Exploitation of *Cryptococcus neoformans* isolated from Cow dung in bioremediation of medical waste

AKSHAYA RAVINDRAN

17PBT002

A Thesis submitted to Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore – 641 043

In Partial Fulfillment of the Requirement for the Degree of

Master of Science in Biotechnology

April 2019

Certificate

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Signature of the

Head of the department

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Signature of the

Supervisor

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Contents

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

Introduction

1.0 INTRODUCTION

The waste produced during the diagnosis, treatment, immunization of human and animal research activities in the production or testing of biological samples or in health camps is referred to as biomedical waste. Biomedical waste is extremely ultra-hazardous type of waste, if it is not managed properly can lead to chronic health and environmental problems (Zeeshan *et* al.,2018). Waste indicates any useless, unwanted, rejected substance or material which cannot be used further. This includes any substance that is spilled, leaked, pumped, poured, emitted or dumped onto the land, water and air. Biomedical waste generated in the hospitals falls under two major categories – nonhazardous waste and bio hazardous waste (Datta *et al.,*2018).

Non-hazardous waste includes non-infected plastic, cardboard, packaging material, paper, etc., while bio hazardous waste are either infectious wastes such as sharps, non-sharps, plastics, liquid wastes, etc., or non-infectious wastes such as radioactive waste, discarded glass, chemical waste, cytotoxic waste, incinerated waste, etc (Pullishery *et al*.,2016). Waste generated by health care activities also includes cultures, infectious agents, human tissues, organs, body parts or blood, used and unused sharp objects such as broken glass wares and lancets that have been used to puncture or cut the body as well as body fluids or wastes (Yawson, 2015).

The waste generated by the hospitals pose a serious threat to the person who handles those wastes. It can be injurious to humans or animals and deleterious to environment. When the hazardous and non-hazardous wastes are mixed together then the whole waste becomes harmful. The microbes present in the waste can leach out and contaminate the environment (Rajan *et al.,* 2018).

Different sources of liquid waste in the hospital include waste disposed from operation theatres, laboratories of microbiology, biochemistry, histopathology, radiology, blood bank, etc. Health care waste has both organic and inorganic substances that enhance the growth of pathogenic microorganisms (Alam *et al*.,2019). Disposal of hospital waste into the municipal sewage may create problems such as health issues and imbalance of the microbial community in the sewage systems, which affects the biological treatment process. It is important to be aware of the sources of waste and treat the waste before being dumped into the environment (Radhakrishnan and Nagarajan, 2015).

The indiscriminate dumping of medical wastes by hospitals and nursing homes was a source of pollution that caused dangers to the health and surroundings. In order to overcome this crisis, the Bio Medical waste (Handling and Management) Rules, were notified in July 1998. The rules introduced medical waste disposal practices in India. The emphasis is on ensuring a process change that will enable health care facilities to handle their waste through proper training and capacity building (Berihun and Solomon,2017). These rules are applicable to all or any persons, who generate, collect, receive, store, transport, treat, dispose or handle biomedical wastes. This includes hospitals, nursing homes, clinics, dispensaries, veterinary institutions, animal homes, pathological laboratories and blood banks. Although the bio-medical waste (Management and Handling) rules have already been introduced a few years back, not much attention has been paid to manage them (Hirani *et al*.,2014).

Biomedical waste is hazardous since it has an inherent potential for dissemination of infection, both noscomial within health care settings as well as risk of infection to persons working outside health care facilities, like waste handlers, scavenging workers and also to the general public (Orgev and Utka,2017). It is reported that 60% of all hospital staff sustain injuries from sharps during various procedures undertaken in health care facilities. Cytotoxic and chemical waste is mutagenic and teratogenic. Additional hazard includes recycling of disposables without being even washed.Safe disposal of biomedical waste is also a legal requirement in India. The objectives of BMW management are:

• To stop transmission and spreading of pathogens and diseases

• To prevent injury to individuals in health care services and workers who handle BMW

• To stop general exposure to the harmful effects of the cytotoxic, genotoxic and chemical biomedical waste

• To prevent environmental degradation

The currently adopted procedures to treat biomedical waste include chemical process, thermal process, mechanical process, irradiation process and biological process. Chemical disinfectants are usually used for killing microorganisms and inactivating hazardous pathogens (Datta *et al.,*2018). Chemical process use chemicals such as sodium hypochlorite, hydrogen peroxide, chlorine etc. that acts as disinfectants for biomedical waste. Thermal process utilizes

heat to disinfectant (Hirani *et al.,*2014). Autoclaving is a low heat thermal method and it uses steam for medical aid of waste. It is commonly used for the human body fluid waste, sharps, and microbiology laboratory waste. This system needs extreme temperature (thermal) that produces steam to decontaminate the medical waste (Airlina,2019). Microwave is an emerging technology to treat bio hazardous waste, including material from healthcare facilities (Zimmermann,2017). It potentially kills the pathogens present in the biomedical waste (Voudrias,2016).

Incineration is an engineered method that is designed to treat health care waste that uses thermal decomposition via thermal oxidation at high temperatures between 900 and 1200ºC to destroy the organic fraction of the waste (Ghasemi and Yusuff,2016). Compaction and shredding is the mechanical process done to reduce the quantity of waste and also to destroy plastic and paper waste (Prasad and Reddy,2017). Reverse polymerization uses microwave energy to break down complex molecules and in this way to treat medical waste. Irradiation process exposes wastes to ultraviolet or radiation in an enclosed chamber. The wave can generate heat to the waste materials and kill all the microorganism (Airlina,2015). They completely lose their physical form and reduce in volume (Zimmermann,2017). Hot air ovens have been used to sterilise glassware and other reusable instruments and infectious health waste (Capoor and Bhowmik,2017). Gas/vapor sterilization uses gaseous or vaporized chemicals as the sterilizing agents. Ethylene oxide is the most commonly used agent (Patan and Mathur,2015).

Biological process use biological enzymes for treating medical waste. It is claimed that biological reactions will not only clean the waste but also cause the destruction of all the organic constituents, so that only plastics, glass, and other inert will remain within the residues (Ghasemi and Yusuff,2016).

Proper medical waste management is the mainstay of hospital cleanliness, hospital hygiene and maintenance activities. Appropriate hospital waste management system is an important element of quality assurance in hospitals. Ultimate aim of waste management is the prevention of disease and protection of environment (Joshi *et al*., 2015). It is the moral duty of health care workers to prevent hospitals from becoming centers of disease rather than center of cure. Raising awareness on public health and environment hazards associated with inappropriate segregation, collection, storage, transport, handling, treatment and disposal of health care waste.

Regular training program for all the sections of health care workers with special emphasis on waste handlers is essential (Kumar *et al*.,2015). Identifying safe, efficient, sustainable economic and culturally acceptable waste management practices and technologies and enabling the participants to spot the systems according to their specific needs is an important area to be focused (Rajan *et al.,*2018).

Cow dung, excreta of bovine animal, is a cheap and easily available bio resource in our planet. Many traditional uses of cow dung such as burning of fuel, mosquito repellent and as cleansing agent are already known in India (Gupta *et al*.,2016). Synthetic and semi synthetic pharmaceuticals are known to pollute the aquatic, terrestrial, and atmospheric environment (Singh and Sharma,2016).

Cow dung is a most important source of bio-fertilizer but at the same time cow's urine, cow's horn and a dead body of a cow can be used for preparing effective bio-fertilizer. There are a variety of cow dung and cow's excreta product, which are being used as fertilizers and pest repellent respectively in agricultural practice (Raj *et al*.,2014). Cow dung is very effective manure for reducing the bacterial and fungal pathogenic disease. Cow dung slurry consists of bacteria, fungi and actinomycetes (Idham *et al*.,2016). It showed positive response in suppression of mycelia growth of plant pathogenic fungi like *Fusarium solani, Fusarium oxysporum* and *Sclerotinia sclerotiorum*. Therefore, application of cow dung in proper and sustainable way to enhance not only productivity of yield but also minimizing the possibilities of disease (Raj *et al.,*2014).

Cow dung is not a waste material, but it is a purifier of all wastes in the nature. *Periconiella* species of fungus isolated from cow dung was found to be an excellent degrader of plastics. It was found to be cheap, safe, and environment friendly method of medical waste disposal (Prashanthi *et al*., 2017). Biomedical waste especially catheter, IV tubes are made up of polyethylene, Teflon, latex, polyurethane and thermoplastic elastomers. The biomedical waste like IV tubes, catheters are disposed off without prior treatment which will lead to spread of infections (Deb *et al*.,2019). Benzene is one of the compounds released from municipal sludge which is carcinogenic and is not bactericidal in nature. Bioremediation of benzene can be done using cow dung micro flora. *Pseudomonas putida* is a potential benzene degrader isolated from the cow dung micro flora and it has its ability to degrade benzene at various time intervals.

Pseudomonas plecoglossicida is a novel organism for bioremediation of hazardous compounds like cypermethrin and chlorpyrifos by *Pseudomonas aeruginosa*. These microorganism obtained from cow dung though have the ability for bioremediation in laboratory setups, can also be applied in pesticide contaminated soil and water. Cow dung slurry can also be effectively used in degrading phenol and also used as a source of microbial consortium for bioremediation of fenvalerate soil. It converts the toxic materials into nutrient, biomass and carbon dioxide through bio degradation (Godambe and Fulekar, 2016).

With this background, the present study was formulated with the following objectives.

- To isolate the microbial species from cow dung.
- To characterize the microbial species and identify the species.
- To evaluate the effect of the identified species in treating the biomedical waste.
- To check the efficiency to biodegradation of biomedical waste using microbes.

Review of literature

2.0 REVIEW OF LITERATURE

Biomedical waste means any useless, unwanted or discarded substance or material which cannot be used further. Biomedical waste is generated from hospitals, health care teaching institutes, research institutions, radioactive materials, chemicals, blood banks, clinics, laboratories, pharmaceuticals, veterinary institutes and animal houses etc (Hameed *et al*.,2017). Waste produced in the health care activities carries a higher potential for injury and infection when compared to the other types of waste. This include any material that is spilled, leaked, pumped, poured, emitted or dumped onto the land, water or air (Rajan *et al*.,2018).

Medical waste management is one of the many complex and demanding challenges faced nowadays. Medical waste that is not properly handled and disposed of contains high risk of infection or injury to healthcare personnel. Most of the medical wastes are considered as hazardous that may be infectious, toxic or radioactive (Janagi *et al*.,2015). At hospitals and other health care facilities, medical waste is generally sorted into colour – coded bins or bags (Rai *et al*.,2017). Medical waste contains a significantly higher plastic content and as a result the combustion of medical waste leads to the formation of polychlorinated dibenzo-p-dioxins and polychlorinated dibenzofurans which are highly toxic substances (Windfeld and Brooks,2015).

The review of literature pertaining to the study entitled, **"Exploitation of *Cryptococcus neoformans* isolated from Cow dung in bioremediation of medical waste"** is discussed under the following headings:

2.1. Biomedical waste

2.2. Categories of biomedical waste

2.3. Classification of biomedical waste

2.4. Health hazard from biomedical waste

2.5. Problems relating to biomedical waste

2.6. Salient features of biomedical waste rules 2016

2.7. Biomedical waste management and its practice

2.8. Biomedical waste treatment and disposal

2.9. Cow dung as a biodegradable agent of medical waste

2.1. BIOMEDICAL WASTE

Medical waste can be defined as the materials accumulated after diagnosis, treatment or immunization of patients. If the medical wastes are not collected separately then majority of the wastes become infectious (Acharya *et al.,*2014). Public health is meant to be protected by the health sector through clinics and hospitals which provide health services to the public, help in the management of infections and gives relevant information regarding health problems. The wastes generated by the health care contains high risk of infections due to their hazardous content (Odumosu*,*2016).

Biomedical waste is any solid or liquid waste that may cause harm to human beings and environment. The waste includes cotton, bandages, hypodermic needles, syringes, tubing such as intravenous sets and catheters etc. It is also known as clinical waste, medical waste and health care waste in different parts of world. Such waste comprises only 15 to 25% of total waste generated in a hospital, the remaining waste is a general waste which includes waste paper, wrapper of drugs, cardboard and left-over food, etc (Kumar *et al.,* 2014).

Health care facilities are the place for patients for diagnosis, analysis and treatment medical issues. They generate a number of solid wastes which is unavoidable. These solid wastes are referred to as "healthcare wastes" which includes all biological and non-biological wastes that is disposed and never will be used again (Acharya *et al.,*2014).

Hospital is not only the source of generating biomedical waste, there are also many other healthcare facilities such as medical clinics, veterinarian centres and medical laboratories that generate hazardous and infectious waste across the world. Some of these sites generate medical waste more quicker than certain hospitals because of the poor waste management (Odumosu*,*2016).

Waste is classified as general, medical or clinical and sharps. General waste does not pose any immediate danger to humans or the environment. Some examples of general waste include packaging materials such as cardboard, paper, cans, etc. Pathological waste includes

tissues, organs, and other body parts. Infectious waste is defined as waste that contains pathogens which result in diseases. Examples are culture plates, surgical and theatre wastes, contaminated plastic, etc. Sharps like needles, syringes, scalpels, knives are the materials that could cause a cut or puncture leading to wound (Mmereki *et al*.,2017).

Autoclaving is suitable for sterilizing hazardous waste. However, the volume of waste cannot be reduced in this process. It is not suitable for recognized body parts (Jaseem *et al*.,2017). On the other hand, the hydropulping, an oxidation technique, has the required water content of approximately 80% as a result of an increase in weight; however, the resultant volume can be as little as 30% of the original volume. The use of a pulping system is highly controversial for clinical waste treatment (Krishnamoorthy,2017).

2.2 CATEGORIES OF BIOMEDICAL WASTE

Health Care Facilities (HCFs) are primarily responsible for management of the healthcare waste generated within the facilities, including activities undertaken by them in the community. Waste generated from the healthcare facility is classified as (Windfeld and Brooks,2015).

- Bio Medical Waste
- General Waste
- Other Wastes

Bio Medical Waste Management Rules, 2016 categorizes the bio-medical waste generated from the health care facility into four categories based on the segregation pathway and colour code. Various types of bio medical waste are further assigned to each one of the categories, as detailed below (Cesaro and Belgiorno,2017).

1. Yellow Category

2. Red Category

3. White Category

4. Blue Category

Table 1: Categories of biomedical waste

CATEGORIES	TYPES OF WASTE
YELLOW	**Human Anatomical waste** Human tissues, organs, body parts and fetus below the viability period. **Animal Anatomical waste** Experimental animal carcasses, body parts, organs, tissues, including the waste generated from animals used in experiments or testing in veterinary hospitals or colleges or animal houses. **Soiled waste** Items contaminated with blood, body fluids like dressings, plaster casts, cotton swabs and bags containing residual or discarded blood and blood components. **Discarded or Expired medicine** Pharmaceutical waste like antibiotics, cytotoxic drugs including all items contaminated with cytotoxic drugs along with glass or plastic ampoules, vials etc. **Chemical waste** Chemicals used in production of biological and used or discarded disinfectants. **Chemical liquid waste** Liquid waste generated due to use of chemicals in production of biological and used or discarded disinfectants. **Discarded linen, mattresses, beddings**

	contaminated with blood or body fluid, routine mask and gown. **Microbiology, Biotechnology and other clinical laboratory waste** Microbiology, Biotechnology and other clinical laboratory waste: Blood bags, Laboratory cultures, stocks or specimens of microorganisms, live or attenuated vaccines, human and animal cell cultures used in research, industrial laboratories, and devices used for cultures.
RED	Wastes generated from disposable items such as tubing, bottles, intravenous tubes and sets, catheters, urine bags, syringes, needles, and gloves.
WHITE	Waste Sharps including metals Needles, syringes with fixed needles, needles from needle tip cutter or burner, scalpels, blades, or any other contaminated sharp object that may cause puncture

	and cuts. This includes both used, discarded and contaminated metal sharps
BLUE	Broken or discarded and contaminated glass including medicine vials and ampoules except those contaminated with cytotoxic wastes.

2.3 CLASSIFICATION OF BIOMEDICAL WASTE

The biomedical wastes are regulated and managed according to various standards and protocols in different countries. In health care facilities, the wastes are generated during improper management, which causes a direct health impact in the community, the environment and the health care workers (Pandey *et al*.,2016). BMW is a dangerous health hazard to the public, hospital, health care units, flora and fauna of the area. BMW must be stored in a secure environment at all times, whenever possible, BMW should not be mixed with chemical, radioactive or other laboratory trash. Containers for BMW must be appropriate for its contents, there are different kinds of containers, and bags are available for the containment and disposal of BMW. The Government of India specifies that BMW is a part of hospital hygiene and maintenance activities (Achuthan and Madangopal,2016).

Biomedical waste can be classified into hazardous and non-hazardous waste. Nearly 75-90% of the medical waste is harmless and non-hazardous. The remaining 10-25% is harmful and hazardous and also can be injurious to humans or animals and pose a serious threat to environment. When both these wastes are mixed together then the whole waste becomes harmful (Deshmukh and Rathod,2016). Medical wastes can be differentiated into risk and non-risk waste. The portion of infectious and hazardous waste is relatively small, but improper management of this waste can lead to contamination of greater volume of general waste (Jaafari *et al*.,2015).

2.2.1. Risk waste

Risk waste is considered to be dangerous to the public because of the presence of certain toxic components. It is further subdivided into many types based on its components, sources and the risk it causes (Skinner *et al.,*2018).

1. Infectious waste
2. Pathological waste
3. Sharps
4. Microbiological / clinical waste
5. Pharmaceutical waste
6. Genotoxic waste
7. Chemical waste
8. Radioactive waste

2.2.1. a) Infectious waste

Infectious waste is biomedical waste that has been previously contaminated with any kind of pathogen with sufficient virulence capable of infecting a susceptible host thereby resulting in an infectious disease. Laboratory wastes, such as culture, surgical and autopsy cases of waste, waste isolation wards that infected patients are hospitalised. Wastes that are produced from parts of infectious patient's hemodialysis, such as pipes and filters, disposable towels, gowns, bands and gloves and waste that are in contact with animals inoculated with infectious agents (Odumosu,2016).

2.2.1. b) Pathological waste

This includes anatomical waste from human and animals usually generated from hospitals and veterinary hospitals. Examples embody blood, body parts such as amputated legs or arms, placenta, and tissues. They are probably dangerous with a high risk of illness and infection in a prone individual who has direct contact with them (Banerjee *et al*.,2019).

2.2.1. c) Sharps

Sharps contains both used and unused sharp objects like hypodermic needles, syringes, Scalpels , broken ampoules, and glassware. They are considered extremely dangerous because they are not only capable of inflicting punctures and cuts on the skin however they also harbor dangerous infective bacterium, which may be introduced into the body via wounds or punctures. Sharps are usually generated within the operation theatre wherever surgical activities are carried out. Alternative areas where they are generated include all wards and laboratories (Padmanabhan and Barik,2019).

2.2.1. d) Microbiological / Clinical wastes

This is closely related to pathological waste in terms of high risk but the contents are quite different from pathological waste. It is usually generated from laboratories during the course of experiments and clinical trials. Examples include human and animal cell cultures from clinical experiments, microorganisms, blood collection tubes, body fluid, drainage bags, vials, used culture dishes, and other materials that were previously in contact with infectious agents (Letcher and Vallero,2019).

2.2.1. e) Pharmaceutical waste

Pharmaceutical waste is generated by various means such as chemicals, expired drugs, preparations of drugs added to an intravenous solution, pharmaceutical products such as drugs (Dubey and Upmanyu,2017). It is very important to note that pharmaceutical waste does not include empty glass ampoules, drugs, and other metabolic products excreted by patient undergoing therapy, empty pills bottles or strip packages from where the drug/capsules have been previously removed (Odumosu,2016).

2.2.1. f) Cytotoxic and Genotoxic wastes

These include unused cytotoxic medicine, solid materials like sharp objects, tissues, IV bags, and other items which may come into contact with a medicinal drug or carcinogenic matter (Samant *et al*.,2018).

2.2.1. g) Chemical wastes

Chemical waste materials include those that are generated during the production of biological preparations such as disinfectants and insecticides, in medical, dental, and veterinary laboratories (Shikoska *et al*.,2016).

2.2.1. h) Radioactive wastes

These are solid, liquid, or gaseous forms of waste generated from the medical or research use of radiology, radionuclide (e.g., during radioimmunoassay), and other radiological procedures which are capable of emission of radiation at above the level set by regulatory authorities as exempt. Examples are found in nuclear medicine treatments, cancer related therapies, and medical devices that use radioactive isotopes (Mmereki *et al*.,2017).

2.2.2. Non risk waste

Non-risk waste is similar to normal domestic garbage and presents no larger risk, than the waste from a normal home. This waste is generated by nearly everyone within the hospital, ie., administration, patient's risk, cafeterias and nursing station. The wastes such as paper and cardboard, packaging, food waste and aerosols are non – risk wastes (Woolridge and Hoboy,2019).

2.4 HEALTH HAZARD FROM BIOMEDICAL WASTE

According to World Health Organization, 85% of medical wastes are non – hazardous, only 10% are infectious and 5% are non – infectious which is considered as hazardous wastes. The hospital wastes such as organs, tissues, body parts, blood and body fluids with cotton,

bandage and plaster casts from infected and contaminated areas need to be properly collected, segregated, stored, transported, treated and disposed of in a safe manner to prevent infections (Padmanabhan and Barik,2019).

A cross-sectional study was conducted on hospital waste management in 8 hospitals of Karachi. Data was collected using sampling technique, self-administered questionnaire with 4 sections, relating to the information of institution, health waste management information and hospital waste management information. Out of 8 hospitals, 2 hospitals provided essential protective materials to the waste handlers. Only 1 hospital arranged training session for waste handling staff and 5 hospitals had storage area. This study concluded that there should be a proper training and management regarding awareness for the waste handlers (Othman *et al*.,2017).

A study was conducted on medical waste management in 60 health care hospitals of Bangladesh. The information collected was regarding the different types of medical waste generated. The results have shown that total health care waste generated is 5562 kg/day, of which about 77.4% are non-hazardous and about 22.6% are hazardous. This study concluded that lack of awareness is responsible for improper management of biomedical waste (Mathur *et al*.,2012).

About 53 nursing homes and smaller hospitals in Delhi a study was carried on biomedical waste management. The appropriate data was collected through field visit. The survey results show that there is a development in segregation practices of medical waste in nursing homes and small private hospitals (Haifete *et al*,2017).

A cross-sectional study was conducted on the biomedical waste management at Balarampur hospital, Lucknow, India. The study shows that within the hospital both infectious and non-infectious waste is dumped together. The treatment facility for infectious waste is not provided in the hospitable. The result of the study demonstrates better environmental system for the disposal of biomedical waste in the hospital (Manar *et al*.,2014).

2.5. PROBLEMS RELATING TO BIOMEDICAL WASTE

1. The scope of the rules has been expanded to include various health camps such as vaccination camps, blood donation camps, and surgical camps.
2. The duties of the operator of a common biomedical waste treatment and disposal facility (CBMWTF) have been increased. They should assist in training of HCW from where the waste is being collected. Furthermore, there should be barcoding and global positioning system established for handling of BMW.
3. Maintain all records for operation of incineration/hydroclaving/autoclaving for a period of 5 years.
4. The segregation, packaging, transportation, and storage of BMW have been improved.
5. Biomedical waste has been classified into four categories based on color code-type of waste and treatment options. In addition, untreated human anatomical waste, animal anatomical waste, soiled waste, and biotechnology waste should not be stored beyond a period of 48 h. In case, there is a need to store beyond 48 h, the occupier should take all appropriate measures to ensure that the waste does not adversely affect human health and the environment.
6. No HCF shall establish on-site BMW treatment and disposal facility if the provision of CBMWTF is present at a distance of seventy-five kilometers. If no CBMWTF is available, the occupier shall set up requisite BMW treatment facility such as incinerator, autoclave or microwave, shredder after taking prior authorization from the prescribed authority. After confirming treatment of plastics and glassware by autoclaving or microwaving followed by mutilation/shredding, these recyclables should be given to authorized recyclers.
7. Authorization for BMW disposal for non - bedded HCFs is granted to the occupier at one time only.
8. Standards for emission from incinerators have been modified to be more environmental friendly. These are permissible limit for SPM-50 mg/nm^3; residence time in secondary chamber of incinerator – two seconds; standard for dioxin and furans – 0.1 ng TEQ/Nm^3.
9. Ministry of Environment, Forest, and Climate change will monitor the implementation of rules yearly (Datta *et al*.,2018).

2.6 SALIENT FEATURES OF BIOMEDICAL WASTE RULES 2016

A major issue associated with current Bio-Medical waste management in several hospitals is that the implementation of Bio-Waste regulation is disappointing as some hospitals are eliminating waste during a haphazard, improper and indiscriminate manner (Deshmukh and Rathod,2016). Lack of segregation practices leads to mixing of hospital wastes with general waste making the entire waste unsafe. Inappropriate segregation ultimately results in an incorrect technique of waste disposal (Debalkie and Kumie,2017).

Environmental Hazard

Inappropriate treatment and disposal of bio-medical waste contributes to environmental pollution, uncontrolled incineration causes air pollution, dumping in tanks and along the river bed causes water pollution and land filling cause's soil pollution. Inadequate Bio-Medical waste management therefore can cause environmental pollution, unpleasant smell, growth and multiplication of vectors like insects, rodents and worms and may lead to the transmission of diseases like typhoid, cholera, liver disease and AIDS through injuries from syringes and needles contaminated with human (Diallo,2016).

Occupational hazard

Various communicable diseases, that spread through water, sweat, blood, body fluids and contaminated organs, are important to be prevented. The Bio Medical Waste scattered in and around the hospitals invites flies, insects, rodents, cats and dogs that are liable for the spread of communication illness like plague and rabies (Mathur *et al*.,2012). Rag pickers within the hospital, checking out the garbage are at a risk of getting tetanus and HIV infections. The utilization of disposable syringes, needles, IV sets and other article like glass bottles without correct sterilization are responsible for infectious disease, HIV, and other viral diseases. It becomes primary responsibility of Health directors to manage hospital waste in most safe and eco-friendly manner (Deshmukh and Rathod,2016).

Public Health Hazard

Poor management of bio-medical waste can cause serious disease to health-care personnel, to workers, patients and to the general public. The greatest risk posed by infectious

waste is needle injuries, which can cause hepatitis B and hepatitis C and HIV infection. There are numerous other diseases which could be transmitted by contact with infectious bio-medical wastes. During the handling of wastes, injuries occur when syringe, needles or other sharps have not been collected in puncture proof containers. The reuse of infectious syringes represents a major threat to public health (Muralikrishan e*t al*.,2017).

2.7 BIOMEDICAL WASTE MANAGEMENT AND ITS PRACTICE

Waste treatment may be defined as any method, technique, or process designed to change the biological character or composition of any medical waste to reduce or eliminate its potential for causing disease. Effective hospital waste disposal systems are essential in ensuring the safety of health workers and the public (Awodele *et al*.,2016). The public has a right to a safe and healthy environment that is free from infectious diseases and hazardous materials arising from hospital waste. The need to spend so much money on waste disposal is to ensure public safety especially from sharps that may cause injuries leading to serious infections. The risk of contracting diseases from nosocomial pathogens, exposure to hazardous chemicals, and many more have been previously established through research studies. Hence, a proper method for disposing hospital waste is essential to safeguard the life of humans and animals (Kumar *et al*.,2015).

The first step towards an effective waste management process is to segregate the different types of waste at the initial source of generation. Through segregation different types of waste can be identified, labelled, and placed in different waste containers and can be treated differently. The aim of this process is to avoid mixing together the risk and non-risk waste, especially the infectious part of waste which represents a lower percentage, which is to be carefully separated and treated differently since it contains dangerous waste matter (Windfeld and Brooks,2015).

In collection of waste, certain procedures are usually followed. According to several regulatory bodies, waste collected at room temperature should not be allowed to stay at room temperature for more than 24 h. This is important to prevent the incubation of pathogens and the deterioration of such waste matter. Generally, different wastes are categorized based on the level of risk they carry and are assigned specific containers with designated colours. Human anatomical waste are collected in yellow plastic containers, microbiological waste in yellow/red, waste sharps in plastic bag puncture-proof in translucent blue/white containers, domestic hospital

waste in green bags, etc. Health care waste is very difficult to manage as it is a heterogenous mixture. If a proper management system is planned the problem can be reduced (Mani and Singh,2016).

Incineration technology

Incineration is the preferred option in many areas for the treatment of biomedical waste. The burning of solid and liquid wastes generated by health care creates many problems. Medical waste incinerators release toxic gases in the environment and cause a serious threat to the humans and animals. Medical waste ashes contain extent level of heavy metals and dioxins. Chlorinated dioxins are extremely toxic and produce effects in humans and animals even at very low dose. Medical waste contains many plastics such as polyvinyl chloride (PVC), which is rich in chlorine (Ghasemi and Yusuff,2016).

Autoclaving

Thermal treatment is usually used for sharps and certain other forms of infectious waste. An autoclave is a large sterilizer that uses high temperatures and steam to deeply penetrate all materials and kill any microorganisms. Depending on the type and quantity of waste, sterilization can be done. Modern autoclaves are automated and thus reduce contamination. Decontaminated sharps and other medical waste that's been autoclaved will then be disposed of as non-infectious waste. Medical wastes such as chemical waste, including therapy waste, as well as pharmaceutical waste cannot be decontaminated in an autoclave (Datta *et al*.,2018).

Chemical treatment

Chemical treatment is used to deactivate liquid waste on site rather than packaging and sending them to a separate facility. Since liquids are extremely liable to spills, it is generally best to have them treated as close to the generation site as possible. Depending on the kind of waste, chemicals like chlorine, sodium hydroxide or calcium oxide can be used. These chemicals might usually produce undesirable by products when applied. Chemical treatment has to be executed carefully and by knowledgeable workers (Capoor and Bhowmik,2017).

Thermal inactivation

Thermal inactivation involves the treatment of waste with high temperatures to eliminate infectious agents. This method is usually used for large volumes of liquid waste. The types of pathogens in the waste determine the temperature and duration of treatment. This method requires higher temperatures and longer treatment cycles than steam treatment (Patan and Mathur., 2015).

Gas / Vapour sterilization

Gas/vapor sterilization uses gaseous or vaporized chemicals as the sterilizing agents. Ethylene oxide is the most commonly used agent, but should be used with caution since it is a suspected human carcinogen. Because ethylene oxide may be adsorbed on the surface of treated materials, the potential exists for worker exposure when sterilized materials are handled (Patan and Mathur, 2015).

Chemical disinfection

Chemical disinfection is the preferred treatment for liquid infectious wastes. It is commonly used to kill microorganisms on medical equipment and on floors and walls in healthcare facilities (Priya *et al*.,2019).

2.8 BIOMEDICAL WASTE TREATMENT AND DISPOSAL

Biodegradation is the process in which organic substances are broken down by living organisms particularly microbes. Microorganisms play a vital role in the degradation of plastic medical waste. The microorganisms that are responsible for biodegradation of medical waste is bacteria and fungi which differ from each other and have their own optimal growth conditions (Devi *et al.,*2016).

There are many plastic wastes generated in hospitals which are disposed of through open, uncontrolled burning and land filling. As a result of open burning of these wastes various health

issues can occur. The organic compounds such as furans and dioxins are produced by burning of polyvinyl chloride plastics which leads to immune disorders and lung diseases (Alshehrei,2017).

Cow dung is a most important source of bio fertilizer. Different products obtained from cow dung are widely used in many formulations. Cow dung increases the resistance of plant against pests and diseases, increases the soil fertility and stimulate plant growth (Rajeswari *et al.,*2016).

Cow dung is not a waste product, but it is a purifier of all wastes in the nature. *Periconiella* species of fungus isolated from cow dung was found to be an excellent degrader of biomedical waste. Fifty grams of biomedical waste, kept in the form of used bandages and cotton in culture media, were effectively and completely reduced by 50th day. It was found to be cheap, safe, and environment friendly method of biomedical waste disposal (Bali *et al*.,2017).

2.9 COW DUNG HAS A BIODEGRADABLE AGENT OF MEDICAL WASTE

The improper management of bio-medical waste causes serious environmental problems in terms of air, water and land pollution. The nature of pollutants can be classified into biological, chemical and radioactive. Environment problems can arise due to the mere generation of bio-medical waste and from the process of handling, treatment and disposal (Rajeswari *et al.,* 2016).

Bio-Medical Waste that generates air pollution is of three types - Biological, Chemical and Radioactive. Indoor air pollutants like pathogens present in the waste can enter and remain in the air in an institution for a long period in the form of spores or as pathogens itself. Chemical Pollutants that cause outdoor air pollution have two major sources- open burning and incinerators. Open burning of bio-medical waste is the most harmful practice and should be strictly avoided (Ghasemi and Yusuff.,2016).

Water Pollution is another major threat from Bio-medical waste. If the waste is dumped into lakes and water bodies, it can cause severe water pollution. Water pollution can either be caused due to biological, chemicals or radioactive substances. The pathogens present in the waste can leach out and contaminate the ground water or surface water. Harmful chemicals

present in bio-medical waste such as heavy metals can also cause water pollution (Muralikrishan *et al*.,2017).

Land Pollution is caused by the final disposal of all bio-medical waste. Even liquid effluent after treatment is spread on land. Hence, pollution caused to land is inevitable. Open dumping of bio-medical waste is the greatest cause for land pollution (Kumari and Sharma,2018).

Methodology

3.0 MATERIALS AND METHODS

Biomedical waste (BMW) generated in our country on a day to day basis is massive and contains infectious and hazardous materials (Manasi *et al.,*2017). Bio-medical waste means any solid or liquid waste, which is generated during the diagnosis, treatment or immunization of human beings or animals or in research (Karmakar *et al.,*2016). A major issue associated with current biomedical waste management in several hospitals is that the implementation of bio-waste regulation is inadequate as some hospitals are disposing of waste in an improper and indiscriminate manner (Priya *et al*.,2019). Various communicable diseases, which spread through water, sweat, blood, body fluids and contaminated organs need to be prevented. The recycling of disposable syringes, needles, IV sets and glass bottles without proper sterilization are responsible for Hepatitis, HIV, and other viral diseases. It becomes primary responsibility of Health administrators to control hospital waste in most safe and eco-friendly manner (Mishra *et al.,*2015). The hospital waste like body elements, organs, tissues, blood and body fluids along with soiled linen, cotton, bandage and plaster casts from infected and contaminated areas are very essential to be properly collected, segregated, stored, transported, treated and disposed of in safe manner to prevent healthcare facility or hospital in spreading non heritable infection (Mathur *et al.,*2012).

Cow dung is traditionally used as organic fertilizer in India for centuries. The addition of cow dung will increase the mineral status of soil, enhances the resistance of plant against pests and diseases. As per Ayurveda, it may also act as a purifier for all the wastes within the nature (Poongothai *et al.,*2016). *Periconiella* species of fungus isolated from cow dung was found to be good degrader of biomedical waste. Nowadays, there is an increasing research interest in developing the applications of cow dung microorganisms for management of environmental pollutants (Gupta *et al.,*2016).

The methodology adopted for the study entitled **"Exploitation of *Cryptococcus neoformans* isolated from Cow dung in bioremediation of medical waste"** is given below:

3.1. Sample collection

The fresh cow dung samples were collected from the farm and preserved in sterile polythene bags and stored in refrigerator till use. The isolation of microbes was then carried out.

3.2 Microbial analysis of cow dung sample

Serial dilution is a series of sequential dilutions used to reduce a dense culture of cells to a more usable concentration. Each dilution will reduce the concentration of microbial species by a specific amount. Isolation and enumeration of fungi from the cow dung sample was done by agar plate method. The medium used for isolation of fungi was potato dextrose agar. (Thilagam *et al.,*2015). By using serial dilution method, cow dung samples were prepared. Following the serial dilution technique, the samples were preserved and labeled. Out of 10 dilutions, 0.1ml of the last three dilutions was taken for spreading. The method used for isolation of fungi from Cow dung sample is outlined in appendix – I.

3.2.1 Isolation and screening of fungal strains from Cow dung sample

Fungus was isolated from cow dung sample using Potato dextrose agar as per procedure outlined in appendix – I. The fungal colonies were isolated, purified and stained using lacto phenol staining.

3.2.2 Primary screening of fungal strains using morphology characterization

Fungal species are usually been identified on the basis of their morphological characteristics. Colony morphology is a method used to describe the characteristics of an individual colony of fungi growing on agar in petri dish. The morphologies are observable through electron microscope. The morphologies are most important for identification of organisms. Colony morphology was observed after 5 days of incubation. Different types of fungi will produce different looking colonies. Some colonies may be coloured, some colonies are circular in shape and others are irregular. Colony morphology includes form, size, elevation, margin or border, surface, opacity, pigmentation, mycelium and spore. The shape of the colony may vary from circular, irregular, filamentous or rhizoid. The size of the colony varies from large to small and it is measured using ruler. Tiny colonies are referred to as punctiform. The magnified edge shape of the colony is observed using microscope. The margin of the colony

varies from entire, undulate, filiform, curled or lobate. The surface of the colony varies from smooth, rough, glistening, wrinkled or dull. Elevation of the colony describes the side view of the colony. Elevation of the colony varies from raised, corvex, flat and crateriform. That is, the colony rising above the agar can be determined by its height. Opacity of the colony determines whether the colony is transparent, opaque or translucent. Pigmentation of the colonies is observing the color of the colonies such as white, buff, red, purple, etc. Mycelium is the thread – like structure found in fungi, which is more visible to the naked eye. The spores are the microscopic, tough and resistant bodies which are round in shape.

3.2.3 Identification of fungal strains using lacto phenol staining

Lacto phenol cotton blue staining is the most widely used method for the characterization of fungi. Lacto phenol cotton blue stain is formulated with lacto phenol and cotton blue. The slides were observed under the light microscope for the identification of fungi (Basava *et al.,*2016). The method used for characterization of fungi from Cow dung sample is outlined in appendix – II.

3.3 Fungal identification using 18S r-RNA sequencing

18SrRNA sequencing is a common sequencing method used to identify and compare the microorganisms present within the given sample. It is one of the techniques that have been used recently to identify microorganisms, including those that are capable of degrading plastics. This technique is used to identify genus and species name of an organism. The detailed procedure carried out to identify the microorganisms is outlined in appendix – III.

3.4 Screening of medical waste degrading Fungi

Once the fungal strain was identified, we planned to test the ability of fungus to degrade biomedical waste. We chose catheters and IV tubes that are usually disposed off without prior treatment. This may in turn pollute the soil as well as cause spread of pathogens. So the fungus was used to treat the biomedical waste. The ability of the fungal species to degrade the biomedical waste material was carried out. The fungal species which have the ability to degrade medical waste were segregated from other fungal isolates which did not have an ability to

deteriorate medical waste (Ahsan *et al.,*2016). The assay was carried out to screen the biomedical waste degrading fungi as per the procedure outlined in appendix – IV.

3.5 Field Emission Scanning Electron Microscopy

The surface morphology was analyzed through Field Emission Scanning Electron Microscopy (FESEM) to check the structural changes in isolated fungal species after incubation with biomedical waste. The biomedical waste namely catheter tubes, IV tubes were collected from hospitals and powdered (Ahsan *et al.,*2016). The fungal species was grown in different concentrations of medical waste powder. The changes in the morphology was observed using FESEM. The detailed protocol is outlined in appendix – V.

The results obtained from the present study are discussed in the next chapter.

Results and Discussion

4.0 RESULTS AND DISCUSSION

Microorganisms such as bacteria, algae, yeast and fungi have potential to degrade plastics. Indigenous microorganisms with specific metabolic capabilities have played a significant role in biodegradation of wastes. Biological treatment is preferred to physico-chemical processes because of feasibility, rehability and capability.

Cow dung is an eco-friendly source of organic matter. The fungi that germinate, grow and sporulate on dung are termed as 'Coprophilous'. The word Coprophilous literally means "dung loving". The nitrogenous compounds present in the cow dung influence the growth of fungi (Nayak *et al.,* 2015).

Cow dung is employed as an excellent bioremediation technique. Cow dung has many properties which have been in use since ages. It is combined with soil bedding and urine which is used as manure for agricultural purpose. It is also used in the production of biogas which is used to generate electricity and heat. It can also be used to repel mosquitoes and as cheap thermal insulator. Cow dung slurry is a cheap and effective measure to bio remediates the harmful pollutants (Ojedokun and Bello*,* 2016).

Biomedical waste is dangerous since it contains pathogenic bacteria, viruses, toxic chemicals, mould and radioactive materials. This kind of waste could contaminate other waste and is also infectious. The studies on degradation of biomedical waste are much rarer than those on other types of waste, most likely due to high risk of danger in handling samples of biomedical waste. However, research on biomedical waste bioremediation should be continuously done because the increasing number of hospitals requires more effective and efficient biomedical waste treatment (Ethica *et al.,* 2018).

In the present study, the fungus was isolated from cow dung to treat the biomedical waste and identified by sequencing technique as *C.neoformans*. Fungal identification was done using 18S r-RNA sequencing to determine the genus and species name of an organism. The structural changes in isolated fungal species was analysed through FESEM, followed by screening of the medical waste degrading fungi by Plate assay method.

4.1 ISOLATION OF PURE FUNGAL CULTURE USING POTATO DEXTROSE AGAR MEDIUM

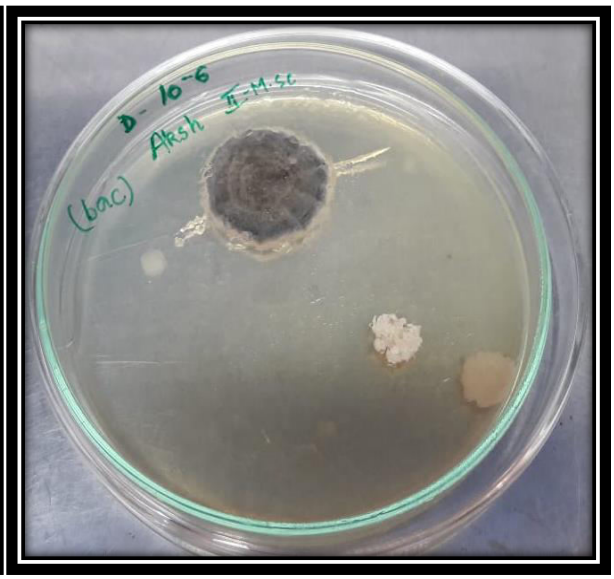
Serial dilution was performed for the growth of microorganisms on agar plate. The medium used for isolation of fungi was potato dextrose agar. The separated colonies were observed and inoculated on the plate. The plates were incubated at 37˚C for 5 days and the purified culture was taken for further analysis.

PLATE 1

Fungal cultures isolated from Cow dung sample using serial dilution method

Dilution factor 10^{-8}

Dilution factor 10^{-9}



Dilution factor 10^{-6}

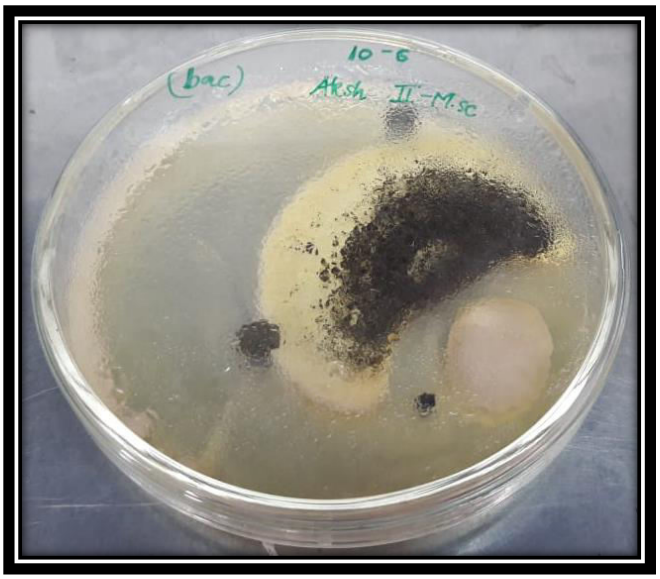


TABLE 1

Microbial analysis of Cow dung sample

Dilution factor	Viable Cell Count	CFU/ml
(10-6)	3	3×10^{-5}
(10-8)	4	4×10^{-10}
(10-9)	3	3×10^{-11}

Serial dilution was done to estimate the concentration of unknown sample by counting the number of colonies cultured in the petri dishes. By knowing the aliquot volume, colony counts were converted into concentrations (CFU/ml). Out of 10 dilutions 0.1ml of the last three dilutions (10^{-6}, 10^{-8} and 10^{-9}) were taken for spreading. The dilution factor 10^{-6} contains three colonies, dilution factor 10^{-8} contains four colonies and the dilution factor 10^{-9} contains three colonies cultured in the petri dishes. The results presented in the Table 1 represents the viable cell counts of the Colony forming units (CFU) averaged 3×10^{-5} CFU/ml, 4×10^{-10} CFU/ml and 3×10^{-11}.

Olawepo *et al*.,(2018) reported that fungal species identified in the cow dung were *Aspergillus niger, Aspergillus flavus, Penicillium chrysogenum* and *Neurospora crazza*. Among the fungi *Penicillium* and *Aspergillus* species are capable of bioremediation of hydrocarbon.

Total fungi counts were determined for the 95 cocoa bean samples by the pour plating technique. The growth of the fungal colonies was consistent on PDA media inoculated with the dilution factors 10^{1} and 10^{3} was reported by Kutsanedzie *et al*.,(2018).

Isolation of fungal strains by serial dilution is a common method. Hence we too employed this to isolate the fungus.

4.2 PRIMARY SCREENING OF FUNGAL STRAINS USING MORPHOLOGY CHARACTERIZATION

Fungal species are usually identified on the basis of their morphology characteristics. The colony appearance of each fungal isolate was characterized on Potato dextrose agar media. Microscopic characteristics of the fungal isolates were examined using an electron microscope. The morphologies are most important for identification of organisms.

TABLE 2

Colony characterization

Dilution	size	shape	margin	Diffusion in media	pigmentation	Mycelium	spore
10^{-6}	small	circular	undulate	Raised	black	-	-
10^{-6}	moderate	circular	Entire	Flat	buff	-	-
10^{-6}	large	irregular	Entire	Raised	buff	-	yes
10^{-9}	moderate	circular	undulate	Raised	buff	-	-
10^{-9}	small	irregular	undulate	Flat	black	-	-
10^{-9}	moderate	circular	Entire	Flat	buff	-	-
10^{-9}	small	circular	Entire	Flat	white	-	-
10^{-8}	large	circular	undulate	Raised	black	-	yes
10^{-8}	small	irregular	undulate	Flat	buff	-	-
10^{-8}	small	irregular	undulate	Flat	white	-	-

Colony morphology of the fungal colonies were observed after 5 days of incubation. Table 2 indicates the morphological appearances of the fungal colonies. The size of the fungal colony observed was small, moderate and large and it was measured using ruler. Shape of the colony observed was circular and irregular. The magnified edges of the colony were observed as entire and undulate. The fungal colony was raised above the agar as well as diffused in media. Pigmentation of the colonies was observed as black and buff. The spores are observed on the colony but no mycelium. Among the colonies from different dilutions these results were noticed in the dilution factor 10^{-6}. Size of the colony identified was moderate and small. The shape of the fungal colony observed was circular and irregular. Margin of the colony observed was entire and undulate. The fungal colony observed was diffused in the agar and few colonies were raised above the media. The colour of the colonies was observed as buff, black and white. Mycelium was not observed; spores appeared on the single colony. No mycelium and spore formation was observed. These results were noticed in the dilution factor 10^{-9}. The size of the fungal colony observed was large and small. The shape of the colony identified was circular and irregular. The magnified edge of the colony was observed as undulate in all the three colonies. The fungal colony observed was raised and flat in the agar. Colour of the colonies was observed as buff, black and white. Mycelium was not observed; spores appeared on the single colony. Among the colonies from different dilutions these results were noticed in the dilution factor 10^{-8}.

Thiruneelakantan *et al*., (2001) reported the fungal isolate MTF1 was pale yellow in colour and the colour of MTF2 was grey green to parrot green and dull yellowish colour. The colour of MTF3 was initially white but soon turned black on the top side but the bottom side remained pale yellow in colour. The appearance of MTF1 was powdery and thick in texture. The MTF2 sample was found to have wrinkled surface whereas MTF3 showed a thread like appearance and a soft texture. The shapes of MTF1, MTF2 and MTF3 were round and the appearance was thick.

A total of 15 fungal cultures were isolated from the collected soil samples. The morphological properties showed variability between the different cultures and media used. All the isolates were also subjected to microscopic analysis for their characterization and identification. The isolate demonstrated cultural and micro morphological features which are similar to those of *Penicillium raistrickii*. Other characteristics were different from those of P.

raistrickii, such as the production of colonies with velutinous texture, the production of a dark yellowish green diffusible pigment in all culture media and the absence of true sclerotia in the mycelium was reported by Mushimiyimana,(2016).

Qayyum *et* al.,(2016) reported that six resistant fungal isolates were identified in soil sample. The overall morphologies of colony and cells physiological of the fungal strain F1 and F2 were identical. Based on microscopic investigations, it was proved that fungal isolates F3, F4, F5 and F6 were A. *flavus*, A. *terreus*, A. *tubingensis*, N. *hiratsukae*, respectively.

Pan *et al*., (2016) reported that the strain 28 colonies were similarly flattened, black-gray, white and lobed on the edge, and they grew relatively slowly. The reverse sides of the colonies were dark yellow to aurantium and the ascocarps were olive or green-yellow with ascomal hairs. Moreover, the spores were pale brown and lemon-shaped. Therefore, these characteristics corresponded well with those of *Chaetomium globosum*.

Malapi-Wight *et* al.,(2016) reported that the *Sarcococca hookeriana* and *Buxus sempervirens,* both fungal isolates grew as puffy colonies on PDA, with the centre of the colony light to dark brown and edges white.

All these studies are in agreement with our results obtained. In our study too, the isolated fungal species could be partially identified based on their colony morphology.

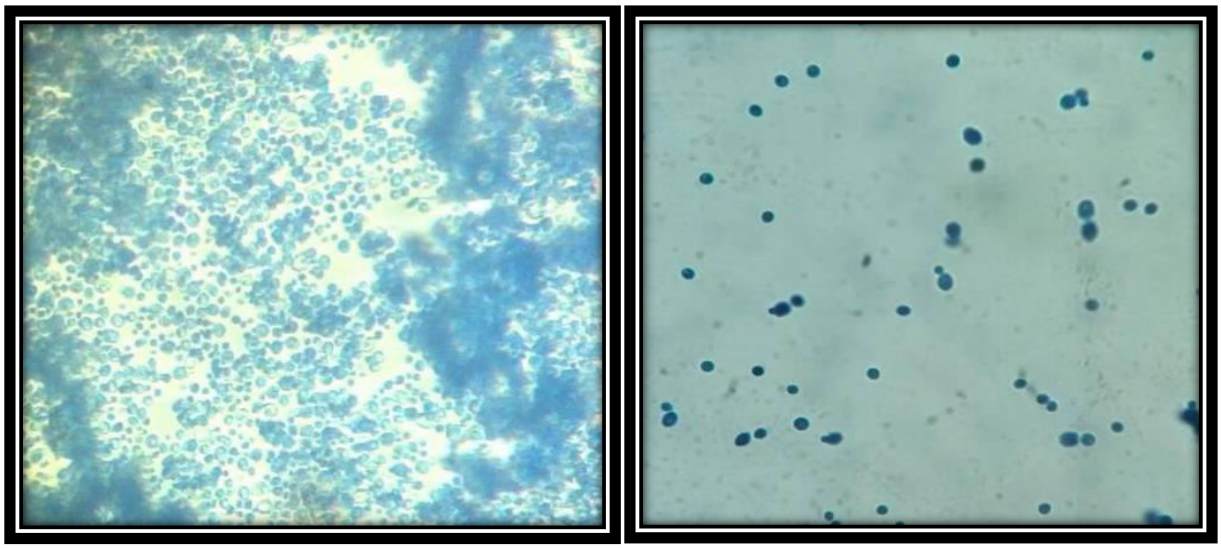
4.3 LACTOPHENOL COTTON BLUE STAINING

Microscopic observation of wet mounts remains the most widely used method in clinical microbiology laboratories for identifying filamentous fungi based on their characteristic morphological features. The standard technique for the microscopic examination of fungal cultures is the lacto phenol cotton blue (LPCB) slide method. Lacto phenol cotton blue is preferred universally for its usage as a fixative, staining and mounting medium. Lacto phenol cotton blue is widely used to study the morphological features of fungal isolates. When observed in the microscopic slide, conidiophores were heavy walled, uncoloured and coarsely roughed. Conidial moses were also observed.

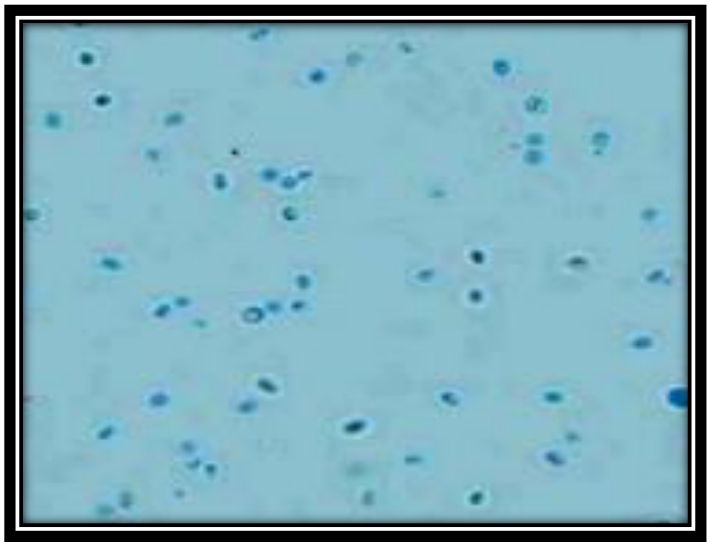
PLATE 2

Morphology of fungus by lacto phenol cotton blue staining

10^{-6} **10^{-8}**



10^{-9}



Lacto phenol cotton blue stain was used to identify the isolated microorganism. Slides mounted with cotton blue dye stained the spores of fungal species in blue. Plate 2 depicts the clear photographic image of the isolated fungal strains from cow dung. The results show that LPCB stains the cell wall intensely blue. Staining results were confirmed by 18S r-RNA sequencing.

Research findings on how to make polyethylene material more readily biodegradable have been reported by several workers. The assimilation of synthetic polyolefin by soil microorganisms at a substantial level however with long incubation period are being carried out.

The isolated fungal strain was identified using 18S r-RNA sequencing method. This helped us to identify the species.

4.4 MOLECULAR IDENTIFICATION OF FUNGAL STRAINS

The isolated fungal species were subjected to 18S r-RNA sequencing. 18S r-RNA sequencing is one of the techniques that have been used to identify microorganisms. It is used to identify genus and species name of an organism. It helps in classification and quantitation of microbes within complex biological mixture. Positive samples of 18S r-RNA PCR were selected for further species confirmation by DNA sequencing. Primers ITS1 and ITS4 were used to amplify the region by PCR. The PCR products were sequenced using a DNA sequencer and analyzed with the BLAST program provided by the National Centre for Biotechnology Information (NCBI) to confirm the fungal species.

This method consists of DNA extraction followed with PCR primer design, then PCR was performed with primer pairs targeted to the 18S r-RNA gene. The nucleotide sequences of the primers used in this study are shown in Table 3. The fungal DNA was isolated and amplified using universal primers ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3'). PCR reaction volume of 25 µl containing Taq DNA polymerase, 0.4mM dNTPs, 3.2mM $MgCl_2$, 5 µl of isolated DNA, 1.5 µl of forward and reverse primer, 5 µL of deionized water, and 12 µL of Taq Master Mix. Amplification was carried out in PCR programmed with an initial denaturation at 94 ˚C for 3 minutes, followed by 30 cycles at 94˚C for 30 seconds (Denaturation), 60˚C for 30 seconds (Annealing), 72˚C for 1 minute

(Extension), and final extension at 72˚C for 10 minutes. PCR product was electrophoresed in 1% agarose gel.

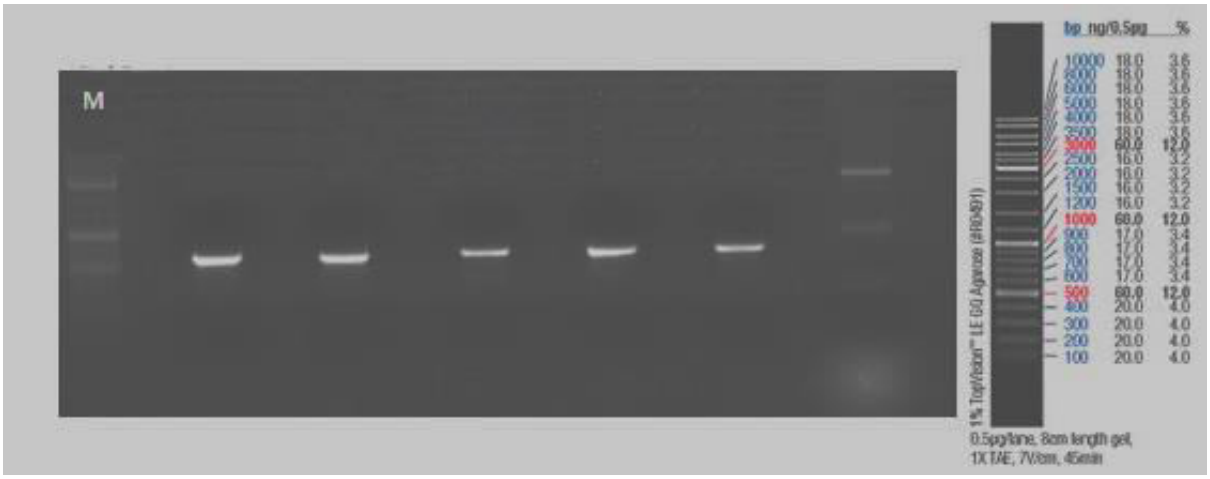
TABLE 3

Sequences of the nucleotide primers used in this study

Primer name	Sequence Details	Number of Base
ITS1	5' TCCGTAGGTGAACCTGCGG 3'	19
ITS4	5' TCCTCCGCTTATTGATATGC 3'	20

FIGURE 2

GEL IMAGE OF PCR PRODUCT



The purified product was sequenced using ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3'). The gel image of the PCR product is shown in the figure 2. Sequencing reactions were performed using a ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems).

Single-pass sequencing was performed on each template using below 16s rRNA universal primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

SEQUENCE DATA IN FASTA FORMAT

>Contig

GAGAATATTGGACTTTGGACCATTTATCTACCCATCTACACCTGTGAACTGTTTATGTGCTTCGGCACGT

TTTAGACAAACTTCTAAATGTAGTGAATGTAATCATATTATAACAATAATAAAACTTTCAACAACGGATC

TCTTGGCTTCCACATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAA

TCATCGAGTCTTTGAACGCAACTTGCGCCCTTTGGTATTCCGAAGGGCATGCCTGTTTGAGAGTCATGAA

AATCTCAATCCCTCGGGTTTTATTACCTGTTGGACTTGGATTTGGGTGTTTGCCGCGACCTGCAAAGGAC

GTCGGCTCGCCTTAAATGTGTAAGTGGGAAGGTGATTACCTGTCAGCCCGGCGTAATAAGTTTCGCTGGG

CCAATGGGGTAGTCTTCGGCTTGCTGATAACAACCATCTCTTTTTGT

Organism: *Cryptococcus neoformans*

The 18s rRNA sequence was blast using NCBI blast similarity search tool. The phylogeny analysis of query sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment. The program MUSCLE 3.7 was used for multiple alignments of sequences. Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as Substitution model.

FIGURE 3

PHYLOGENY ANALYSIS OF *Crptococcus neoformans*

18S r-RNA sequencing results confirmed that the organism belongs to genus *Cryptococcus* and species *C.neoformans*. The phylogenetic relation of this isolate to related fungi is shown in figure 3. Thus this strain was identified as *C.neoformans* based on its morphological characteristics and the r-DNA sequencing of its region data. This tree was constructed on the basis of the r-DNA sequence (ITS1 and ITS4) by neighbor joining method.

Dou *et* al., (2017) identified 81 isolates of *Cryptococcus neoformans* AFLP1/VNI from 435 samples of pigeon droppings. The phylogenetic tree was constructed by using the maximum likelihood method. The sequences of 7MLST loci from environmental and clinical isolates along with those of cryptococcal strains were aligned.

Guerra *et* al., (2019) reported that nine fungal species were isolated and identified through DNA analysis: *A. niger; T. atroviride; T.harzianum; Trichoderma* sp.; *C. sphaerospermum; Cladosporium sp.; Lecanicillium sp.;Penicillium oxalicum;* and *Purpureocillium lilacinum.* The ITS region was amplified in some of the samples, separating the 18S and 28S regions from the fungal r-DNA using the primers ITS1 (TCCGTAGGTGAACCTCGCG) and ITS4 (TCCTCCGCTTATTGATATGC). The fragments were processed and analyzed with the ABI prism 3130 automatics genetic analyser. The resulting sequences were compared with data from the GenBank.

Valenzuela-Lopez *et* al., (2017) reported that a level of identity ≥ 98% was considered for species-level identification. The total genomic DNA was extracted and was amplified with the primer pair LROR and LR5. The sequences were obtained using the SeqMan software version 7.0.0.

Fakruddin *et al*., (2016) extracted and quantified DNA from the *Aspergillus flavus* isolates. Amplification of the region was performed by PCR using the primers nu – SSU – 0817 – 5' (TTAGCATGGAATAATRRAATAGGA) and nu – SSU – 1196 – 3' (TCTGGACCTGGTGAGTTTCC). Sequencing reactions were carried out using ABI-Prism Big dye terminator cycle sequencing ready reaction kit. The purified cycle sequenced products were analyzed with an ABI-Prism 310 genetic analyzer.

Qayyum *et al*., (2016) reported that heavy metal resistant fungal strains were extracted and purified from the soil sample. PCR products were separated by agarose gel electrophoresis and recovered by using UNIQ column DNA gel recovery kits. The phylogenetic analysis confirmed that the fungal isolates were *Rhizomucor pusillus*, *Aspergillus flavus*, *Aspergillus terreus*, *Aspergillus tubingensis* and *Neosartorya hiratsukae*.

In our study too, we isolated the fungal strain and identified using 18S r-RNA sequencing method as reported by all the studies quoted.

Next we attempted to see if the biomedical waste powder could be degraded by the isolated fungi.

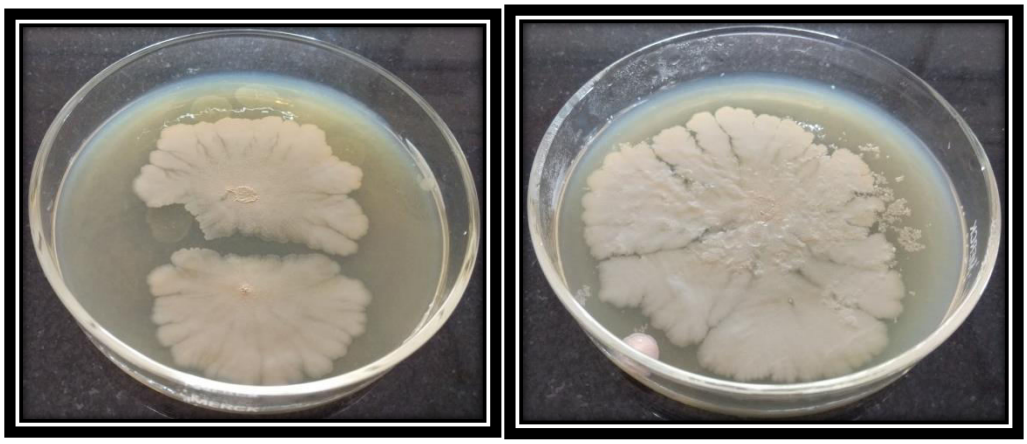
4.5 FESEM CHARACTERIZATION OF FUNGAL STRAIN

Field Emission Scanning Electron Microscopy was used to examine the topography of fungal species (*Cryptococcus neoformans*) treated with medical waste. FESEM provides the images at 100 and 10nm scales. The images were acquired using FESEM and the surface texture and morphology of medical wastes like catheter, IV tubes was analysed to check for any structural changes after incubating for 5 days. A typical topographic FESEM image, which represents the whole surface morphology of *C.neoformans* before and after treatment with medical waste, is shown in figure 4. Different quantities of biomedical waste were grown along with the inoculated fungi. Among this 0.02g of medical waste treated with fungal species showed better result. So it was chosen for FESEM analysis to examine the morphological changes before and after treatment with medical waste.

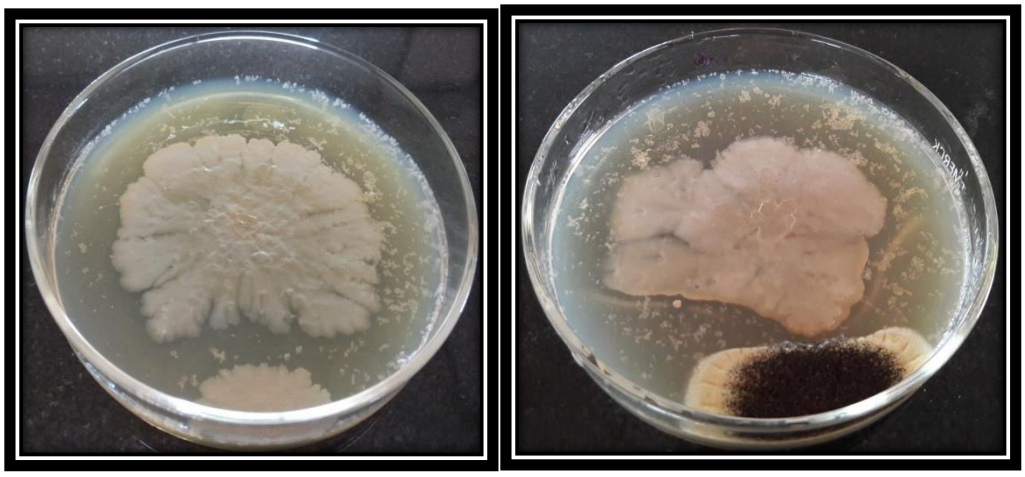
PLATE 4

FESEM analysis of sample before and after treatment with medical waste

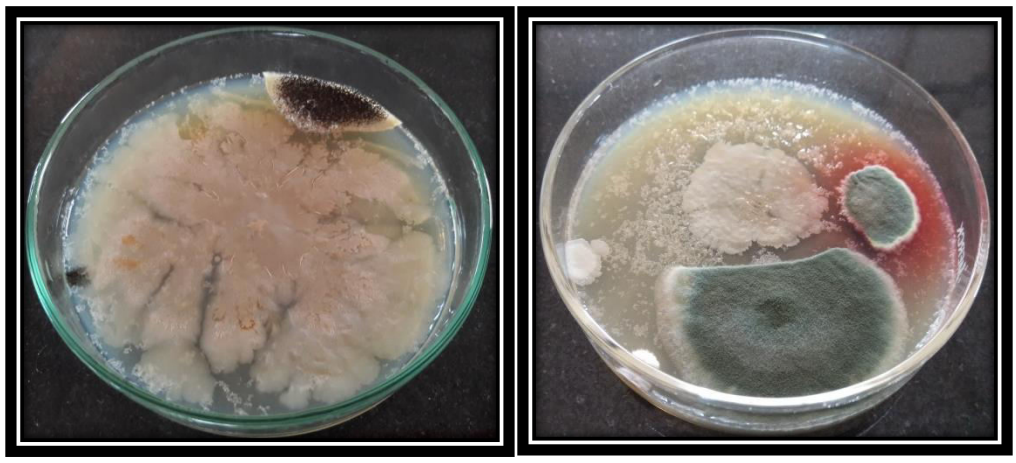
Control **0.02g**



0.04g **0.06g**



0.08g **0.10g**



The fungal species were grown in the biomedical waste powder and the ability to grow and absorb the waste was tested by observing the morphology of the cultures using FESEM.

FIGURE 5a

FESEM analysis of *C.neoformans*

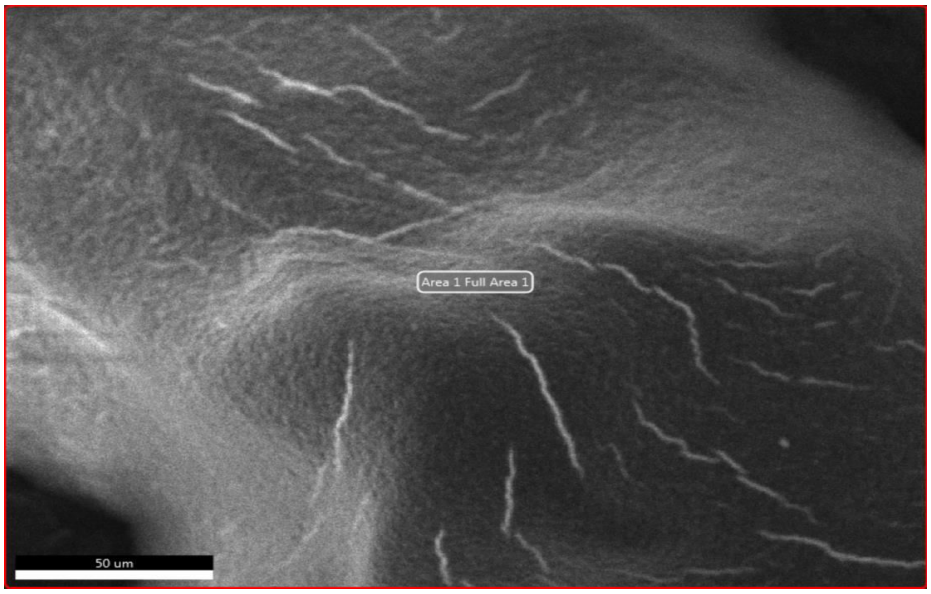
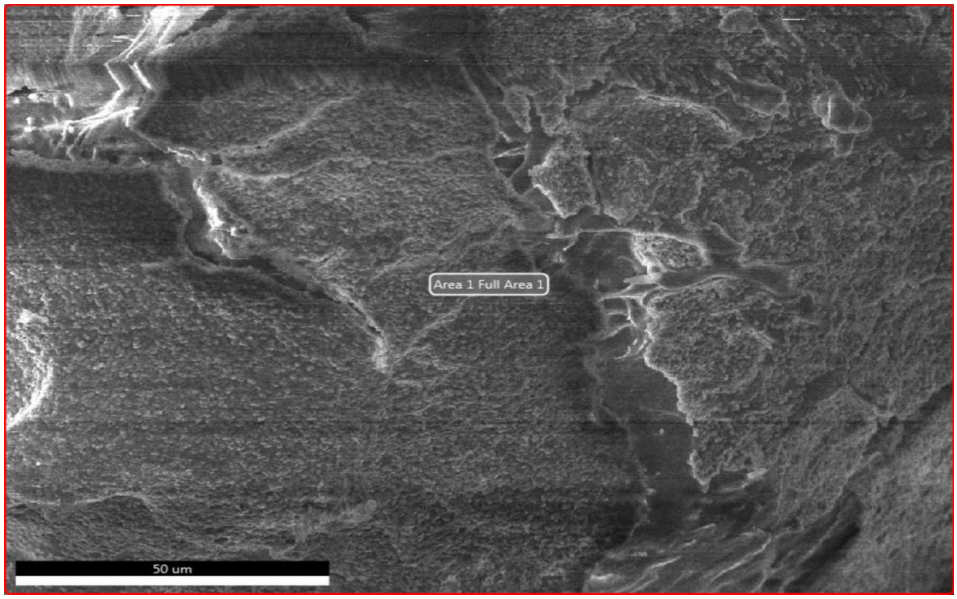


FIGURE 5b

FESEM analysis of *C.neoformans* treated with medical waste



FESEM images are captured at different magnifications 5µm, 10µm, 15µm, 25µm and 50µm. The clear image was observed at 50µm magnification showing structural changes in the fungal strain with treated and untreated medical waste. Figure 4.a and b shows the surface morphology of *C.neoformans* before and after treatment with medical waste. The image shows that *C.neoformans* strongly adheres onto the surface of polyethylene powder indicating the utilization of medical waste. The results of FESEM analysis showed that *C.neoformans* has the ability to deteriorate medical wastes like catheter, IV tubes, etc.

The surface morphology of the activated carbon by FESEM before and after treatment of triclosan was reported by MohdKhori *et al*.,(2018). The activated carbon from coconut pulp waste before adsorption of triclosan showed valley-like depressions and many visible pores on its surface, which increased the percentage removal. The activated carbon after adsorption of triclosan was filled up by many particles. Hence he proved that activated carbon from coconut pulp waste can act as adsorbent for triclosan in water.

The surface morphology of the LDPE films was analysed using FESEM analysis before and after the fungal treatment. Das *et al*.,(2018) reported that control LDPE strip displayed smooth surface view as compared to the fungal treated ones. It was observed that isolated fungi strongly adhere to the polymer surface indicate the utilization of LDPE.

Majumder *et al*.,(2018) reported that the attachment of the spores and hyphae to the surface may be an important step in the biodegradation of polyurethane. The ability of *Leucoagaricus sp.*to degrade polyurethane was confirmed by the presence of numerous holes in solid PU. The surface of materials has turned from smooth to rough with cracking.

FESEM analysis was performed on the control biomass, compared with the biomass treated with 50mg/l, 100mg/l and 150mg/l of lead to visualize the changes in morphological characteristics.Gururajan *et al*.,(2018) reported that visual damage was minimal in control when compared to the fungal biomass treated with lead.

Biodegradation of low density polyethylene was studied using mixed culture of *Pseudomonas sp, Aspergillus sp,* and *Rhizopus arrhizus* isolated from polyethylene dumped area. Bhave *et al*.,(2017) reported that LDPE showed structural changes such as splitting polymers, cracks and holes.

All these reports are supportive of our findings.

Researchers on the reduction of biomedical waste by microorganism have been extremely carried out. The materials we used in the present study were catheter tubes and IV tubes. They were cut into small bits and inoculated into the medium and fungus *Cryptococcus neoformans* was grown. The ability of the fungus to utilize this as a source and its ability to degrade them was studied.

In our study too, the degradation of medical waste by the fungus *Cryptococcus neoformans* was observed and checked for its changes in the morphological features.

Summary and Conclusion

5.0 SUMMARY AND CONCLUSION

Hospital plays critical role to provide prevention, treatment, rehabilitation and promotion for maintaining and improving public health. The biomedical waste is the waste that is generated during the diagnosis, treatment or immunization of human beings or animals or in production or testing of biological components. The application of proper waste management techniques and its identification helps to improve their understanding of good practice. We also need a cost effective environment friendly technology. The present study was carried to find the significance and to recommend development method of bioremediation using fungal strains isolated from cow dung.

In the present study, cow dung was found to be an excellent degrader of biomedical waste. Cow dung is not a waste product, but it is a purifier of all wastes in the nature. The fungal strain *Cryptococcus neoformans* isolated from cow dung have the ability for degradation of biomedical waste. *C.neoformans* is responsible for the majority of infections found in immunocompromised individuals. Cow dung samples were prepared by using a serial dilution method. The different fungal cultures were purified by using spread plate method on potato dextrose agar medium. The plates were preserved and labelled for the growth of microorganisms at 37˚C for 5 days. The unique colonies were further sub cultured for screening and identification of fungal strains.

By knowing the aliquot volume, colony counts were converted into concentrations (CFU/ml). Out of 10 dilutions 0.1ml of the last three dilutions (10^{-9}, 10^{-8} and 10^{-6}) represented the viable cell counts averaged 4×10^{-11} CFU/ml, 3×10^{-10} CFU/ml and 3×10^{-5}. Based on their morphological characteristics fungal species has been identified and examined using electron microscope. From the observations it was clear that the size of the fungal colony observed was small, moderate and large and it was measured using ruler. Shape of the colony observed was circular and irregular. The magnified edges of the colony were observed as entire and undulate. The fungal colony was raised above the agar as well as diffused in media. Pigmentation of the colonies was observed as black and buff. The spores are observed on the colony but no mycelium. Among the colonies from different dilutions these results were noticed in the dilution factor 10^{-6}. Size of the colony identified was moderate and small. The shape of the fungal colony observed was circular and irregular. Margin of the colony observed was entire and undulate. The

fungal colony observed was diffused in the agar and few colonies were raised above the media. The colour of the colonies was observed as buff, black and white. Mycelium was not observed; spores appeared on the single colony. No mycelium and spore formation was observed. These results were noticed in the dilution factor 10^{-9}. The size of the fungal colony observed was large and small. The shape of the colony identified was circular and irregular. The magnified edge of the colony was observed as undulate in all the three colonies. The fungal colony observed was raised and flat in the agar. Colour of the colonies was observed as buff, black and white. Mycelium was not observed; spores appeared on the single colony. Among the colonies from different dilutions these results were noticed in the dilution factor 10^{-8}.

Microscopic observation of fungal strains using lacto phenol cotton blue staining was done and the results showed that the LPCB stains the cell wall intensely blue. The observations showed clear photographic image of the fungal strains confirming that the conidiophores were heavy walled, uncoloured and coarsely roughed. Further the isolated fungal strain was identified by using 18S r-RNA sequencing method which helped to identify the species.

18S r-RNA sequencing is one of the techniques that have been used to identify microorganisms. It was used to identify genus and species name of an organism. Positive samples of 18S r-RNA PCR were selected for further species confirmation by DNA sequencing. The fungal DNA was isolated and amplified using universal primers ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3'). Amplification was carried out in PCR programmed with an initial denaturation at 94 ˚C for 3 minutes, followed by 30 cycles at 94˚C for 30 seconds (Denaturation), 60˚C for 30 seconds (Annealing), 72˚C for 1 minute (Extension), and final extension at 72˚C for 10 minutes. PCR product was electrophoresed in 1% agarose gel. The 18s rRNA sequence was blast using NCBI blast similarity search tool. The phylogeny analysis of query sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment. The program MUSCLE 3.7 was used for multiple alignments of sequences. Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as Substitution model. 18S r-RNA sequencing results confirmed that the organism belongs to genus *Cryptococcus* and species *C.neoformans*. Thus this strain was identified as *C.neoformans* based on its morphological

characteristics and the r-DNA sequencing of its region data. This tree was constructed on the basis of the r-DNA sequence (ITS1 and ITS4) by neighbor joining method.

In our study, fungal strains were isolated and identified using 18S r-RNA sequencing, followed by FESEM characterization. The surface texture and morphology of medical wastes like catheter, IV tubes was analysed. The fungal strains grown in the biomedical waste powder were noted for its ability to grow and absorb the waste. The results showed that *Cryptococcus neoformans* strongly adhere onto the surface of polyethylene powder indicating the utilization of medical waste. From the results obtained it was clear that *C.neoformans* has the ability to degrade medical wastes like catheter and IV tubes.

The results of the present study revealed that the isolated fungal organism from the cow dung sample has good potentiality to deteriorate the medical wastes like catheter, IV tubes because of the adherence of this powder to fungal strains. Bioremediation of the medical wastes can reduce pollutants in the soil and atmosphere and cause less harmful effects to the plants and animals.

Suggestions for future research

- The degradative capacity of the fungal species needs to be further tested using other assays.
- The fungal species can be isolated from other sources and check for its ability to degrade medical wastes.
- The effect of the liquid medical wastes can also be tested using various methods.
- Composting can also be done to degrade the medical waste.

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Appendices

APPENDIX I

ISOLATION OF PURE FUNGAL CULTURE USING POTATO DEXTROSE AGAR MEDIUM

(Tiwari and Saraf, 2017)

Principle

Potato Dextrose Agar (PDA) is a common microbial growth media made from an infusion of potato and dextrose. PDA is one of the most widely used media for the isolation of fungus as well as for its maintenance. PDA is composed of dehydrated Potato infusion and Dextrose that stimulate the fungal growth. Dextrose acts as a carbohydrate source which serves as a growth stimulant and potato infusion that provides a nutrient base for the growth of fungi.

Materials required

1. Potato Dextrose Agar
2. Agar – agar type I
3. Serially diluted test tubes
4. Petriplates
5. Pipettes
6. L-rod
7. Forceps

Procedure

Potato Dextrose Agar comprises 200g of potato infusion which serves as a nutrient base, 20g of dextrose as carbohydrate source and 20g of agar as solidifying agent, distilled water is used to make up the media and finally pH is adjusted to 7. The media was autoclaved at 121˚C temperature and 15 lbs pressure for a period of 15-30 minutes. 1g of the suspension from the Cow dung sample was mixed with 10ml sterilized distilled water. To ensure the proper mixing, these samples were vigorously shook in vortex for 2 minutes. In order to activate the microorganisms, all the samples were incubated at 37˚C for 30-40 minutes in an incubator prior

to plating. The dilutions of each sample were prepared after incubation by using standard dilution method with the help of sterilized pipette. Serial dilution from 10^{-0} to 10^{-9} of Cow dung sample was done and inoculated into the plates. In the test tube stand the labelled tubes were placed, from which 1ml of sample is transferred to test tube number 1 and further 1ml of sample is transferred to test tube number 2 and the process is repeated for each dilution. The medium was then sterilized by autoclaving at 121˚C for 20 minutes. Then, the medium was left to cool and then poured into petri dishes to solidify. 100µl of the sample (10^{-8} and 10^{-9}) was inoculated to the sterile petri plates. The L-rod was sterilized and spread over the surface of the agar medium by rotating the petri plate manually. The plates were incubated in an inverted position for 5-7 days at 37˚C for the growth of fungi. The incubated plates were inspected daily for the fungal growth. The separated colonies were observed and picked up with sterile forceps and inoculated on the plate. The plates were incubated at 37˚C for 5 – 7 days and the purified culture was taken for further analysis.

APPENDIX II

IDENTIFICATION OF MICROBIAL STRAINS USING LACTO PHENOL STAINING

(Basava *et al.,* 2015)

Principle

The Lacto phenol Cotton Blue (LPCB) wet mount is most widely used method for the staining and observation of fungi. It is used in the preparation of slides for microscopic examination and characterization of fungi. Fungal elements are stained intensely blue. Lacto phenol cotton blue stain is formulated with lactic acid which preserves the fungal structure and clears the tissue, phenol acts as a disinfectant and cotton blue imparts blue coloration to the fungal spores and hyphae.

Materials required

1. Lacto phenol cotton blue
2. 95% ethanol
3. Glass slide
4. Cover slip

Procedure

Place a drop of 95% ethanol on a clean microscopic glass slide. Remove a fragment of the fungus colony 2 – 3 mm from the colony edge using an inoculation loop or adhesive strips. Place the fragment in the middle of the slide. A drop of cotton blue solution was placed on the fragment using a Pasteur pipette, followed by spreading of fungal culture. A coverslip was placed on the drop and left for 5 minutes. Then, the slides were observed under the light microscope for the identification of fungi.

APPENDIX III

FUNGAL IDENTIFICATION USING 18S r-RNA SEQUENCING

DNA ISOLATION

Principle

The isolation of DNA from fungi is a relatively simple process. The organism to be used should be grown in favourable medium at an optimal temperature. The genomic DNA isolation needs to separate total DNA from RNA, protein, lipid, etc.

Procedure

Lysis/homogenization: Cells grown in monolayer were lysed by suspending1-3 colonies aseptically and mixed with 450 µl of "B Cube" lysis buffer in a 2 ml micro centrifuge tube and lyse the cells by repeated pipetting. Added 4 µl of RNAse A and 250 µl of "B Cube" neutralization buffer. Vortexed the content and incubated the tubes for 30 minutes at 65˚C in a water bath. To minimize shearing the DNA molecules mixed DNA solutions by inversion. Centrifuged the tubes for 20 minutes at 14,000 rpm at 10 ˚C. Following centrifugation transferred the resulting viscous supernatant into a fresh 2 ml micro centrifuge tube without disturbing the pellet. Added 600 µl of "B Cube" binding buffer to the content and mixed thoroughly by pipetting and incubated the content at room temperature for 5 minutes. Transferred 600 µl of the contents to a spin column placed in 2 ml collection tube. Centrifuged for 2 minutes at 14,000 rpm and discarded flow-through. Reassembled the spin column and the collection tube then transferred the remaining 600 µl of the lysate. Centrifuged for 2 minutes at 14,000 rpm and discarded flow-through. Added 500 µL "B Cube" washing buffer I to the spin column. Centrifuged at 14,000 rpm for 2 mins and discard flow-through. Reassembled the spin column and added 500 µl "B Cube" washing buffer II and centrifuged at 14,000 rpm for 2 mins and discarded flow-through. Transferred the spin column to a sterile 1.5-ml micro centrifuge tube. Added 100 µl of "B Cube" Elution buffer at the middle of spin column. Care was taken to avoid touch with the filter. Incubated the tubes for 5 minutes at room temperature and centrifuged at 6000 rpm for 1min. Repeated the above mentioned step 14 and 15 for complete elution. The buffer in the micro centrifuge tube contained the DNA. DNA concentrations were

measured by running aliquots on 1% agarose gel. The DNA samples were stored at -20°C until further use.

POLYMERASE CHAIN REACTION (PCR)

Principle

Polymerase Chain Reaction (PCR) is a process that uses primers to amplify specific cloned or genomic DNA sequences with the help of a very unique enzyme. PCR uses the enzyme DNA polymerase that directs the synthesis of DNA from deoxynucleotide substrates on a single-stranded DNA template. DNA polymerase adds nucleotides to the 3` end of a custom-designed oligonucleotide when it is annealed to a longer template DNA. Thus, if a synthetic oligonucleotide is annealed to a single-stranded template that contains a region complementary to the oligonucleotide, DNA polymerase can use the oligonucleotide as a primer and elongate its 3` end to generate an extended region of double stranded DNA.

Composition of the Taq Master Mix

1. Taq DNA polymerase is supplied in 2X Taq buffer
2. 0.4mM dNTPs,
3. 3.2mM $MgCl_2$ and
4. 0.02% bromophenol blue.

Primers used

Primer name	Sequence Details	Number of Base
ITS1	5' TCCGTAGGTGAACCTGCGG 3'	19
ITS4	5' TCCTCCGCTTATTGATATGC 3'	20

Procedure

Added 5 µL of isolated DNA in 25 µL of PCR reaction solution (1.5 µL of Forward Primer and Reverse Primer, 5 µL of deionized water, and 12 µL of Taq Master Mix). Performed PCR using the following thermal cycling conditions.

1. Denaturation

The DNA template was heated to 94°C. This broke the weak hydrogen bonds that holded DNA strands together in a helix, allowed the strands to separate creating single stranded DNA.

2. Annealing

The mixture was cooled to anywhere from 60℃ . This allowed the primers to bind (anneal) to their complementary sequence in the template DNA.

3. Extension

The reaction was then heated to 72° C , the optimal temperature for DNA polymerase to act. DNA polymerase extended the primers, added nucleotides onto the primer in a sequential manner, using the target DNA as a template.

PCR CONDITION

STAGES	TEMPERATURE	TIME	
Initial Denaturation	94˚C	3 min	
Denaturation	94˚C	30 sec	
Annealing	60˚C	30 sec	30 cycles
Extension	72˚C	1 min	
Final extension	72˚C	10 min	
Hold	4˚C	∞	

Purification of PCR Production

Removed unincorporated PCR primers and dNTPs from PCR products by using Montage PCR Clean up kit (Millipore). The PCR product was sequenced using the primers. Sequencing

reactions were performed using a ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems).

Sequencing protocol

Single-pass sequencing was performed on each template using below 16s rRNA universal primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

Bioinformatics protocol

1. The 16s rRNA sequence was blast using NCBI blast similarity search tool. The phylogeny analysis of query sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment.

2. The program MUSCLE 3.7 was used for multiple alignments of sequences (Edgar 2004).The resulting aligned sequences were cured using the program Gblocks 0.91b.ThisGblocks eliminates poorly aligned positions and divergent regions (removes alignment noise) (Talavera and Castresana 2007).Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as Substitution model.

3. PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data, while being one order of magnitude faster. PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data, while being one order of magnitude faster. The program Tree Dyn 198.3 was used for tree rendering. (Dereeper *et al*., 2008).

APPENDIX IV

BIODEGRADATIVE CAPACITY OF MEDICAL WASTE BY ISOLATED FUNGI

(Ahsan *et al.,* 2016)

Principle

Plate assay is the technique performed to test the ability of fungi to degrade the medical waste. The fungal species which have potential to degrade medical waste were separated from other fungal isolates which did not have an ability to deteriorate medical waste.

Materials required

1. Potato Dextrose Agar
2. Agar – agar type I
3. Medical waste – Catheter and IV tube
4. Petri plates

Procedure

Potato Dextrose Agar was prepared and medical waste was mixed with it. The media was autoclaved at 121˚C temperature and 15 lbs pressure for a period of 30 minutes. It was shook for one hour so that medical waste got mixed properly in the fungal medium. The fungal species was inoculated into medium containing medical waste. The plates were then incubated for 3 – 5 days at room temperature to observe the ability of the fungus to grow in the medium containing biomedical waste.

APPENDIX V

FIELD EMISSION SCANNING ELECTRON MICROSCOPY

(Ahsan *et al.*, 2016)

Principle

Field Emission Scanning Electron Microscopy (FESEM) is often used for imaging and characterization purposes. It is a type of electron microscope that images a sample by scanning it with a high – energy beam of electrons. The electrons interact with the atoms that make up the sample producing signals that contain information about the samples surface topography, composition and other properties such as electrical conductivity. FESEM can produce very high – resolution images of a sample surface revealing details about less than 1 to 5 nm in size.

Procedure

The 5 day old fungal culture on Potato Dextrose Agar medium treated with medical waste was used for FESEM observations. About 5 × 10 mm segments were cut from cultures growing on PDA plates and promptly placed in vials for fixation. For the FESEM analysis, control plate and the plate containing medical waste treated with fungal cultures were analyzed. The structural differences between the treated and untreated waste plates were observed.