after 30 Minutes.

4(b) Dye decolourization by H_2SO_4 modified and unmodified TG and TL after 60 Minutes.

4(c) Dye decolourization by H_2SO_4 modified and unmodified TG and TL after 90 minutes.

4(d) Dye decolourization by H_2SO_4 modified and unmodified TG and TL after 90 minutes.

4(e) Dye decolourization by H_2SO_4 modified and unmodified TG and TL after 150 minutes.

Picture 4. Effect of contact time on dye decolourization by H_2SO_4 modified tea leaves and granules (a- e)

5(a) Dye decolourization by H_2SO_4 modified and unmodified TG and TL with adsorbent dose of 0.25 gm.

5(b) Dye decolourization by H_2SO_4 modified and unmodified TG and TL with adsorbent dose of 0.50 gm.

5(c) Dye decolourization by H_2SO_4 modified and unmodified TG and TL with adsorbent dose of 0.75 gm.

5(d) Dye decolourization by H_2SO_4 modified and unmodified TG and TL with adsorbent dose of 1.0 gm.

5(e) Dye decolourization by H_2SO_4 modified and unmodified TG and TL with adsorbent dose of 1.25 gm**.**

5(f) Dye decolourization by H_2SO_4 modified and unmodified TG and TL with adsorbent dose of 1.50 gm.

Picture 5. Effect of adsorbent dose on dye decolourization by H_2SO_4 modified tea leaves and granules (a-f)

6(a). On modified tea leaves Agar

6(b). On modified tea granules agar

6(c). On Malt extract agar

6(d). On Potato dextrose agar

Picture 6 (a-d). Effect of the substrate / nutrient medium composition on the growth of *Phanerochaete chrysosporium*

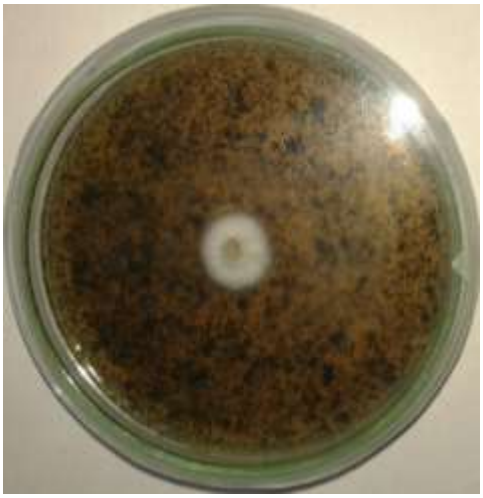
7.(a)

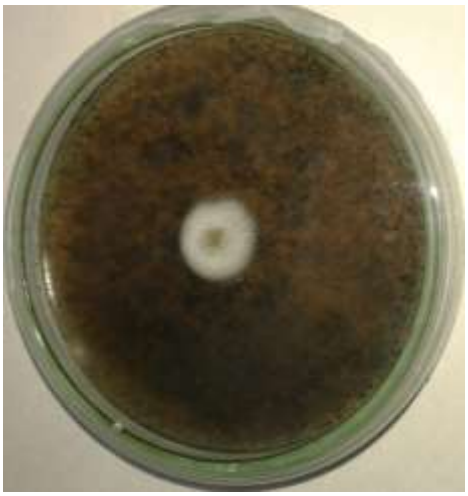
7.(b)

7 (c)

7 (d)

Picture 7 (a-d) Effect of incubation temperature on the production of laccase by *Phanerochaete chrysosporium*





8 (a) Dye absorbed tea leaves agar **8 (b) Dye absorbed tea granules agar**

Picture 8 (a-b) Laccase production by *Phanerochaete chrysosporium* in the H_2SO_4 modified tea leaves and granules SSF.

Chapter 7

Conclusion and Future Scope

Today pollution is one of the major problem faced by the environmental resources. And unfortunately it is increasing day by day due to the increase in the human activities and industrialization. The main cause of the water pollution is the dyes discharged from the industrial effluents and causing harmful effects on the human life. As a results of this, most research has been done to find the cost effective methods for the removal of these types of dyes. This work was done on the improvement of methylene blue decolourization by modifying tea sample with H_2SO_4 and Potassium acetate and comparing it by testing along with unmodified tea samples. And finally to use it as a substrate for *Phanerochaete chrysosporium* for the further checking of methylene blue decolourization process in search of producing significant results.

From the data it was observed that the potassium acetate modified tea samples were observed as very less effective in the adsorption and decolourization of methylene blue as even the control of unmodified tea leaves and granules were also more effective than potassium acetate modified sample. Where, H_2SO_4 modified tea leaves and granules were effective in adsorption and dye decolourization of methylene blue leading to 94 % and 86 % decolourization as compared to the unmodified tea leaves and granules. And as a tea waste is commonly available household waste, thus it is cheaper substrate that can be utilized at large scale and could be modified with H_2SO_4 to increase the decolourization process and in the removal of the dyes.

Beside this, many microorganisms mainly white rot fungi have been found capable of decolourizing and degrading the numerous dyes by producing different classes of modified lignin enzymes. Furthermore in this study dye adsorbed H_2SO_4 modified tea waste was evaluated as a substrate for the growth and laccase activity by *Phanerochaete chrysosporium*, it gave positive results but showed very less laccase production may be due to modification done. These shows that the H_2SO_4 modified tea leaves and granules could not be used further as a substrate or laccase production for the *Phanerochaete chrysosporium* growth and for the further aid in the process of the methylene blue dye decolourization.

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Appendix

1. **Media**: Media which was used in the project work: **Malt Extract Agar** (MEA).

Composition:

Peptic digest of animal tissue:		0.78 gram /L
Maltose (Sugar)	:	12.75 gram /L
Dextrin (Sugar)	:	2.75 gram /L
Final pH	:	4.7 ± 0.2
Agar	:	15 gram / L

2. **Nutrient salt solution**:

Composition:

$(NH_4)_2SO_4$: 0.14 %

KH_2PO_4 : 0.2 %

$MgSO_4.7H_2O$: 0.3 %

$CaCl_2$: 0.03%

$FeSO_4.7H_2O$: 0.002 %

$ZnSO_4.7H_2O$: 0.002 %

$MnSO_4.7H_2O$: 0.002 %

$COCl_2$: 0.002 %

pH : 4.5