BIOETHANOL PRODUCTION FROM TEA FUNGAL BIOMASS GROWN ON TEA MANUFACTURE WASTE

Thesis submitted to Department of life science for the partial fulfillment of the M.Sc. Degree in Life science

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CERTIFICATE

This is to certify that the thesis entitled "**BIOETHANOL PRODUCTION FROM TEA FUNGAL BIOMASS GROWN ON TEA MANUFACTURE WASTE"** which is being submitted by **Mr. Manoj Narayani**, Roll No. **411LS2055**, for the award of the degree of Master of Science from National Institute of Technology, Rourkela, is absolutely based upon his work carried out under my supervision. The results embodied in this thesis are new and have not been submitted to any other university or institution for the award of any degree/diploma.

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DECLARATION

I do hereby declare that the Project Work entitled **"BIOETHANOL PRODUCTION FROM TEA FUNGAL BIOMASS GROWN ON TEA MANUFACTURE WASTE"**, submitted to the Department of Life Science, National Institute of Technology, Rourkela is a faithful record of bonafide and original research work carried out by me under the guidance and supervision of Dr. Rasu Jayabaln, Asst. Professor, Department of Life Science, National Institute of Technology, Rourkela, Odisha.

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Although theory is heard in many ways which seems to be very simple and effortless but when it comes to real ground then it matters and competes to retrospect the system in each and every way to realize the real retention process which leads to perfection. I feel myself speechless before them to enumerate their help and guidance who are real pedagogues.

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ABBREVIATION

KT: Kombucha tea

TF: Tea fungus

SG: Specific gravity

FTIR: Fourier transform infrared spectroscopy

SEM: Scanning electron microscope

KBr: Potassium Bromide

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ABSTRACT

Tea fungus which is used to prepare Kombucha tea (KT) is a symbiotic growth of acetic acid bacteria and osmophilic yeast strains in a thick jelly membrane which has to be cultured in sugared tea. KT is composed of fermented tea broth and cellulosic pellicle layer. A portion of cellulosic pellicle layer (tea fungus or kombucha) is used to start next batch of fermentation and the remaining portion is thrown as waste. Cellulose available in tea fungal biomass can be utilized as a substrate for bioethanol production. Tea waste material is the by-product produced during the process of black tea manufacture which will be dumped in tea industries as waste material. It has been suggested that tea waste material can be utilized as a substrate for the growth of tea fungus. The growth of tea fungus is influenced by concentration of sucrose, tea manufacture waste material and surface area to depth ratio of culture medium. It was experimentally found that 7% of sucrose and 1% of tea manufacture waste is having optimum yield of tea fungus growing in shallow container with a very wide opening. Extract of tea waste material can be utilized as an inexpensive substrate for bioethanol production from tea fungal biomass. Today, the economics are more favourable towards development of alternative fuel sources with particular reference to alcohols. Bioethanol is a suitable candidate which can be used as biodegradable fuel additive as ethanol/diesel blend to combat the global fuel crisis by replacing the fossil fuel based fuels.

1. INTRODUCTION

1.1. Bioethanol

Depletion of fossil fuels, hike in price of petroleum-based fuels and global warming are gaining great concern and this situation demanded the search for alternative, sustainable, renewable, efficient and cost-effective alternative sources with lesser greenhouse gas emissions. Bioethanol is a suitable candidate to replace the gasoline, but it is extremely important to make sure that the development of bioethanol is not obstructed by raw materials constraints. Bioethanol and biodiesel are emerging as a potential alternative fuels which can replace the conventional energy fuels like petrol and diesel. However, bioethanol is expected to be the most widely used future fuel around the globe which can be produced from starch/cellulose biomass. Bioethanol has several advantages over gasoline. Bioethanol is less toxic, is readily biodegradable, and produces fewer air-borne pollutants than petroleum fuel. It can also reduce the greenhouse gas levels. Not only as fuel, bioethanol can also be used as a fuel for electric power generation, in fuel-cells (thermo-chemical action), in power co-generation systems, as a raw material in chemical industries and as a main component in beverage industries. Bioethanol can be employed to replace octane enhancers such as methylcyclopentadienyl manganese tricarbonyl (MMT) and aromatic hydrocarbons such as benzene or oxygenates such as methyl tertiary butyl ether (MTBE). Currently, industrial scale production of bioethanol utilizes feedstock crops. But, it has raised doubts about its potential impact on food supply and security in the future. Hence, research on alternative, sustainable and economic resource to replace food-based feedstock has been triggered.

1.2. Tea fungus (TF)

Tea fungus (Medusomycesgisevii) is the most usual name for a symbiotic growth of acetic acid bacteria and osmophilic yeast strains in a thick jelly membrane (zoogleal mat) which has to be cultured in sugared tea to produce kombucha tea which is used as the ultimate health and refreshing beverage. Sugared tea is the substratum of tea fungus for its growth and existence. Tea fungus ferments the sugared tea by converting the added sugar into organic acids and ethanol. Tea fungus utilizes sugar as its carbon source and forms a new jelly membrane during fermentation. Only a portion of the newly formed tea fungus membrane is used for further

fermentation and the remaining portion is thrown as waste [1]. Acetic acid bacteria produce cellulose net on the surface of the tea where the cell mass of bacteria and yeasts are attached. The net is the secondary metabolite of tea fungus fermentation, but also one of the main characteristics of the culture. Tea fungus is not a mushroom. It has no spores and reproduced by vegetative sprouting. The name 'tea fungus' is a misnomer and arises from the unique ability of bacteria to synthesize a floating cellulose network that resembles a surface mold on non-agitated medium. Bacteria and yeast strains present in kombucha form a powerful symbiosis that can inhibit the growth of potential contaminating bacteria. Cellulose produced during the fermentation of sugared tea appears as a thin film on top of the tea where the cell mass of bacteria and yeast is attached (Fig. 1).

1.3. Kombucha tea (KT)

Kombucha tea is slightly sweet, slightly acidic refreshing beverage consumed worldwide, obtained by the fermentation of sugared tea by a symbiotic association of bacteria and yeasts, forming "tea fungus" [2]. The tea fungus broth is composed of two portions, a floating cellulosic pellicle layer and the sour liquid broth. This refreshing beverage tasting like sparkling apple cider is often produced in the home by fermentation using a tea fungus passed from home to home. Black tea and white sugar are the best substrates for the preparation of kombucha, although green tea can also be used.

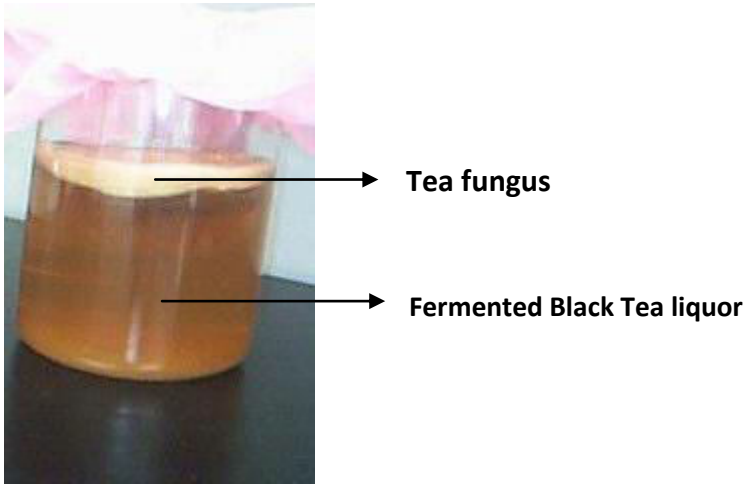


Fig. 1 : **Tea fungus growing in black tea liquor during kombucha fermentation**

1.4. Microbiology of tea fungus

The main acetic acid bacteria found in the tea fungus are A. xylium, A. xylinoides, Bacterium gluconicum, A. aceti and A. pasteurianus. The yeasts were identified as Schizosaccharomycespombe, Saccharomyces ludwigii, Kloeckeraapiculata, Saccharomyces cerevisiae, Zygosaccharomycesrouxii, Z. bailii, Brettanomycesbruxellensis, B. lambicus, B. custersii, Pichiamembranaefaciens, Torulopsis and Candida [3, 4]. The exact microbiological composition also depends on the source of inoculum of the tea fermentation. Growth patterns of these microorganisms during the fermentation process of kombucha are not well documented [5].

1.5. Symbiosis

Bacteria utilize ethanol to grow and produce acetic acid and the presence of acetic acid stimulates the yeasts to produce ethanol. Such a symbiotic interaction was also observed between the yeast Saccharomyces cerevisiae and the bacterium Gluconobacteroxydans in natural fermentation of reconstituted orange juice [2]. Acetobacter bacteria can be found in symbiotic relationships with many different plants, such as sugarcane and coffee as well as in fermenting vinegar. Many of these bacteria have been observed to promote plant growth, but the mechanisms behind this relationship are not yet fully understood. Acetobacteris strictly aerobic bacteria using the hexose monophosphate pathway and the tricarboxylic acid cycle for the breakdown of sugars. It oxidises sugars, alcohols and steroids directly. It can grow on ethanol and acetate as sole source of carbon, but not methanol. Some strains require growth factors. It performs ketogenesis from glycerol. Ethanol and acetic acid are well-known growth inhibitors and are used as antimicrobial agents. On the other hand, they are products, by-products or substrates of fermentation. Ethanol toxicity and mechanism of adaptation of yeast cells have been extensively studied. Ethanol inhibits cell growth, viability, solute accumulation and proton fluxes. It primarily affects the plasma membrane resulting in an altered membrane organization and permeability. Yeasts cells in tea fungus are found to be resistant to ethanol which is formed during kombucha fermentation. Several reports on increased ethanol tolerance of immobilized yeast cells have been published [6, 7]. Acetic acid is a by-product of ethanol production and inhibits fermentation in an exponential way. The mechanism of its toxicity involves the acidification of the cytoplasm and modifying certain enzymes of glycolysis [8]. As glycolysis is

absent in Acetobacters, acetic acid exerts its toxic effect probably by blocking the enzymes of tricarboxylic acid cycle.

1.6. Preparation of kombucha tea

Kombucha tea is prepared by placing the kombucha culture in a solution of tea and sugar and allowing to ferment. If tea fungus is cultivated according to the standard recipe on black tea, sweetened with sucrose, it turns this substrate into a refreshing beverage called tea fungus beverage with high nutritive value and medicinal properties [9]. By virtue of the numerous health-promoting aspects reported and the easy and safe preparation of this beverage at home, it has gained popularity as other traditional beverages. The standard procedure was as follows: Tap water (1L) was boiled and during boiling 50 g of sucrose was stirred into solution. Subsequently 5 g tea was added and removed by filtration after 5 min. After cooling to room temperature (20ºC) the tea was inoculated with 24 g tea fungus and poured into a beaker (1 L) that had been previously sterilized with hot water. The growth of undesired microorganisms was inhibited by addition of 0.2 L fermented kombucha, thus lowering the pH value. The beaker was covered with a paper towel to keep Drosophila flies away. The incubation was carried out at 20 to 22ºC. The optimal temperature is in wide range between 18 and 26ºC. In the next few days the culture will float to the surface towards the air and start to form a clear or translucent thin skin of jelly across the entire available surface. This is the newly formed daughter culture beginning as a new layer above the mother. The tea will start to smell fermented and a few gas bubbles appear from the carbonic acid formed during fermentation. The mother culture will remain in the same size as it went in and stay under the daughter culture. After 10-14 days, a new tea fungus had developed on the surface of the tea as a disc of 2 cm thickness covering the whole diameter of the beaker. The tea fungus is removed from the surface and kept in a small volume of fermented tea. The beverage is passed through cheesecloth and stored in capped bottles at 4ºC [3]. The taste of the kombucha changes during fermentation from a pleasant fruit sour-like lightly sparkling flavour after a few days, to a mild vinegar-like taste with prolonged incubation. It is remarkable that 50 g sucrose/L gave the optimal concentrations of ethanol and lactic acid and this sugar concentration has been used in traditional recipes for the preparation of teakwass for a long time [3]. An optimum fermentation time is required for the production of

drinkable kombucha. Longer fermentation often results in the production of too high levels of acids (like mild vinegar) that may pose potential risks when consumed [5].

1.7. Cellulose network

Cellulose produced during the fermentation of A. xylinum appears as a thin film on top of the tea where the cell mass of bacteria and yeasts is attached. This fungus like mixture of microorganisms and cellulose is likely why kombucha is also called "tea fungus" [5]. Bacterial cellulose prepared from pellicles of A. xylinum (Gluconacetobacterxylinus) is a unique biopolymer in terms of its molecular structure, mechanical strength and chemical stability [10]. A similar cellulose network floating on the surface of various fruit juices fermented by a symbiotic culture composed of A. xylinum and yeasts and named "nata" is consumed in Philippines as a delicacy. In Brazil, this cellulose network is used for the treatment of skin burns and other dermal injuries and is produced by a pure culture of A. xylinum grown on a medium composed mainly of sucrose and tea xanthenes [11]. Caffeine and related compounds (theophylline and theobromine) are identified as activators for cellulose production in A.xylinum [12] In ancient days, this cellulose biofilm has been used for the treatment of wounds. Microbial cellulose synthesized in abundance by Acetobacterxylinum shows vast potential as a novel wound healing system. The high mechanical strength and remarkable physical properties result from the unique nanostructure of the never-dried membrane [13].

1.8. Tea fungus – cellulose rich biomass

Tea fungus is an excellent example for biofilm which consists of bacteria and yeasts. A part of the fungal mat produced during black tea fermentation is used as starter culture, while the remaining goes as a waste. Recycling of waste tea fungal mat is one of the most important means of utilizing it. The waste tea fungal biomass obtained from kombucha fermentation is rich in fibre, which includes cellulose and hemi-cellulose with good quality of protein [14]. Microbialcellulose has several practical implications in biotechnology and other fields of biomedical sciences. It is produced comparatively in larger quantities unlike other microbial polymers. In the recent past, cellulose membranes/sheets have been suggested for use as biobased packaging materials for food contact applications. In ancient days, this cellulose biofilm has been used for the treatment of wounds. Microbial cellulose synthesized in abundance

by A. xylinum shows vast potential as a novel wound healing system [13]. Fontana et al. [15] [15] reported that cellulose pellicle produced by Acetobacter can be utilized as temporary skin substitute in the treatment of skin wounds, such as burns, ulcers, grafts, and as an adjuvant indermal abrasions. Also, this dried tea fungal mat can be used as biosorbent for the removal of heavy metals. The use of nonliving biomass of yeast Saccharomyces as a suitable biosorbent of metal ions (lead, zinc, copper, and nickel) is also reported.

1.9. Bioethanol from tea fungus

Cellulose biomass in tea fungus can be converted to monosaccharides by saccharification process aided by cellulase enzyme. Glucose molecules produced during saccharification process will be converted to ethanol by yeast fermentation. Tea fungal biomass is usually grown in sugared tea decoction. Tea fungus is able to grow in green tea, black tea and tea waste material. Tea waste material is fiber like by product which is a voluminous waste obtained during black tea manufacturing process in tea industries. Tea fungus requires at least 7 days forming a thick jelly membrane. Cellulose from this jelly membrane can be extracted in wet form or in dry form. Dried membrane is the suitable form for cellulose extraction. Extraction of cellulose can be done using hot water. The extract will be saccharified using cellulase enzyme. Saccharified product will be the source for ethanol fermentation by yeasts.

1.10. Economic viability of ethanol production from tea fungus

Since tea fungus is the byproduct during kombucha tea preparation and can be grown even in decoction prepared from tea waste material, the cost of raw materials can be eliminated. Cost of sugar can also be excluded by finding some waste carbohydrate rich materials. Hence, the only cost involved is extraction process, cellulase enzyme and distillation technique. Growing tea fungus in decoction prepared from tea waste material and waste carbohydrate rich materials will be a highly economical way for producing bioethanol.

1.11. Alternative sources of sugar

An alternative source of sugar can be supplemented by several residues from agro-forestry industries, namely grape skins aqueous extract, cheese whey, crude glycerol and sulfite pulping liquor were evaluated as economic carbon and nutrient sources for the production of bacterial cellulose [16]. These residues possess high organic loads and are rich in nutrients suitable for microbial growth from the aforementioned residues, only cheese whey was tested as a carbon and nutrient source for the production of bacterial cellulose. However, the use of complex substrates for bacterial cellulose production did not affect the quality of cellulose mats, since no significant differences were detected by the characterization techniques applied [16]. Hence, it is clear that bacterial cellulose can be produced by using by products from agro-forestry industries. Tea fungus also involves the bacterial cellulose production by Acetobacter species. Thus, it is possible to grow the tea fungus in tea decoction supplemented with carbon sources from byproducts of agro-forestry industries.

2. REVIEW OF LITERATURE

2.1. Kombucha tea

Kombucha tea is sugared black tea fermented for about 14 days with a consortium of acetic acid bacteria and yeasts, named as "tea fungus". The name tea fungus is a misnomer since there is no fungus involved in the fermentation [17]. Like green tea and black tea, kombucha black tea can also be bottled for commercialization. The findings of various health benefits of kombucha tea have led to a general consumer's appreciation for its functional properties. Thus, kombucha tea is consumed not only to satisfy consumers' fine taste buds but also to impart health benefits. Tea fungus is an excellent example of a biofilm that consists of bacteria and yeasts. Several bacterial and yeast species are reported to be present in the tea fungal consortium [18]. After fermentation, the kombucha tea is filtered through a cheese cloth and is consumed as a health drink. When kombucha tea is stored at 20 °C, the biofilm continues to form due to the presence of microorganisms in it.

2.2. History of Kombucha tea

Kombucha is the internationally used Germanized form of the Japanese name for slightly fermented tea beverage. It was first used in the orient for its healing benefits. Kombucha known by many names, was originated in Northeast China (Manchuria) where the "Divine Che" was prized during Tsin Dynasty ("Ling Chi"), 220 B.C. for its detoxifying and energizing properties. In 414 A.D., the physician Kombu brought the tea fungus to Japan from Korea to cure the digestive troubles of the Emperor Inkyo. As trade routes expanded, Kombucha (former trade name "Mo-Gu") found its way first into Russian (Cainii grib, Cainii kvass, Japonski grib, Kambucha, Jsakvasska) then into eastern European countries, appearing in Germany (Heldenpelz, Kombuchaschwamm) around the turn of the 20th century. During World war II, this beverage was introduced into Germany, then in the 50's, it arrived in France and also in France-dominated north Africa where its consumption was quite popular. The habit of drinking fermented tea became acceptable throughout Europe until World War II brought widespread shortages of the necessary tea and sugar ingredients. In the postwar years, Italian society's passion for the beverage ("Funko cinese") peaked in the 1950s. Then, in the 1960s, scientific

research in Switzerland reported that drinking kombucha was similarly beneficial like eating yogurt, and kombucha's popularity increased. Today in the United States Kombucha is soldnationwide in retail food markets as part of an herbal tea blend, Sun Luck green tea with kombucha (San Francisco, CA, USA) and the kombucha journal is electronically published worldwide in several languages [19]. Currently kombucha is alternately praised as "the ultimate health drink" or damned as "unsafe medicinal tea" [11, 20].

2.3. Changes in biochemical constituents of tea during kombucha fermentation

Catechins are one of the few groups of flavanoid compounds possessing a significant degree of bioavailability [21]. Some bacteria may degrade many phenolic compounds including catechins and catechin degradation products like catechol and protocatechuic acid [22-25]. Lewis et al. [26] isolated three species of Pseudomonas from soil which utilized catechin and also reported that catechin was degraded within 12 days in forest soils. Deschamps et al. [22] isolated catechin degrading Bacillus, Staphylococcus and Kelbsiella. Species of Rhizobium, such as Rhizobium japonicum, R. leguminosarum, R. phaseoli and R. trifolii utilized catechin as sole carbon source [27, 28]. Arunakumari et al. [29] reported that Pseudomonas solanacearum utilized spectrum of phenolic compounds such as tannic acid, catechin, tannin, phenol, catechol, resorcinol, phloroglucinol and protocatechuic acid. The catabolism of catechin by Bradyrhizobium was investigated by [30]. Recently, degradation of catechin by Acinetobacter calcoaceticus was investigated by [31].

Although catechins degradation in green tea, canned and bottled tea drinks have been reported [32-34] there is no study to date that has examined the stability of tea catechins and theaflavin during kombucha fermentation. The beneficial effects of kombucha tea are depending on its biochemical composition which includes polyphenols, organic acids and micronutrients produced during fermentation. Since tea fungus is a consortium of bacteria and yeasts, it is expected that there will be some influence of microorganisms on biochemical constituents of tea during kombucha fermentation. As the tea polyphenols are important in preventing cancer and other biochemical constituents are important for beneficial effects of kombucha tea, it is

therefore necessary to study the changes in biochemical constituents of tea during kombucha fermentation.

2.4. Antimicrobial activity of kombucha tea

Recent research on kombucha has proved that its antimicrobial activity against pathogenic microorganisms is largely attributable to acetic acid. Acetic acid is known to inhibit number of Gram positive and Gram negative microorganisms. Sreeramulu et al. [35] reported that kombucha tea could inhibit the growth of the pathogens, Entamoeba cloacae, Psuedomonas aeruginosa, Bacillus cereus, Escherichia coli, Aeromonas hydrophila, Salmonella typhimurium, Salmonella enteritidis, Staphylococcus epidermis, Leuconostoc monocytogenes, Yersinia enterolytica, Staphylococcus aureus, Shigella soneei,Campylobacter jejuni, Helicobacter pylori and Candida albicans. Their findings suggest other than acetic acid, large proteins and catechins in kombucha also contribute to antimicrobial properties of kombucha. According to their results, the antimicrobial activity of kombucha increased with fermentation time.

2.5. Antioxidant properties of kombucha tea

In recent years, there has been a global trend towards the use of phytochemicals present in natural resources, such as fruits, vegetables, oilseeds, and herbs as antioxidants and functional foods. Natural antioxidants can be used in the food industry, and there is evidence that these substances may exert their antioxidant effects within the human body. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are various forms of activated oxygen and nitrogen which include free radicals such as superoxide ions ($O2^{\bullet-}$), hydroxyl ($OH^{\bullet}$) and nitric oxide radicals ($NO^{\bullet}$) as well as non-free radical species such as hydrogen peroxide (H_2O_2) and nitrous acid (HNO_2). In living organisms, various ROS and RNS can be formed by different ways. Aerobic respiration stimulated polymorpho nuclear leukocytes, macrophages and peroxisomes and is the main endogenous sources of most of the oxidants produced by cells. Exogenous sources of free radicals include tobacco smoke, ionizing radiation, certain pollutants, organic solvents and pesticides. In vivo, some of the ROS play a positive role such as energy production, phagocytosis, regulation of cell growth, and intercellular signaling, or synthesis of biologically important compounds. Free radicals can cause lipid peroxidation not only in foods but also in cell membranes, which leads to deterioration of foods and decrease in membrane fluidity. ROS

and RNS may cause DNA damage in terms of mutation that could lead to cancer. In addition, ROS and RNS have been implicated in >100 diseases, including malaria, acquired immunodeficiency syndrome, heart disease, stroke, arteriosclerosis, diabetes and cancer. When produced in excess, ROS can cause tissue injury. However, tissue injury can itself cause ROS generation [36].

Lipid peroxidation has many deleterious effects on membrane structure and function [37] since it disrupts membrane order and generates many potentially cytotoxic products such as unsaturated aldehydes and lipid hydroperoxides that are highly toxic in vivo and in vitro. These compounds are able to inactivate enzymes[38], modify biomolecules by covalent binding of their fragments and initiate free radical-mediated modification of proteins and lipid peroxidation [39].

A potent scavenger of these species may serve as a possible preventive intervention for free radical-mediated diseases. All aerobic organisms, including human beings have antioxidant defenses that protect against oxidative damages and repair damaged molecules. However, this natural antioxidant mechanism can be inefficient, hence, dietary intake of antioxidant compounds will become important. Recent studies showed that a number of plant products including polyphenolic substances (flavonoids and tannins) and various plant or herb extracts exert antioxidant actions [40]. Phenolic compounds, like vitamin E and synthetic antioxidants (butylated hydroxyl anisole and bytulated hydroxyl toluene), are used to protect cosmetics, drugs and foods from oxidative degradation. It has been reported that synthetic antioxidant compounds have some side effects. Therefore, a research into the determination of the natural antioxidant source is important [41].

Components of black tea whose antioxidative properties have been explicitly proved are the catechins: (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG) as well as (-)- epigallocatechin gallate (EGCG). These compounds may be arranged according to their antioxidative properties: epigallocatechin = epigallocatechin gallate >> epicatechin gallate = epicatechin > catechin [42]. Antioxidative properties of catechins are manifested particularly by

their abilities to inhibit free radical generation, scavenge free radicals and chelate transition metal ions, mainly Fe and Cu, which are catalysts of free radical reactions. Based on the standard one-electron reduction potential (E8V) values, catechins should scavenge free radicals generated in an organism such as hydroxyl, superoxide and lipid radicals (alkoxyl radical, peroxyl radical, and alkyl radical). They also prevent free radical generation by inhibiting activity of existing enzymes participating in their generation, in particular, xanthine oxidase or by increasing the activity of enzymes with antioxidative properties probably by the way of induction of protein molecule biosynthesis [43]. Independently of catechins, theaflavins contained in black tea also possess antioxidative properties; for example, TF3 has been proved to show higher antioxidative activity than EGCG, which is the strongest antioxidant among all catechins and a precursor of TF3 [44, 45]. Theaflavins have more hydroxyl (OH) groups, which are considered to be necessary for exerting radical scavenging activity (antioxidative properties), than do catechins, since theaflavins are dimers of catechins.

More specific investigations concerning theaflavins structure have shown that depending on the amount and position of hydroxyl groups within their molecules, antioxidative properties change in the following way: TF3 > TF2 > TF1 [45]. A number of studies have shown that the antioxidative properties of theaflavins manifest themselves in their abilities to scavenge reactive oxygen species and to inhibit their generation [44]. The ability to scavenge free radical is partially influenced by the value of standard one-electron reduction potential E8V, characteristic of a particular chemical compound. A lower E8V indicates that less energy is required for hydrogen or electron donation and is one factor in determinating antioxidant activity. Tea catechins and theaflavins have E8V values comparable to that of vitamin E value, but higher than vitamin C, which is a superior hydrogen donor (antioxidant) to tea polyphenols [45].

Even in cases of very large quantities of ingested tea, the concentrations of catechins and other polyphenols in human blood plasma are from 100 to 1000 times lower than the concentrations of other physiological antioxidants such as ascorbate or glutathione. TF3, TF2, TF1 and EGCG have been found however be able to scavenge the superoxide radical, with TF3 most effectively [46]. In addition, theaflavins were shown to react with superoxide radical over 10 times faster than EGCG. In vitro investigations have shown that black tea was able to scavenge other reactive

oxygen species such as singlet oxygen and hydroxyl radical [47]. Another manifestation of the antioxidative properties of the theaflavins is inhibition of prooxidative enzymes activity. An experiment on human leukemia cells HL-60 proved that TF3 effectively inhibited xanthine oxidase activity, which catalyses oxidation of hypoxanthine and xanthine to uric acid accompanied by oxygen reduction to superoxide radical and hydrogen superoxide [48]. Moreover, applying 2-amino-3-methylimidazo[4,5]quinoline as a substrate in a free radical-generating system, it was shown in in vitro studies that the black tea EGCG influenced free radicals generation through reduction of NADPH-cytochrome P-450 reductase activity [49]. Studies on macrophages revealed that theaflavins present in black tea inhibited the activity of nitric oxide synthase (NOS), preventing NO generation [50]. Black tea also inhibited the activity of cyclooxygenase-2 and 5, 12 and 15-lipoxygenase and enzymes participating in enzymatic lipid peroxidation in human colon mucosa and colon tumor tissues [51].

Antioxidative properties of black tea are also determined by the strong interaction of its polyphenols with transition metals, which may form complexes with iron or copper ions, preventing free radicals generation, and inhibiting, among other actions, the lipid peroxidation process. In vitro studies have confirmed that inhibition of lipid peroxidation induced by processes involving participation of metal ions resulted from the ability of the polyphenols to chelate these metal ions. Inhibition of this process was also confirmed by experiments in vivo. It is not known whether the antioxidative properties of black tea under physiological conditions are determined by chelating properties of its components because most of the transition metal ions in vivo are tightly bound to proteins in forms not able to participate in free radical generation. Tea polyphenols have a strong interaction with transition metal ions and form insoluble complexes with iron [52]. This binding in the gastrointestinal tract strongly inhibits iron absorption. Black tea was more inhibiting than green tea [53]. The binding affects non-haem iron only and can be overcome by the presence of ascorbic acid, which is iron absorption enhancing factor [54].

Antioxidant activity is dependent on the structure of the free radical scavenging compounds, the substituents present on the rings of flavonoids and the degree of polymerization. Although there

is some debate as to whether the degree of polymerization increases the antioxidant capacity, it appears that epicatechin and epicatechin polymers are better antioxidants than the catechin and catechin polymers [55, 56]. The structural criteria for the potent free radical scavengers are that these should possess (i) a 3-hydroxy group on a unsaturated C ring or (ii) a 2,3-double bond with the 3-OH group and 4-one in the C ring or (iii) an ortho-OH substitution pattern in the B ring where the OH groups are not glycated [57, 58]. The major polyphenolic components, catechin and epicatechin, fulfill the first and third structural criteria for being a good antioxidant.

Many claimed beneficial effects of kombucha such as alleviation of inflammation and arthritis, cancer prevention and immunity enhancement may be associated to its anti-oxidant activities [59]. Dufresne et al. [19] proposed that some curative effects of kombucha tea might come from fermentation process but the mechanism remained unclear. Kombucha was usually prepared statically at ambient temperature for up to 10 - 14 days but the roles of fermentation time were not seriously considered. It was therefore necessary to elucidate the relationship between the fermentation time and antioxidant activities of kombucha.

2.6. Chemical composition of kombucha

Chemical analysis of tea fungus beverage showed the presence of sugars, gluconic, glucuronic, L-lactic, acetic, malic, tartaric, malonic, citric, oxalic, succinic, pyruvic, usnic acids, ethanol, purines, pigments, lipids, fourteen amino acids, water soluble vitamins, biogenic amines, monosaccharides, proteins, vitamin C, antibiotically active matters, carbon (IV) oxide, some hydrolytic enzymes as well as insufficiently known products of yeasts and bacterial metabolism [9, 60]. In the course of metabolic activities, yeast and bacteria in the tea fungus make use of substrates by different and complementary ways. Yeast cells hydrolyse sucrose into glucose and fructose by yeast invertase and produce ethanol via glycolysis, with a preference for fructose as a substrate. Acetic bacteria utilize glucose to produce gluconic acid and ethanol to produce acetic acid. The pH value of kombucha decreases during the fermentation process following the increase in the organic acid content. Part of the glucose was directed toward the production of organic acids such as gluconic acid by pentose phosphate pathway and to biosynthesis of cellulose by acetic bacteria. Most Acetobacter strains are known to oxidize glucose and produce gluconate. Fructose never turned into gluconic acid. One of the possible ways of glucose

transformation is also its oxidation into glucuronic acid. The presence of glucuronic acid in tea fungus beverage was quantitatively proved by [9] on different concentrations of sucrose. In contrast to glucose, fructose was poorly metabolized by A. xylinum, and thus, accumulated in the broth. Phosphofurctokinase was absent in A. xylinum, rendering glycolysis by this bacterium either absent or very weak. Furthermore, A. xylinum was incapable of utilizing sucrose to produce acid. Water-soluble vitamins also rank among the metabolites of the tea fungus. It has been reported that kombucha contains vitamins B1, B2, B6, B12 and vitamin C [61].

2.7. Toxicity

Although kombucha tea has been reported to have curative effects, there is some evidence of toxicity associated with kombucha tea. Some people report dizziness and nausea after consuming kombucha tea. Two cases of unexplained severe illness have also been reported following kombucha tea consumption (MMWR, 1996). Kombucha tea is contra-indicated in pregnant women and lactating women. The tea has been found to cause lead poisoning and gastrointestinal toxicity in two people. Further, Sadjadi[62] reported the presence of anthrax Bacillus in kombucha tea fermented in unhygienic condition. However, all of these cases are very isolated and involve only a small number of (two or four) people. Moreover, there is no substantial evidence to confirm the toxicity of the tea or the occurrence of illness by these studies [63].

2.8. Kombucha - Non-toxic drink

The Food and Drug Administration, USA and Kappa Laboratories, Miami, Florida (1995) have also carried out microbiological and biochemical tests and reported the tea is safe for human consumption. More recently, [63] carried out sub-acute oral toxicity studies with kombucha tea on Wistar rats and found that the tea did not alter any of the biochemical and histopathological parameters studied.

2.9. Hepatoprotective and curative effects of kombucha tea against aflatoxin B_1 induced hepatotoxicity in rats

One of the most serious problems to deal with the quality of food products is the presence of mycotoxins which has become a worldwide concern. Among mycotoxins, aflatoxins are of

greatest concern as they are highly toxic, mutagenic, teratogenic and carcinogenic compounds that have been implicated as causative agents in human hepatic and extrahepatic carcinogenesis [64-68]. The contamination of food and feed materials with aflatoxins causes important health problems and economic losses [69]. Until now, 19 different toxic derivatives of aflatoxins have been reported. Amongst these, aflatoxin B1 (AFB1) is the most naturally occurring compound of toxigenic isolates of Aspergillus flavus and Aspergillus parasiticus. Due to the high toxic action of aflatoxins, many countries have established maximum residue levels, generally lying between 4 and 50 mg/kg, of these toxins in food items. The European Union has one of the strictest regulations with the maximum tolerated limit of aflatoxin in consumable items of 2 mg/kg for AFB_1 [70]. Aflatoxins not only contaminate food stuffs but are also found in edible tissues, milk and eggs after consumption of contaminated feed by farm animals [71].

The positive correlation between the consumption of AFB_1 contaminated foods and the increased incidence of liver cancer in several Asian and African populations has led to the classification of AFB_1 as a group IA carcinogen by the International Agency for Research on Cancer (IARC) of World Health Organisation (WHO) in 1993. Several epidemiological studies have implicated aflatoxins in the increased incidence of human gastrointestinal and hepatic neoplasms in Africa, the Philippines and China. AFB_1 also has been implicated in human liver cell carcinoma [72]. Recently, aflatoxins outbreaks affecting a large geographical area and over 123 deaths were reported in Kenya (CDC, 2004). A number of survey and monitoring programs have been carried out in several countries attempting to obtain a general pattern of the extent of food contamination. Aflatoxins may contaminate a number of granular foods, including cereals, grains and groundnuts. The incidence of aflatoxins and their concentration in contaminated products depend on the conditions of temperature and humidity during crop growth and storage [73] . Several survey studies showed that, AFB_1 can be found in sesame seeds and tahin which is the main constituent of helva [74]. The study of Nguyen and his co-workers (2007) demonstrated that the contamination of mycotoxins in rice in five provinces of the central region of Vietnam was alarmingly high, especially AFB_1. Var et al. [75] found that AFB_1 was in excess of Turkish legal limit of 5 µg/Kg in 4 of 102 helva samples. Juan et al. [76] reported the natural occurrence of aflatoxins in dried fruits and nuts available in Rabat-Sale´ area in Morocco.

Recently, there has been renewed interest in hepatocellular carcinoma (HCC) in developed as well as in developing countries, because it accounts for 15% of total cancer mortality burden. Accumulating epidemiological and experimental evidence has revealed the influence of a number of naturally occurring and synthetic compounds on drug detoxification and HCC incidence [77]. It has been shown that AFB_1 is activated by hepatic cytochrome P450 enzyme system to produce a highly reactive intermediate, AFB_1-8,9-epoxide, which subsequently binds to nucleophilic sites in DNA, and the major adduct 8,9-dihydro-8-(N7guanyl)-9-hydroxy-AFB_1 (AFB_1 N7-Gua) is formed. The formation of AFB_1-DNA adducts is regarded as a critical step in the initiation of AFB_1-induced hepatocarcinogenesis [78]. Although, the mechanism underlying the hepatotoxicity of aflatoxins is not fully understood, several reports suggest that toxicity may ensue through the generation of intracellular reactive oxygen species (ROS) like superoxide anion, hydroxyl radical and hydrogen peroxide (H_2O_2) during the metabolic processing of AFB_1 by cytochrome P450 in the liver . These species may attack soluble cell compounds as well as membranes, eventually leading to the impairment of cell functioning and cytolysis. Peroxidative damages induced in the cell are encountered by elaborate defense mechanisms, including enzymic and nonenzymic antioxidants. Biological compounds with antioxidant properties contribute to the protection of cells and tissues against deleterious effects of ROS and other free radicals [79].

Opportunities for primary prevention against aflatoxin toxicity and carcinogenicity include drugs that interfere with carcinogenic process through pharmacologic interventions. Since the increase in the use of synthetic chemicals in cancer therapy has led to many side effects and undesirable hazards, there is a worldwide trend to go back to natural resources which are therapeutically effective, culturally acceptable and economically within the reach of even the poor people. Traditional and indigenous systems of medicines have persisted for many centuries even where modern healthcare is readily available. WHO has called the attention of many countries to the ever increasing interest of the public in the use of herbal medicines and encourages countries to identify and exploit those aspects of traditional medicine that provide safe and effective remedies. During recent years, active principles with diverse chemical structures have been isolated from plants reportedly possessing hepatoprotective effects. For instance, various

triterpenes like, oleanolic acid, ursolic acid and celastrol are effective in protecting against liver disorders [80]. Lupeol, a structurally similar pentacyclic triterpene, isolated from the medicinal plant Crataeva nurvala Buch-Ham (Capparidaceae), has been shown to exhibit antihepatotoxic, antioxidant and antitumor activities in rats. Silymarin, a purified extract of Silybum marianum Gaertn, is frequently used in liver diseases where it is capable of protecting liver cells directly by stabilizing the membrane permeability through inhibiting lipid peroxidation and preventing liver glutathione depletion. Liu et al. [81] demonstrated the hepatoprotective property of Salvia miltiorrhiza against AFB_1 induced hepatotoxicity in Fischer 344 rats as evidenced by decrease in AFB1 -DNA adducts formation as well as AFB_1-induced oxidative DNA damage (8-hydroxydeoxyguanosine) in rat liver. Jodynis-Liebert et al. [82] reported the hepatoprotective effect of Aquilegia vulgris L. on AFB_1 induced hepatic damage in rats as evidenced by inhibition of lipid peroxidation and preventing reduced glutathione depletion. Preetha et al. [83] assessed the hepatoprotective effect of lupeol, a pentacyclic triterpene, isolated from the stem bark of Crataeva nurvala, on AFB_1 induced hepatotoxicity in a rat model.

Recent studies have suggested that kombucha tea prevents paracetamol induced hepatotoxicity [60] and chromate (VI) induced oxidative stress in albino rat [84]. Our previous investigation has demonstrated that kombucha tea could protect the liver against CCl_4 induced hepatotoxicity in rats as revealed by unaltered transaminases and lipid peroxidation and also by the normal histology of hepatocytes[85]. As kombucha tea is rich in compounds known to be strong antioxidants, it is expected to ameliorate liver damage induced by AFB_1.

2.10. Effect of tea fungal mat inclusion in rabbit feed

The demand for human food from animal products (meat, egg and milk) is increasing year by year but it is predicted and that there will be a world shortage of cereal grain due to the competing needs of expanding human and livestock populations [86]. The rabbit (Oryctotaguscuniculus) is a meat-producing animal which is well adapted to utilization of protein-rich foliages due to the nature of its digestive system in which enzymatic digestion (in the stomach) precedes microbial fermentation (in the caecum). The rabbit is a non-ruminant herbivore and can consume high fibre diets and are regarded as hind gut digesters. Fermentation of cellulose and other fibrous components is post gastric. This occurs in the caecum and colon

which are well developed in rabbit and harbour a considerable amount of microbial population [87]. These microorganisms are involved in digestion of starch and cellulose.

Rabbit production contributes to improved nutrition and economy in the family as a source of animal protein, as well as extra income by sale of animals. Rabbits can be fed by many kinds of grasses (guinea grass), legumes (stylosanthes), vegetables (water spinach and sweet potato vines), leaves from trees, fruits (bananas), roots and tubers (cassava, sweet-potatoes) and by-products from the kitchen. Concerning the nutritional requirement of rabbits, they require diets with moderate to high levels of fibre (at least 15 to 20% in the dry matter), moderate levels of protein (12 to 15% in dry matter) and low fat [88]. Rabbit meat is very nutritious. The meat is rich in protein and low in fat and cholesterol. On the other hand, rabbits have the ability to consume directly forage proteins and convert this to animal protein, while swine and poultry rely mainly on cereal grains to meet their dietary protein needs. Hence, rabbit meat is often referred to as an inexpensive protein source [89]. In recent years, rabbit husbandry in south east Asia has developed quickly due to demand of fresh meat for human consumption. Furthermore, it is becoming a popular animal because of specific characteristics of feeding behaviour that favours its role in integrated farming systems.

One major limiting factor to livestock production is the high cost of feedstuffs such as groundnut cake (GNC), soybean cake and fishmeal. However, many feedstuffs especially the agro-industrial by-products which are usually of no feeding value to humans could alternatively be fed at cheaper cost to monogastric animals [90] . In the light of predictions of future protein shortages, the non-agricultural routes to protein production have become an industrial reality. These routes are based on the continuous fermentation of micro-organisms to produce single cell proteins (SCP). Seen as only a long-term possibility a few years ago, many commercial enterprises now have successful SCP production facilities, while research and development in this field continues at a rapid pace. There is therefore a need for research in order to develop systems of animal production based on locally available resources.

Tea fungus (Medusomyces gisevii), can be used as a rich non-conventional source of microbial protein in animal feeds. Tea fungus is an excellent example for biofilm which consists of

bacteria and yeasts. A part of the fungal mat produced during black tea fermentation is used as starter culture, while the remaining goes as a waste. Recycling of waste tea fungal mat is one of the most important means of utilizing it. The waste tea fungal biomass obtained from kombucha fermentation is rich in fibre, which includes cellulose, hemi-cellulose and silica with good quality of protein. Tea fungal mat has been successfully tried as a protein source in poultry feed [85]. The fungal mat is a hard cellulosic pellicle containing rich nutrients similar to the single cell protein produced by other yeasts. Based on the biochemical constituents of the tea fungus, an attempt has tried out to investigate its performance on weaner rabbits as a supplementary diet.

2.11. Shelf life study of kombucha tea

As the growth of the beverage industry enables the massive production of tea products, the market for canned tea products has expanded rapidly during the past few years. Ready-to-drink black and green teas are now increasingly consumed in the world, especially in Japan and China, because of their health benefits [1, 91, 92]. Like green tea and black tea available in bottles, kombucha black tea can also be bottled for commercialization. The findings of various health benefits of kombucha tea have led to a general consumer's appreciation for the functional properties of it. Thus, kombucha tea is consumed not only to satisfy consumers' fine taste buds but also to acquire health benefit. Tea fungus is an excellent example for biofilm which consists of bacteria and yeasts. Several bacterial and yeast species were reported to present in the tea fungal consortium [3] [35]. After fermentation, the kombucha tea is filtered through a cheese cloth and is consumed as health drink. If the kombucha tea is stored at ≥20ºC, the biofilm is continued to form due to the presence of microorganisms in the kombucha tea.

Food products should not contain microorganisms, their toxins, or metabolites in quantities that present an unacceptable risk for human health [93]. Regulation (EC) No. 178/2002 sets down general food safety requirements, according to which, food must not be placed on the market if it is unsafe [94]. The shelf-life of food products is an integral part of food safety. The Codex Alimentarius defines shelf-life as the period during which a food product maintains its microbiological safety and suitability at a specified storage temperature and, where appropriate, specified storage and handling conditions [95]. In legislative terms, the term "date of minimum

durability" will describe a food product's shelf-life and is the date until which a food product retains its specific properties when properly stored. The date of minimum durability must be indicated by a 'best-before' date or a 'use-by' date. The 'best-before' date will reflect the quality e.g. taste, aroma, appearance rather than safety of a food product. A food which is past its 'best-before' date may not necessarily be unsafe to consume but it may no longer be of optimum quality. Typically, a 'best-before' date is required on products such as canned, dried and frozen foods. Food products which, from a microbiological point of view, are highly perishable and are therefore likely, after a short period of time, to constitute a danger to human health must have a 'use-by' date [96]. The 'use-by' date will indicate the date until which the product can be safely consumed. Therefore, unlike the 'best-before' date, the accurate determination of the 'use-by' date to ensure product safety is critical. Shelf-life means either the period corresponding to the period preceding the 'use-by' date or the 'best-before' date [93, 96] .

Yeasts are significant as spoilage microorganisms, especially in food of low pH, high sugar content, high salt content and in those containing sorbate or benzoate preservatives. Many environmental factors affect the growth of yeasts but the response to any particular condition varies with the species [97, 98].

Zygosaccharomyces bailii is the major spoilage yeast in acidic foods, being noted for its strong fermentative activity and ability to grow in hostile environments containing relatively high concentrations of food preservatives such as benzoic and sorbic acids. When spoilage of soft drinks, fruit juices, mayonnaise, salad dressings and syrups containing these preservatives are encountered, Z. bailii is often the cause. Many strains of Z. bailii are capable of growing in the presence of >600 µg/mL benzoic or sorbic acids and in media containing 2% acetic acid. These acids have been shown to inhibit various metabolic processes in yeasts, particularly those associated with uncoupling the active transport system necessary for cellular energy supply. Membranes of preservative-adapted yeasts are characterized by altered permeability to acid anions, which enable them to maintain an intracellular pH that is not lethal [99].

Brettanomyces bruxellensis, which is similar to B. claussenii, is considered a wine spoilage yeast species due to its ability to produce volatile phenols conferring off-odours and losses of fruity sensorial qualities in wines. It is a strong resistant species standing nutrients deprivation and high ethanol degrees. SO_2 is generally used to control microbial spoilage. Its effectiveness depends on the pH and on the phenolic compounds level. Only molecular SO_2 is active against microbial growth and required a minimum molecular SO_2 concentration of 0.625 mg/L to exert significant impact on B. bruxellensis [100].

Among the molecules tested, sorbic acid has a significant impact on cell viability by disrupting homeostasis pH. But its degradation by certain lactic acid bacteria leads to the formation of 2-ethoxycarbonyl-3,5-hexadiene, which cause disagreeable ‘‘geranium’’ tastes. Chitosan interacts with anionic groups on the yeast cell surface and limits the diffusion of essential solutes, such as sugars and heavy metal cations (copper, cobalt and cadmium) [101]. However its effectiveness is strongly variable according to the pH.

Vanillin is used against spoilage yeast in fruit juices and dairy products. It inhibits enzymes implicated in cell energy production. It also disrupts membrane functions [102]. However, some species are less sensitive to vanillin due to their ability to convert it to its alcohol and acid derivatives. It is notably the case for Brettanomyces sp. Moreover to insure an effective anti-microbial action, the level of vanillin required (30–100 mg/L) is one thousand times higher than usual wine concentrations [103]. Its excessive use would lead to a modification of wine aroma. Nisin is a natural product of the bacteria Lactococcus lactis exhibiting anti-microbial activities towards a wide range of Gram positive bacteria by forming pores into the cytoplasmic membrane and allowing the efflux of essential cellular materials. But some strains present high tolerance due to nisinase activity. A similar resistance phenomenon may occur for yeast for which the inhibitory effect of nisin is limited [104] .

Heat treatment is an effective method to inactivate spoilage yeasts. However, exposure of yeast cells to heat without causing death can result in metabolic and structural debilitation [105].

Resistance of yeasts to heat inactivation and injury can be influenced by a large number of factors, including inherent differences among strains and species [106] and the composition of the medium in which yeasts are grown before and during heat treatment. The ability of tea fungal microbes to form biofilm is a big problem when the kombucha tea is being stored and when it is being commercialized. So, it is essential to kill or to remove the microbes in the kombucha tea after fermentation, thus preventing the biofilm formation during storage period.

2.12. Anticancer properties of kombucha tea

Cancer is a group of diseases characterized by uncontrolled growth and spread of abnormal cells. If the spread is not controlled, it can result in death. Cancer is caused by both external factors (tobacco, radiation, chemicals and infectious organisms) and internal factors (inherited mutations, hormones, immune conditions and mutations that occur from metabolism). These causal factors may act together or in sequence to initiate or promote carcinogenesis.

The progression of a tumor from being in situ to invasive is a major prerequisite for cancer metastasis [107] and involves the acquisition of cell motility, surface adhesion properties, and activity of extracellular proteases. For invasion, a cancer cell requires increased migration, various cytophysiological changes including loss of cell–cell adhesion along with a gain of cell–matrix adhesion and increased expression and activation of extracellular proteases to degrade the extracellular matrix (ECM) and allow cell invasion and metastasis [107-109]. Extracellular proteases play an important role in immune functions, wound healing, fetus implantation, angiogenesis and cancer cell invasion. Degradation of ECM by cancer cells via protease, such as serine proteinase, metalloproteinases (MMPs), cathepsins, may lead to the separation of intercellular matrix to promote the mobility of cancer cells and eventually result in metastasis. Of these proteases, serine proteinase and MMPs are the most vital ones for degradation of basement membrane and therefore involved in cancer migration and invasion . The MMPs, a group of zinc-dependent ECM degrading enzymes for the proteolysis of ECM proteins such as collagen, proteoglycan, fibronectin, elastin and laminin [110], are thought to be critical in tumor invasion, metastasis and angiogenesis. MMPs are secreted by cells as proenzymes that must be cleaved in order to become functional. This latency of the MMP zymogens is due to the presence of the N-

terminal pro-domain that shields the catalytic cleft through coordination of the catalytic zinc molecule [111]. Cleavage of the pro-domains of MMPs is mediated in most cases by soluble MMPs or by proteases of the serine families such as plasmin, plasma kallikrein and neutrophil elastase. Both MMP-2 and MMP-9 are abundantly expressed in various malignant tumors [110] and contribute to invasion and metastasis as documented in many reports. Furthermore, a serine proteinase, urokinase-type plasminogen activator (u-PA), may promote a proteolytic cascade by converting plasminogen to its active form, plasmin, which in turn may cleave and activate MMPs to enhance tissue remodeling by degrading ECM components. Meanwhile, the activities of MMPs and u-PA are prone to the inhibition of endogenous tissue inhibitor of metalloproteinases (TIMPs) and plasminogen activator inhibitor (PAI), which are specific inhibitors of MMPs and PAI, respectively, and the imbalance between MMPs and TIMPs may contribute to degradation or deposition of ECM [112] .

Carcinogenesis is a multifactorial and multistage process in which numerous genes are affected. Many of these genes are prime targets for chemopreventive agents because they regulate intracellular, cell-surface, or extracellular functions. Chemoprevention can be defined as the use of substances to interfere with the process of cancer development. Although substantial progress has been made in elucidating the basis of carcinogenesis, further advances are needed to identify molecular and cellular targets for effective use of chemopreventive agents. Hundreds of compounds have been identified as potential chemopreventive agents. However, the safety and efficacy of each substance must be thoroughly investigated.

From the number of scientific papers being published, interest in the concept and practice of chemoprevention as an approach to the control of cancer has increased greatly in the past few years. This increased interest is probably due to the lack of an effective decrease in mortality from the most common cancers (lung, colon, breast and prostate) since the ‘War on Cancer’ was declared by US President Richard Nixon in 1971. Chemoprevention is defined currently as a pharmacological approach used to arrest or reverses the process of cancer development (carcinogenesis) before invasion and metastasis occur. Many review articles (1999 – 2000)

focus on the issues, rationale, progress and promise of chemoprevention as a new anticancer strategy.

Natural products are considered to have little or no toxicity because they are present in commonly consumed foods and beverages. Chemoprevention by dietary agents has evolved as a promising approach to control the incidence of different types of cancer, an important contributor to morbidity and mortality [113]. Of late, chemoprevention by a combination of dietary phytochemicals with distinct molecular mechanisms has received growing consideration as a means to achieve higher efficacy and potency with reduced toxicity.

Frequent consumption of fruits and vegetables has been associated with lower incidence of cancers at different organ sites [114]. Several factors may contribute to this association. First, the nutrients in fruits and vegetables, notably vitamin C, vitamin E, folic acid, provitamin A, selenium, and zinc, are essential for normal cellular functions. A deficiency in these nutrients may enhance the susceptibility of an individual to cancer. Second, some nutrients, such as vitamin C, vitamin E, selenium, and ß-carotene, at levels above nutritional needs, may display inhibitory activities against carcinogenesis. A third factor is that nonnutritive constituents, such as polyphenols, organosulfur compounds, and indoles, have anticarcinogenic activities.

The involvement of the first two factors is supported by the demonstration that supplementation of a high cancer risk population in Linxian, China, with tablets containing ∝-tocopherol, ß-carotene, and selenium for 63 months significantly lowered the mortality rate of gastric cancer [115] .The subjects involved in this intervention study were known to have low micronutrient status .Supplementation with these antioxidant nutrients apparently produced a protective effect against this cancer.

In recent years, extensive studies have been conducted on tea and tea constituents because of their potential beneficial health effects. In particular, combination regimens that use tea polyphenols as one of the constituents have been found to be potentially effective in chemoprevention [116-119] . In most parts of the world, tea is consumed together with milk. Both milk and tea are rich in bioactive compounds and nutraceuticals. Among the tea catechins, EGCG is the most abundant and the most biologically active compound. Tea has been shown to inhibit tumorigenesis in many animal models, including those for cancer of the skin, lung, oral

cavity, esophagus, stomach, small intestine, colon, liver, pancreas, bladder and prostate [120-122]. The mechanisms of the chemopreventive activity, however, are not clearly understood. Although, the cancer preventive activity of tea polyphenols has been demonstrated in many experimental systems [121], caffeine has been shown to be the active ingredient in some other systems; for example, in the inhibition of UV-light induced skin tumorigenesis in mice [123] and chemically induced lung tumorigenesis in F-344 rats [124]. Although the anticarcinogenic activity of tea and polyphenols has been demonstrated in many animal studies, such activity has not been clearly demonstrated in humans. More epidemiological investigations, especially prospective studies, concerning the effect of polyphenol consumption on human cancer risk are needed. Because the causative factors may be different for different cancers and for the same cancer in different populations, the effects may vary in different situations.

OBJECTIVES

1. Utilization of Tea manufacture waste material for production of Tea fungal biomass

2. To study the effect of variation in Tea manufacture waste material and Sucrose concentration for production of Tea fungal biomass.

3. To study the effect of Surface area and depth of culture medium on Tea fungal biomass production.

4. Bioethanol production from tea fungal biomass.

4. MATERIALS AND METHODS

4.1. Materials

4.1.1 Tea Manufacture waste (waste tea)

Tea manufacture waste or waste tea is dry straw and fiber of tea leaves resulting from the black tea production process. This waste material occupies large area in tea industries for its storage. An attempt has been made in this study to utilize the tea manufacture waste to prepare kombucha tea. Tea manufacture waste was collected from Parry Agro Industries Ltd., Valparai, Tamil Nadu, India.

4.1.2 Sucrose

Sucrose utilized to prepare kombucha tea was of food grade.

4.1.3 Starter culture

Starter culture or tea fungal mat of Medusomyces gisevii was obtained from the tribal people of Kolli hills, Tamil Nadu, India and was maintained in sugared black tea [125] [125, 126].

4.1.4 Cellulase (Novozymes, Denmark)

Cellulase (Novozymes, Denmark) was utilized to saccharified tea fungus for fermentation process.

4.1.6 Distillation Unit

Simple distillation unit bearing heater, round bottom flask and condenser was utilized for distillation of fermentation of bioethanol.

4.2. Methods

4.2.1 Maintenance of tea fungus

The tea fungus sample was maintained as described by [125] or activated every 2 weeks as per the designated procedure [2]. Briefly, a known volume of ditilled water was boiled before adding of sucrose and tea manufacture waste material. The mixture was left to steep for 10 min. Then tea infusions were filtered out and the sweetened black tea was immediately dispensed into a beaker. Finally, tea fungus (3% w/v on wet weight basis) and liquid broth (10% v/v) of the tea fungus sample were added to the cooled tea broth. The beaker was covered with a clean paper towel that was held tightly in place with rubber bands. Fermentation was carried out room temperature (25 ± 3˚ C) and in dark and 3% (w/v on wet weight basis) tea fungus was used to inoculate new fermentations.

4.2.2 Preparation of Kombucha tea

The preparation of Kombucha was done as described by [14, 125]. Tea manufacture waste were added to boiling water (1.2%) and allowed to infuse for about 5 min after which the infusions were filtered through sterile sieve. Sucrose (10%) was dissolved in hot tea and the preparation was left to cool. The cooled tea (500 mL) was poured into 1000 mL glass jars that had been previously sterilized at 121°C for 20 min and inoculated with 3% (w/v) of freshly grown tea fungus that had been cultured in the same medium for 14 days and 10% (v/v) of previously fermented liquid tea broth aseptically. The jar was covered with a clean cloth and fastened properly. The fermentation was carried out in dark at 24±3°C for 14 days.

4.2.3 Effect of sucrose concentration on yield of tea fungal biomass

The effect of sucrose concentration on yield of tea fungal biomass was studied as described by [127] varying the concentration of sucrose (1%, 3%, 5%, 7%, and 9%) in 100 mL of tea broth by keeping the concentration of tea manufacture waste constant at 1.2% in all the beaker. Control sample consisiting of 100 mL of tea broth was prepared in the same manner without the addition of sucrose. About 3% (w/v on wet weight basis) of tea fungus and 10% of the previously

fermented tea from the previously activated tea fungus sample were added to all the freshly prepared tea broth. After 2 weeks of fermentation the wet weight of tea fungus was weighed in weighing unit.

4.2.4 Effect of tea manufacture waste material on yield of tea fungal biomass

The effect of tea manufacture waste material concentration on yield of tea fungal biomass was studied by varying the concentration of tea manufacture waste material (0.5%, 1%, 1.1%, 1.2%, and 1.5%) in 100 mL of tea broth by keeping the concentration of sucrose constant at 10% in all the beaker. Control sample consisting of 100 mL of tea broth was prepared in the same manner without the addition of tea manufacture waste material. About 3% (w/v on wet weight basis) of tea fungus and 10% of the previously fermented tea from the previously activated tea fungus sample were added to all the freshly prepared tea broth. After 2 weeks of fermentation the wet weight of tea fungus was weighed in weighing unit.

Fig.2: Tea manufacture waste

4.2.5 Effect of surface area and depth of culture medium on yield of tea fungal biomass

The effect of surface area and depth of culture medium on yield of tea fungal biomass was studied as described by [127] by preparing tea broth for fermentation in dimensionally varying container such as beaker, round container, rectangular container, measuring cylinder. The tea broth of 100 mL was prepared by following the same procedure used for maintenance of the tea fungus sample previously. Containers of different dimension used for study are listed in Table: 1.

Table: 1 Containers with different Surface area:Depth

Depth (cm)	Surface area (cm^2)	Ratio of surface area: Depth (cm)
5.1	**19.60**	**3.80**
34.2	**29.20**	**0.90**
6.4	**78.50**	**12.30**
5.4	**130.50**	**24.20**
6.6	**227.00**	**34.40**

4.2.6 Determination of pH

The pH of the sample was checked before and after fermentation with an electronic pH meter. To take the measurement, a known amount (5 ml) of the samples was withdrawn carefully without disturbing the tea broth.

4.2.7 Fourier transform infrared spectroscopy (FT-IR)

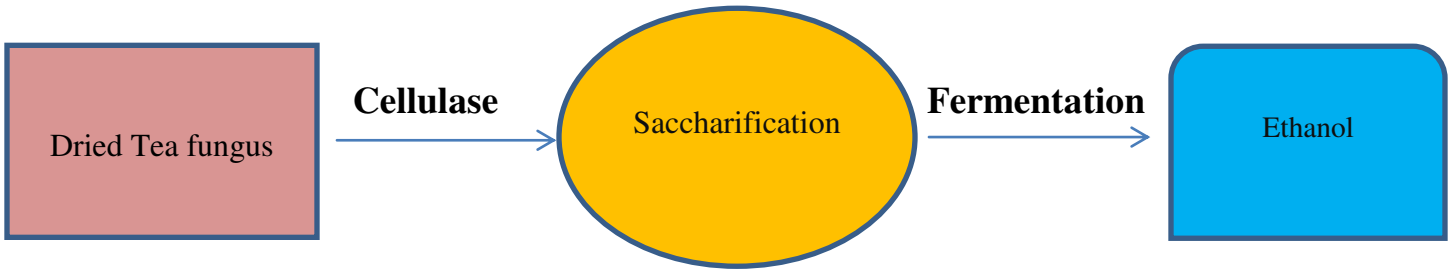
FT-IR spectroscopy of dried tea fungal sample was performed as described by [128] to study its conformational characteristics. The wet tea fungus sample was dried in hot air oven at 60˚C for 8 hours and then the dried sample was crushed into powder form using mortar and pistol. Dried tea fungus was mixed with KBr and pellet was prepared. FT-IR analysis was done from 4000-400 cm^{-1} . A comparative study of tea fungus was done with commercial grade cellulose (Himedia, Mumbai)

4.2.8 Scanning electron microscopy (SEM)

SEM of Tea fungus sample was performed to study the surface topography and composition. The wet tea fungus was dried in hot air oven at 60˚C for 8 hours and then the dried sample was crushed into powder form using mortar and pistol. Dried tea fungus powder was taken in a glass slide and was coated with platinum. SEM image was taken in 500x, 1000x and 2000x resolution.

4.2.9 Fermentation of dried tea fungus biomass

The dried tea fungus biomass was used for fermentation purpose for bioethanol production as described by [129].The wet tea fungus biomass was dried in hot air oven at 60˚C for 8 hours. After drying of tea fungus it was crushed into powder form using mortar and pistol. The dried tea fungus biomass was mixed with Cellulase (Novozyme, Denmark) for saccharification of tea fungus cellulose into glucose monomer. 10 mL of cellulase enzyme was diluted with 90 ml of water i.e. 1:10 dilution. 8 g of dried sample was treated with enzyme at 55˚C for 20 hours. Saccharomyces cerevisiae was cultured in yeast culture medium (1% Yeast extract and 5% Glucose) for fermenting the saccharified tea fungus. The fermentation process was carried out with 10% inoculum of S. cerevisiae for 3 days in anaerobic condition.



4.2.10 Analysis of Ethanol production

The dried tea fungus biomass after 3 days of fermentation was distilled in simple distillation.unit. 100 ml of fermented tea fungus was distilled at 78.6˚ C. The distilled liquid was analyzed for ethanol content in UV-visible spectrophotometer and through gravimetric measurement method. In UV- visible spectrophometer commercial grade ethanol was taken as control for comparative analysis of ethanol produced from tea fungal biomass. Both control and sample ethanol was scanned in UV- visible region (200-700 nm) to study absorbance maxima. In case of gravimetric

measurement method the specific gravity of tea fungus before and after fermentation were calculated and were applied in a mathematical formula to find the percentage of alcohol.

$$\text{Percentage (\%) v/v of alcohol} = \frac{1.50 * (SG1 - SG2) * 100}{SG2}$$

Where, SG1= Initial Specific gravity of sample before fermentation

SG2= Final Specific gravity of sample after fermentation

5. RESULTS AND DISCUSSION

5.1. Production of Tea fungus biomass

After 2 weeks of fermentation of sugared black tea with 3% of starter culture (tea fungus) it was observed that a thick jelly membrane of tea fungus (Fig. 4) has been produced which was floating on the surface of the tea broth (Fig. 3).

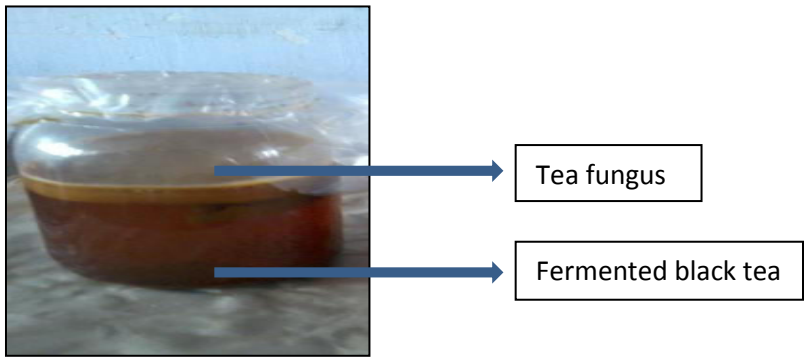


Fig. 3: Tea fungus growing in black tea liquor during Kombucha preparation

Fig. 4: Tea fungus Biomass

The tea fungal biomass is bacterial cellulose produced by the microorganism which depends on carbon and nitrogen source for cellulose production.

5.2. Effect of sucrose concentration on yield of tea fungal biomass

The effect of sucrose concentration on the yield of Tea fungal biomass grown in 100 mL of tea broth (Fig. 5) by keeping tea manufacture waste material concentration constant (1.2%) and varying concentration of sucrose (1%, 3%, 5%, 7%, 9%) was observed after 2 weeks of incubation. Control was taken with 1.2% of tea manufacture waste material and without sucrose. Effect of different concentration of sucrose on tea fungal biomass is depicted in Table 2.

Fig. 5: Tea fungus grown in different concentration of sucrose

The wet weight of Tea fungus grown in different sucrose concentration was measured in weighing machine after 2 weeks of incubation in 100 mL tea broth as shown in Table 2.

Table 2: Effect of different sucrose concentration on the yield of tea fungal biomass

Concentration of sucrose (w/v)	Yield of Tea fungus (g)
Control (0%)	**5.4**
1%	**6.8**
3%	**6.3**
5%	**10.00**
7%	**35.2**
9%	**29.8**

Almost all living microorganisms require carbon source for their general growth and metabolism and culture of Tea fungal biomass depends on the supply of a carbon source (sugar, mainly

sucrose) as it cannot produce the cellulose in adequate quantities on its own. Our preliminary experiments conducted revealed that the concentration of sucrose present in the tea broth affects the synthesis of bacterial cellulose and 7% of sucrose was found to be optimum for tea fungus to grow with maximum yield of tea fungal biomass. This is because during the course of fermentation when a substantial amount of sugar is present in the tea broth more metabolic products would lead to product inhibition.

5.3. Effect of Tea manufacture waste concentration on tea fungal biomass

The effect of tea manufacture waste concentration on the yield of tea fungal biomass grown in 100 mL of tea broth (Fig. 6) by keeping sucrose concentration (7%) and varying concentration of tea manufacture waste was observed after 2 weeks of incubation. Effect of Tea fungal biomass production in different concentration of sucrose is depicted in (Table: 3).

Fig. 6: Tea fungus grown in different concentration of Tea manufacture waste

The wet weight of Tea fungus grown in different concentration of tea manufacture waste was measured in weighing machine after 2 weeks of incubation in 100 ml tea broth (Table: 3)

Table 3: Effect of different concentration of tea manufacture waste on the yield of Tea fungal biomass

Concentration of Tea manufacture waste (w/v)	Yield of Tea fungus (g)
Control (0%)	7.6
0.5%	41.8
1%	45.6
1.1%	42.7

1.2%	**40.5**
1.5%	**34.1**

Tea manufacture waste constitutes Caffeine and related xanthines which stimulate cellulose synthesis by bacteria. Experimentally it was found that 1% of tea manufacture waste is optimum for Tea fungal biomass production as the phenolic compounds present in tea is less absorbed by acetic acid bacteria and yeast present in Tea fungus.

5.4. Effect of surface area and depth of culture medium on yield of Tea fungal biomass

The effect of surface area and depth of culture medium on the yield of tea fungal biomass grown in different volume of tea broth (Fig. 7) by keeping sucrose concentration (7%) and Tea manufacture waste concentration (1%) constant. It was observed after 2 weeks of incubation. Effect of Tea fungal biomass production in different surface area and depth ratio is depicted in (Table: 4).

Fig.7: **Tea fungus grown in different surface area: depth ratio of culture medium**

The wet weight of Tea fungus grown in different surface area : depth ratio of culture medium was measured in weighing machine after 2 weeks of incubation in 100 mL, 500 mL, , 700 mL, 1000 mL, 1500 mL of tea broth (Table: 4)

Table 4: Effect of different surface area: depth ratio on the yield of Tea fungal biomass

Depth (cm)	Surface area (cm^2)	Ratio of surfacearea:Depth (cm)	Yield of Tea fungus (g)
5.1	**19.6**	**3.80**	**7**
34.2	**29.20**	**0.90**	**21.30**
6.4	**78.50**	**12.30**	**41.00**
5.4	**130.50**	**24.20**	**66.90**
6.6	**227.00**	**34.40**	**104.80**

The growth of Tea fungus depends on the surface area and depth ratio of container. Experimental studies reveal that container with maximum surface area and minimum depth has maximum yield of tea fungus. These results can be explained as follows: with the increasing depth of the culture medium the flow of nutrient from broth to tea fungus get slower, hence the growth of tea fungus gets slower and less. But when the depth of culture medium is less than the flow of nutrient occur faster from broth to tea fungus, hence the growth yield of tea fungus is maximum. Cells produce carbon dioxide, which is trapped in the tea fungus pellicle [130] and the deeper the culture medium the more carbon dioxide accumulates in the pellicle. When the inside of the pellicle is less aerobic, cell growth and pellicle formation are inhibited because acetic acid bacteria are strict aerobes.

5.5. Determination of pH

The change in pH of tea broth before and after fermentation of sugared black tea with tea fungus in 7% sucrose and 1% tea manufacture waste material and control with only previous batch fermented tea broth (10%) and tea fungus (3%) was studied. It was found that pH decreases with increasing fermentation time. This decrease in pH decreases tea fungus production as each

microorganism has a typical pH range within which their growth is possible and pH plays an important role in microbial growth and tea fungual biomass production. Change in pH before and after fermentation is shown in Table: 5

Table: 5 showing change in pH before and after fermentation

pH before fermentation	pH after fermentation
Control (5.1)	3.8
4.6	3
4.4	2.5
4.5	2.3
5	2.08
4.1	2.1

5.6. Fourier transform infrared spectroscopy (FT-IR)

As shown in (Fig.9), the conformational characteristics and purity of the tea fungus were determined by FT-IR spectroscopy from 4000-400cm^{-1} . and commercial cellulose was also studied to compare (Fig.8) The FT-IR spectra showed characteristic cellulose peaks around 3430 to 3435 cm-^{1} for hydroxyl groups stretching vibration, at 2927 to 2949 cm-^{1} for C-H stretching vibration, at 1433 to 1456 cm-^{1} for C-H bending vibration and 1045 to 1067 cm-^{1} for C-O-C and C-O-H stretching vibration of the sugar ring. In FT-IR spectra of cellulose, the band at 3400 cm-^{1} was attributed to the intramolecular hydrogen bond for 3O…H5. For β 1-4 glycosidic bond of cellulose peak at 892 cm-^{1} was observed.

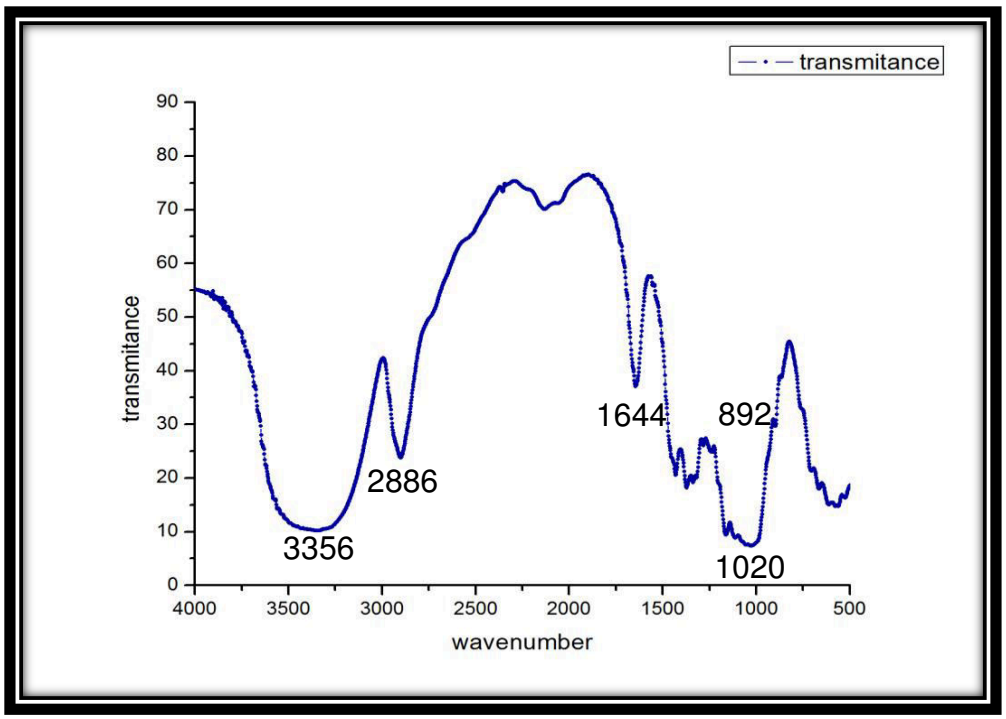


Fig. 8 FT-IR graph of Commercial cellulose powder (control)

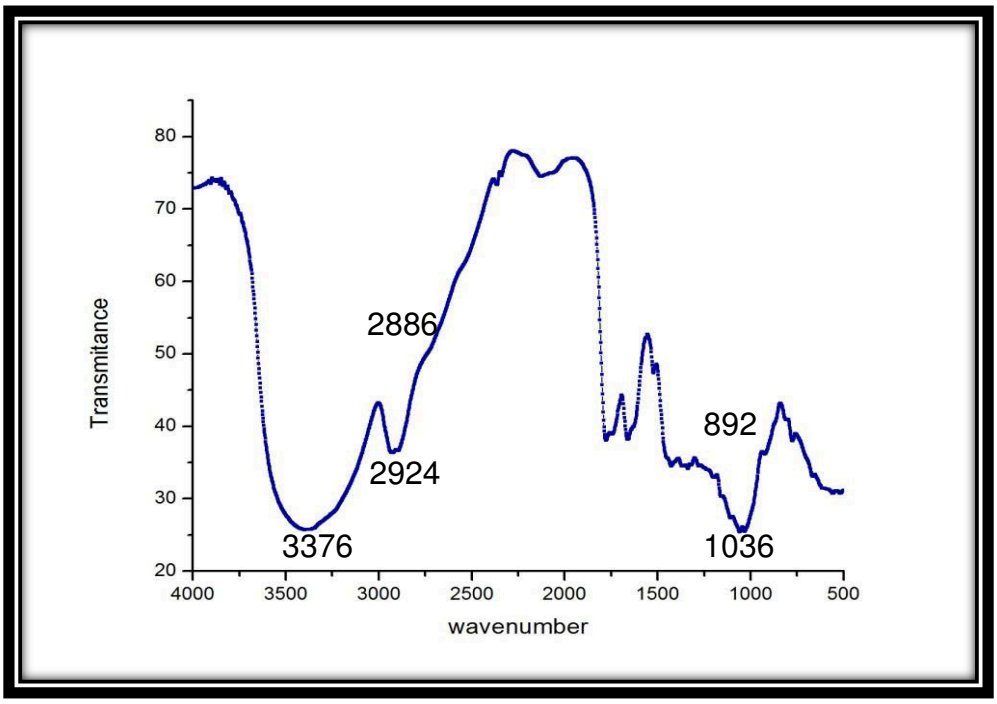


Fig. 9: FT-IR graph of Tea fungus powder

From FT-IR graph it was analysed that tea fungus constitutes cellulose as the peak at 892 cm^{-1} for β 1,4- glycosidic bond was observed in both commercial cellulose and dried tea fungus powder. Hence, tea fungus consists of cellulose and component which might be attributed by tea manufacture waste such as phenolic compounds.

5.7. Scanning electron microscopy

SEM image of Tea fungus powder was taken in 500x (Fig. 10), 1000x (Fig. 11) and 2000x (Fig. 12). SEM image shows that the cellulose in tea fungus are in fibrous form and arranged in bundle. From SEM analysis the topographic conformation of tea fungus was observed to be net fibrous like.

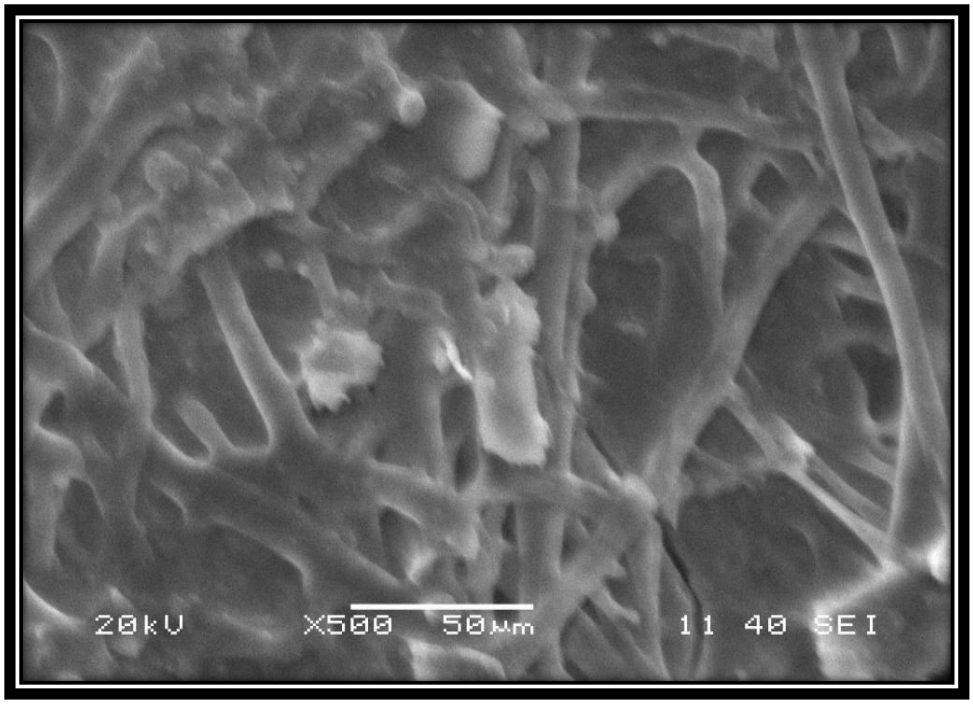


Fig. 10: SEM image of dried tea fungus powder (500x)

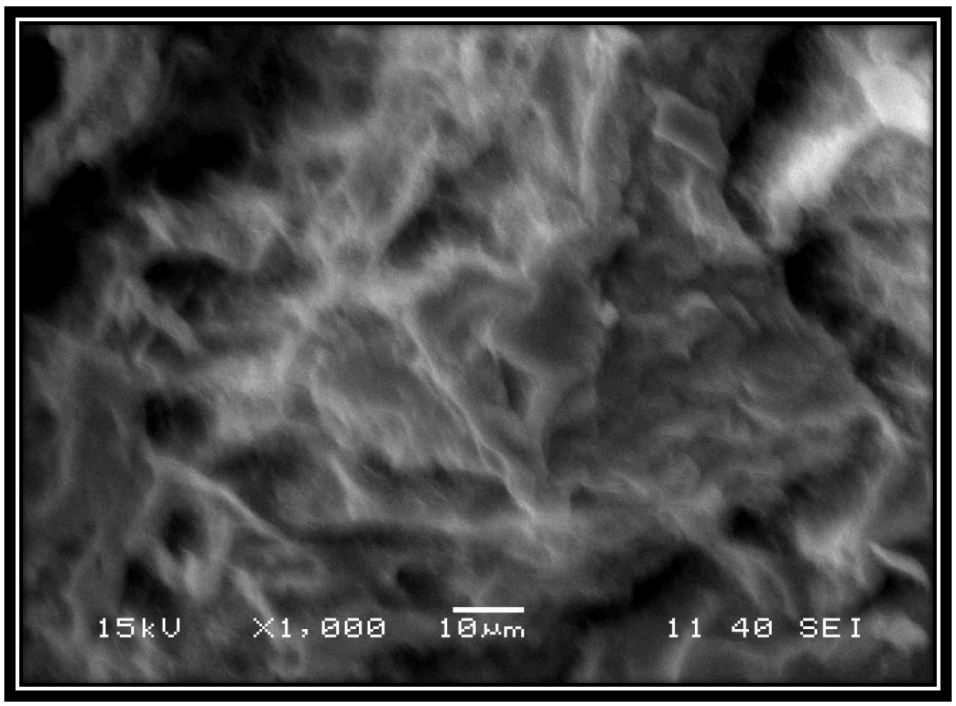


Fig. 11: SEM image of dried tea fungus powder (1000x)

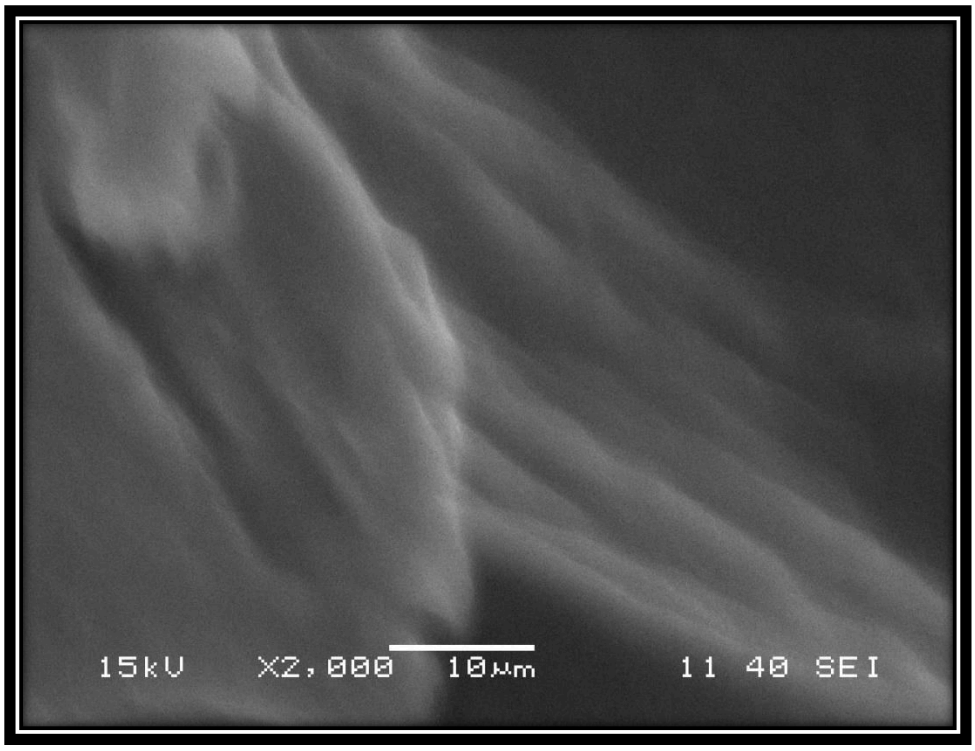


Fig. 12: SEM image of dried tea fungus powder (2000x)

SEM image analysis reveals that cellulose are present in fibrous form as bundles and net like structure of cellulose might entangled by microorganism.

5.8. Analysis of Ethanol production by UV-VISIBLE spectrophotometer

The dried tea fungus powder after 3 days of fermentation was analysed for bioethanol production in UV- visible spectrophotometer taking commercial grade ethanol as control and ethanol produced from tea fungus biomass. The absorbance graph of ethanol in UV-visible range is shown graph (Fig. 13-14)

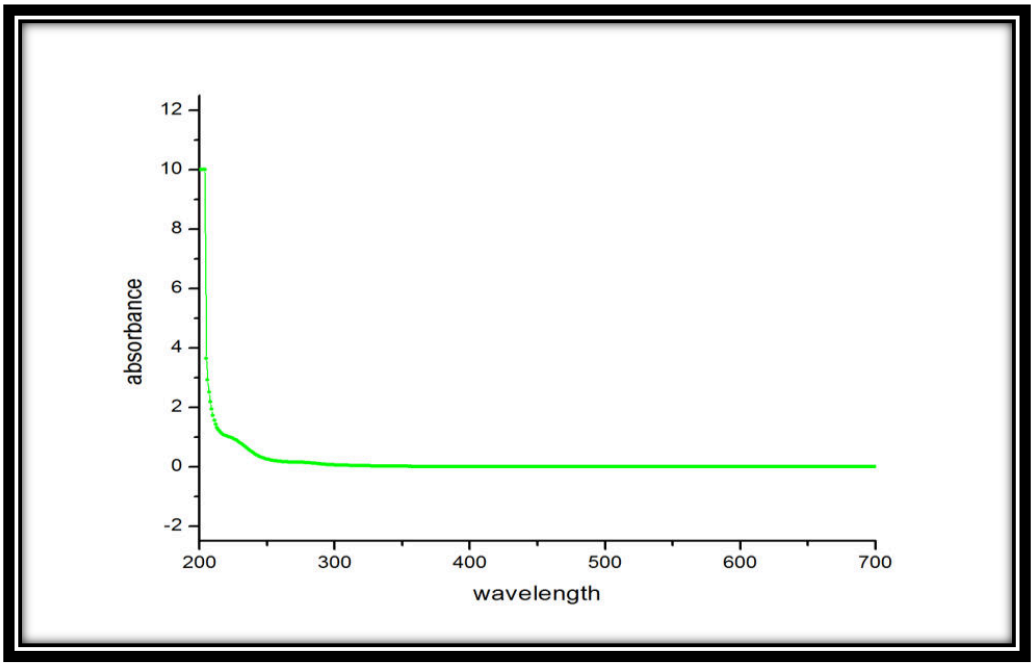


Fig.13: Absorbance graph of commercial Ethanol (control)

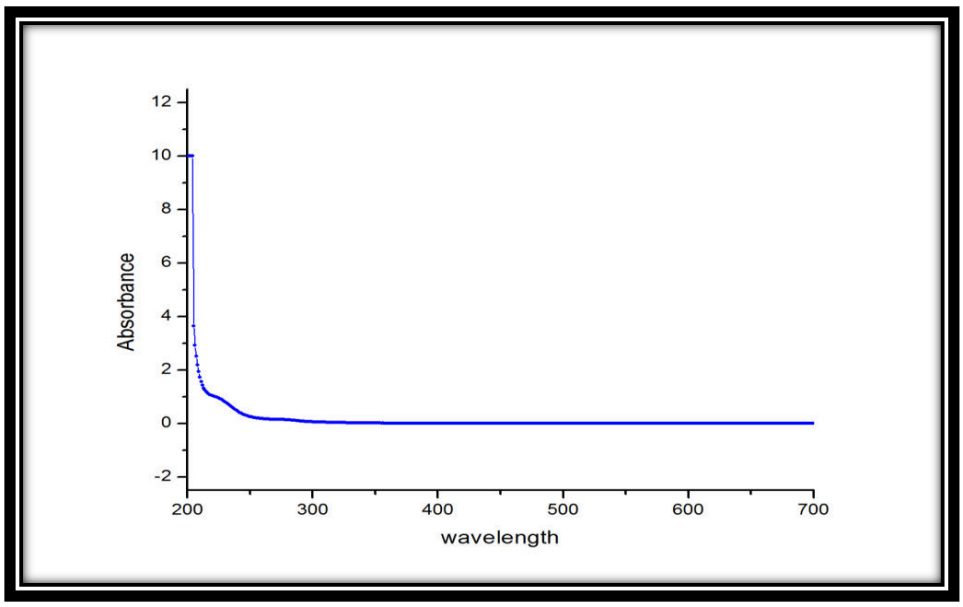


Fig.14: Absorbance graph Ethanol produced from dried tea fungus powder

5.9. Analysis of Ethanol production by gravimetric method

Difference in specific gravity before and after fermentation of dried tea fungus treated with Novozyme was measured to calculate percentage of alcohol. It was calculated that 8 g of dried tea fungus powder yields 13.636% of alcohol.

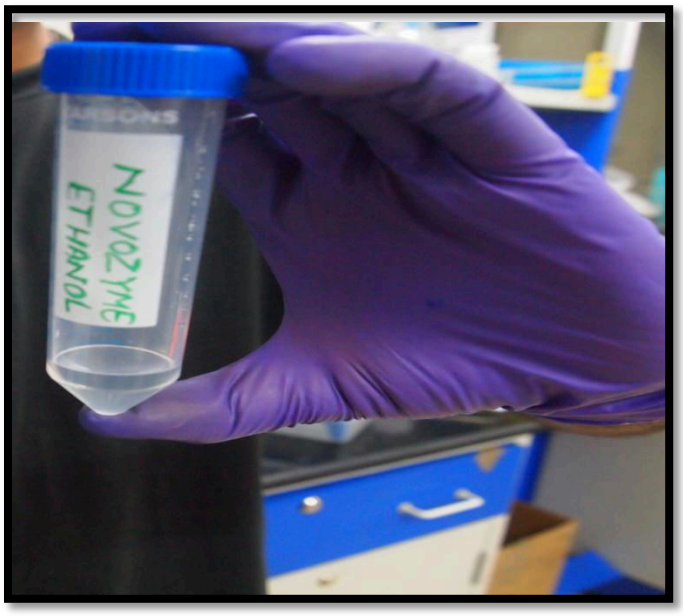


Fig.14 Ethanol

6. CONCLUSION

It is found from the literature survey that the tea fungus has not been utilized as the source of bioethanol in any part of the world. Tea fungus is widely used for the production of Kombucha tea (sugared and fermented black tea beverage) and it is consumed by several people all around the world. Tea fungus is a consortium of acetic acid bacteria and ethanol producing yeasts trapped in cellulose matrix. Tea fungus looks like a thick jelly membrane and only a portion of this is required to start the fermentation process. The remaining tea fungus biomass is thrown as waste. The growth of tea fungus in tea broth is influenced by sucrose concentration and 7% of sucrose was found to be optimum for maximum yield of tea fungus. Tea manufacture waste material which acts as nitrogenous source also influences the growth of tea fungus and it was found that 1% of tea manufacture waste is having maximum yield of tea fungus. The growth of tea fungus is also influenced by surface area to depth ratio of culture medium. It was observed that container with shallow area and wide opening is having maximum yield of tea fungus. It was found that the pH of tea broth decreases with increasing fermentation day which also influences tea fungus growth and it was concluded that at very low pH growth of tea fungus is slower and hence less production of tea fungus occurs. Therefore, if the optimized condition for tea fungus growth is maintained for its pilot production in industrial level then the cellulose available in the tea fungus biomass can be utilized for the production of bioethanol. Hence, tea fungus can be a sustainable source of bioethanol provided with a suitable and optimized process for the conversion.

Challenges in scaling up to industrial level

The production of commercialized bioethanol has been a key interest of researchers to combat the fossil fuel depletion. Bioethanol can be produced from any material which contains sucrose, cellulose or lignocellulose. But the production of bioethanol from Tea fungus biomass is a new hope for researchers. The major raw materials required for its production are tea and sugar as nitrogen and carbon source respectively which are quite expensive for industrial production of ethanol. Hence a cost effective source for carbon and nitrogen to commercially produce tea fungus for bioethanol production is a major challenge for industries. This challenge can be overcome by utilizing tea waste material and by products of agro-forestry industry. Other main challenge is the time required for the growth of tea fungus. The in vitro growth of tea fungus is slow and less, hence optimization of cultural conditions for the maximum growth of tea fungus in short duration of time is very essential. Since tea fungus is growing only on the surface of the tea liquor, larger surface area will be required for getting a huge biomass. This will be possible by growing the tea fungus in large trays with minimum quantity of tea liquor than in cylindrical type vessels. But, growing the tea fungus in horizontally kept trays will occupy a large space. Conditions for extracting the cellulose from tea fungal biomass must be optimized for the highest yield. Since tea fungus is the byproduct during kombucha tea preparation and can be grown even in decoction prepared from tea waste material, the cost of raw materials can be eliminated. Cost of sugar can also be excluded by finding some waste carbohydrate rich materials. Hence, the only cost involved is extraction process, cellulase enzyme and distillation technique. Growing tea fungus in decoction prepared from tea waste material and waste carbohydrate rich materials will be a highly economical way for producing bioethanol.

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