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IMPROVING CIRCULARITY OF BIO-BASED SIDE STREAMS WITH PRE-TREATMENTS

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ABSTRACT

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Agriculture plays a leading role in the African economy, yet its impacts on the environment as well as human living conditions are preventing sustainable development in this continent. A large amount of side stream from agricultural crops ends up in landfills or incinerators without proper management, which is wasteful as biomass residues are known as rich resources of lignocellulose which can be retrieved and recycled for several applications. Lignocellulose, mainly including cellulose, hemicellulose, and lignin, is especially potential in the manufacturing of innovative bio-based materials called biocomposite, which can be further utilized in biopackaging production.

However, the utilization of biomass residues for biocomposite and biopackaging in Africa is limited at present due to the contamination caused by microorganisms, molds in particular. To resolve the issue and enhance the recyclability of crop side streams, appropriate elimination methods for mold must be applied. Thus, the thesis aims at studying and assessing current pretreatment methods of agricultural biomass waste in terms of their mold removal efficiency which is determined via antifungal efficacy/fungicidal effect. The most suitable method will be selected based on applicability, affordability and sustainability concerning the African context besides its removal efficiency. From the literature review, pretreatment with chemicals such as hydrogen peroxide (H_2O_2), organic acids or ethanol (EtOH) was considered the most simple, practical, and eco-friendly among all available methods. Apart from chemical pretreatment, a basic physico-chemical technology i.e. autoclave was also contemplated in this case thanks to its widespread application in medical and food disinfection.

During the experiment, the mold concentration was determined by the plating and culturing method before and after pretreatment. The fungi were also characterized through microscopic examination. The pretreatment testing was implemented with two stages, screening and repetition. The side stream was dipped into H_2O_2 alone at 3-5% for different durations from 10 to 120 minutes, and into H_2O_2 added organic acids (formic and acetic). The vapor form of H_2O_2 was also examined on the residues. With ethanol, the samples were immersed in 35 and 50% EtOH at room temperature and 50 °C for 10 minutes. For autoclave examination, the samples were sterilized in one standard cycle i.e. at 121 °C for 20 minutes.

The processes with H_2O_2 alone and H_2O_2 in addition to acids along with autoclave were selected for further replication. Most of the methods obtained the satisfactory removal efficiency of mold i.e. 4-log or higher. There was no presence of mold growth after the incubation in the cases of autoclave, 5% H_2O_2 in 10 minutes and H_2O_2 in combination with acetic acid, suggesting that the removal efficiency might go up to 99.9999% or above. Yet, due to the uneven distribution of fungi on the side stream, such a high estimated value can be unreliable. The residue portion used in these experiments may contain little to none of molds.

On the other hand, pretreatment with 5% H_2O_2 in 20 minutes showed the most consistent results with an average removal efficiency of ca. 5-log concerning pretest and repetition outcomes. Additionally, the use of H_2O_2 fits all selection criteria as the chemical is affordable and accessible worldwide. The operation of dipping pretreatment is simple. The method is also sustainable as H_2O_2 decomposition is eco-friendly and no residue is left on the side stream, while its wastewater can be either directly released or managed with current wastewater treatment technology. Hence, this process is expected to be further applied in reality.

Keywords: biomass waste pretreatment, mold/fungi detection, antifungal efficacy, fungicidal effect, hydrogen peroxide, ethanol, autoclave

PREFACE

This thesis was conducted as a part of InnoFoodAfrica which is a research, innovation and action project that targets major obstacles in African food value chains. VTT Technical Research Centre of Finland Ltd is the project coordinator in collaboration with several partners from Europe and Africa. As being entrusted with this part of the project, I would like to thank especially my supervisor Janne Keränen from VTT. I could not have finished the work without his immense help and guidance from the beginning, not only with analytical thinking but also with mental support.

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On the whole, my most sincere appreciation goes to my family for always supporting me regardless of what I do. Thank you, Duc, my long-time partner and soon-to-be husband for comforting and cheering me up whenever my mental breakdown kicks in. And I could not forget my two lovely kids, Kiri and Kosho, for their meowing chant that gives me strength.

Tampere, 3 November 2022

Anh Kieu Nguyen

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LIST OF SYMBOLS AND ABBREVIATIONS

AFEX	Ammonia fiber expansion
CFU	Colony forming unit
DNA	Deoxyribonucleic acid
EOs	Extract oils
ESA	Eastern and Southern Africa
GC-MS	Gas chromatography-mass spectrometry
GDP	Gross domestic product
ILs	Ionic liquids
IR	Infrared
ISO	International Organization for Standardization
MFC	Minimal fungicidal concentration
ND	Not determined
NES	Not elsewhere specified
NTP	Nonthermal plasma
OFSP	Orange-fleshed sweet potato
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PS	Peptone saline
RH	Relative humidity
SE	Steam explosion
US	Ultrasound
UV	Ultraviolet
VOC	Volatile organic compound

C	Total colonies counted from 2 successive diluted plates
c_A	Average concentration of H_2O_2 vapor (ppm)
d	Dilution factor corresponding to the first dilution retained
E	Mold removal efficiency (antifungal efficacy)
N	Standard number of mold (CFU/g)
N_e	Estimated number of mold (CFU/g)
N_o	Number of mold in the original sample (CFU/g)
N_t	Number of mold in treated sample (CFU/g)
t_{finish}	Finishing point of H_2O_2 vapor generator (minutes)
t_{start}	Starting point of H_2O_2 vapor generator (minutes)
V	Volume of inoculum placed on the plate (mL)

1. INTRODUCTION

Agriculture is one of the largest industries playing a chief role in the global economy which accounted for 4% of the global gross domestic product (GDP) in 2018, and it is regarded as the cornerstone of economic systems in some developing countries, especially in Africa (World Bank 2022). During the period between 2000 and 2019, agriculture, forestry and fishing have contributed to global value chains with approximately 1.5 trillion United States dollars, reaching 3.5 trillion dollars in 2019 (FAO, 2021, p. 1). Agricultural activities account for around 14the % of the total GDP in sub-Saharan African nations, with employment provision to the majority of the population within this continent (OBG & OCP, 2021, p. 5). In fact, other non-agricultural industries gradually start to surpass agriculture in all regions, except for Africa whose contribution to global value chains by agriculture doubled over the above-mentioned period (FAO, 2021, p. 1). As a consequence of rapid but indiscriminate expansion of agriculture, African countries are facing multiple agricultural waste accumulation, environmental contamination as well as degradation of local living conditions (Onu & Mbohwa, 2021, pp. 21-23).

Agricultural residues, excluding those that were used in other sectors (livestock feed, building materials, etc.), accounted for a great deal of total global waste i.e., approximately 390 million tonnes (Mt) in 2019, of which Africa constituted 55 Mt (FAO, n.d.-a). It is unfortunate that most of the agricultural residues do not receive appropriate postharvest treatment and end up either in incinerators or accumulating in landfills (Pérez et al., 2002, p. 53.; Sadh et al., 2018, pp. 1-2). With the aim towards sustainable development, most African countries recognize that the management of agricultural waste or agrowaste should be improved for the obtainment of value-added products.

Many scientists highly perceive that among components of agricultural residues, lignocellulosic biomass is the most prominent waste type to be retrieved and recycled, whose applications widely vary from bioenergy, biochemical to biomaterial (Sadh et al., 2018). Specifically, lignocellulose, mainly containing three natural polymers – cellulose, hemicellulose, and lignin, has huge potential in the production of novel bio-based materials called biocomposite, which can be further manufactured for biopackaging (Pérez et al., 2002, p. 53). However, there are external factors that limit the side stream utilization in Africa i.e., both average temperature and humidity are relatively high, which generates an ideal environment for microorganisms such as fungi and bacteria to develop on the

biomass residues (Fink-Gemmels, 2008, p. 155). The improper handling of crops and their byproducts after the harvest also distributes the biological contamination widespread among the residues, resulting in their mass disposal. To optimize the utilization of the agricultural side stream, these contaminants must be eliminated or reduced to the safety standards prior to further processes, thus an appropriate pretreatment phase is required. However, the decay of biomass residues has hardly been specifically investigated, so not many studies have documented or reported on the contaminant removal efficiency of typical methods. Furthermore, the applicability of these methods for biocomposite and biopackaging processes remains ambiguous as well due to the lack of data records. Most of African countries are also leaning towards circular economy i.e. a model of economy that utilizes the life cycle of materials or products as long as possible while little waste is generated (European Parliament, 2022), which indicates that the recyclability of agricultural side stream must be enhanced.

Therefore, the thesis mainly aims at studying and evaluating current pretreatment methods for agricultural wastes in terms of their fungicidal activities i.e., ability of killing molds. For these methods to be commercially applicable in African countries, research questions should be concentrated on practical aspects, including:

1. Assessment of mold removal efficiency for such methods.
2. Their applicability in the African context at both domestic and industrial levels, including operational expense, approach to needed materials and facilities, and level of technical expertise required.
3. Their environmental impacts as these methods are expected to contribute to sustainable development in Africa.

In the second chapter, a summary of agricultural statistics in Eastern and Southern Africa (ESA) will be presented to justify the recycling potential of the agricultural side stream and determine the selection of residues for further utilization. This chapter will also assist in understanding the impacts of mold on biomass residues, which will be thoroughly investigated in the third section. Next, a brief review of current pretreatment methods for lignocellulosic material will be demonstrated and the experimental methodology of selected pretreatment procedures will be described successively. After which, the results are evaluated and discussed for future proposals. In the end, the most prominent method will be concluded.

2. SIDE STREAM SELECTION

In the following subchapters, the most potential agricultural side stream for biocomposite and biopackaging processes will be investigated in ESA region, including Ethiopia, Kenya, South Africa and Uganda. The selection analysis is based on the accumulative quantity of biomass residues from the 10 most cultivated crops within the area annually, along with their compatibility with polymers to form biocomposite, which is indicated as processability. The crop processability is determined via its lignocellulosic components.

2.1 Annual availability of side stream

The availability of side stream can be simply understood that whether the residues from these crops can provide an adequate amount for the bio-composite and bio-packaging production periodically, which considerably depends on different biomass resources in combination with different biobased polymers (Chang et al., 2020, p. 17956). For instance, in a study about biochar-based polypropylene bio-composites, biomass constituted 30% of total weight (Das et al., 2018, p. 404). The research of polypropylene hybrid composites is also reported utilizing coir fibers and coir shells at 20% of total weight content (Jawaid et al., 2017, p. 4).

In ESA, the sources of biomass residues are expected to mostly come from cereals, with the tremendous growth in annual production from an average of 109.7 Mt (2007-2009) to 114 Mt (2017-2019) (OBG & OCP, 2021, p. 7). The reasons for such mass cultivation are mainly based on favorable ecological conditions, coupled with the traditional regime of the locals (Reynolds et al., 2015, p. 805). Specifically, the diets in this region are majorly based on maize, sorghum, millet, amaranth, teff in addition to pulses such as Bambara groundnut, cowpea, faba/fava bean, tubers (orange-fleshed sweet potato or OFSP) and plantains (banana) (OBG & OCP, 2021, p. 7; Siame & Nawa, 2008, p. 117).

The obtainability of agricultural biomass can be examined through the total crop production quantity since the agrowaste will be inevitably generated in an equivalent amount as stems, stalks, leaves, and seed shells after the fruits or edible components have been collected (Batista Meneses et al., 2020, p. 548). From Figure 1, it can be seen that maize is the most cultivated plant in ESA from 2018 to 2020 with nearly 40 Mt harvested in South Africa, of which more than 15 Mt was produced in 2020. In addition to its massive production, besides its kernels, other components still have few applications and largely

go to waste, indicating a potential supply of raw materials for bio-composite and bio-packaging processes.

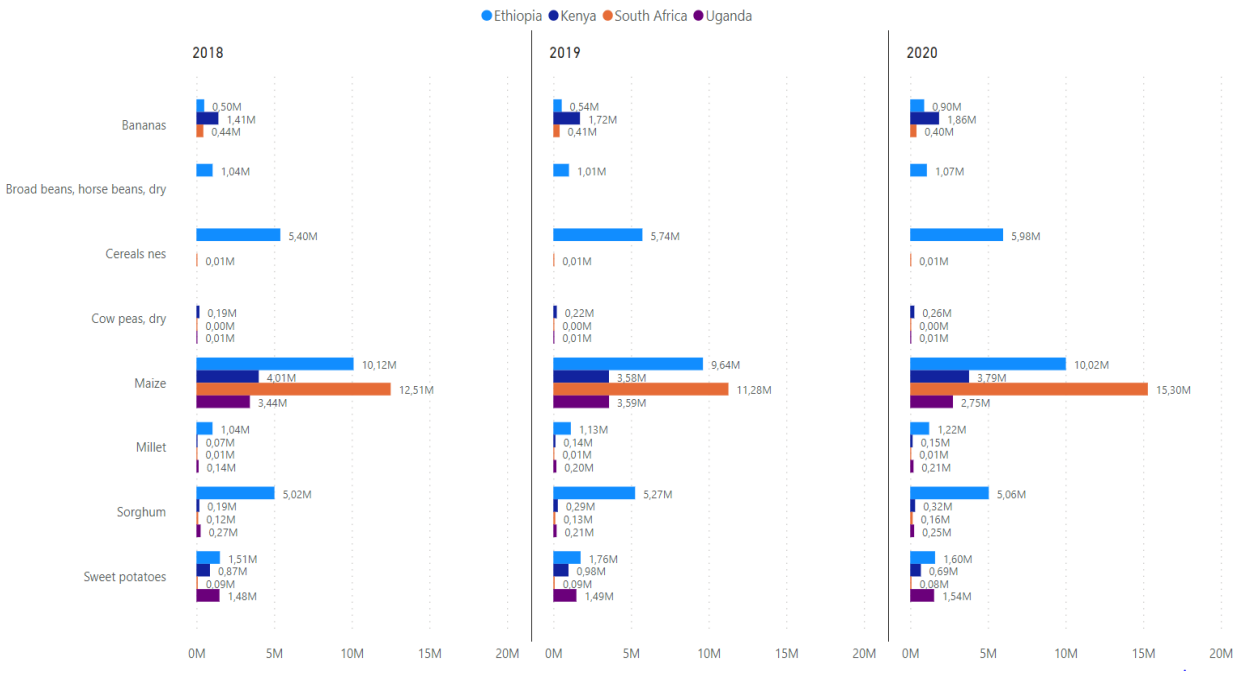

Figure 1. *Agricultural crop production (tonnes) in Ethiopia, Kenya, South Africa and Uganda from 2018 to 2020. The all cereals NES (not elsewhere specified) include amaranth along with other minor cereals, while the millet contains also teff besides finger millet (adapted from FAO, n.d.- b)*

There is no data for Bambara groundnut produced in 4 countries within the period, thus its side stream is not available. The data for amaranth is unreliable as it contains other minor cereals, leaving it undetermined in terms of production quantity. The second largest availability is sorghum with 15 Mt of production coming from Ethiopia during the period. In addition, the dry weight of sorghum residues accounts for ca. 74% of the crop, indicating a great potential for other applications (Bakeer et al., 2013, p. 265). Additionally, crop rotation should also be considered e.g., sorghum is usually harvested 1-2 times per year (National Agricultural Advisory Services, n.d.) while plantains or sweet potatoes can be cultivated all year round thanks to warm weather in Africa (Brodie, n.d.). Thus, a variety of residues from different crops will ensure the supply in a certain period.

The agricultural output was quite constant year by year. For example, sorghum quantity (Ethiopia) in 2018, 2019 and 2020 was 5.02, 5.27 and 5.06 Mt respectively. This also suggests a stable provision of side stream annually. Overall, if only one type of residue is used for biocomposite manufacturing in Africa, maize or sorghum is the most potential crop. In case of various residue combination, the availability of side stream is more highly secured.

2.2 Processability of side stream in biocomposite production

Biocomposites are composite materials that contain one or more phase(s) originating from biological sources (Khalil et al., 2013, p. 673). Specifically, one or more types of biopolymers, which can be produced from renewable resources or synthesized from petroleum-based chemicals, are blended with the addition of reinforcements including recycled wood, wastepaper and especially, byproducts from food crops. The adhesivity of biomass residues with polymers in the composites is enhanced due to their cellulose and lignin properties (Khalil et al., 2013, p. 674). These two components are mostly favored thanks to being the most and second abundant natural polymers all around the world (Batista Meneses et al., 2020, p. 549). Cellulose is consisted of long chains of glucose units tightly linked together, forming bundles which are known as microfibrils (Khalil et al., 2013, p. 675). Cellulosic microfibrils are regarded as the strongest known material with an estimated tensile strength of 7.5 GPa (Šercer et al., 2009, p. 721). Out of three main constituents, lignin has the least affinity for water i.e., it does not tend to be dissolved by water easily. Lignin works as a strengthening agent for cellulose molecules in the fiber cell walls, which can be utilized in tailoring the properties of biocomposite (Khalil et al., 2013, p. 674).

Table 1. *Lignocellulosic composition of agricultural crops in Eastern and Southern Africa (Unit: % Dry matter)*

Crop	Fiber content	Cellulose	Lignin	Ash content	Reference
Sorghum	37	38	5	11	Bartha, 1970; CGIAR, 2009
Finger millet	39	65	6	10	Patel, 1966; Sen, 1938
Teff	34	54	3	9	McCown et al., 2012; Roseberg et al., 2007; Ross et al., 1927; Staniar et al., 2010
Cowpea	28	45	8	12	Koralagama et al., 2008; Sarria et al., 2010; Oluokun, 2005
Faba bean	39	50	10	9	Bruno-Soares et al., 2000; Grimit, 1984
Banana	24	N/A	N/A	11	Sen, 1938
OFSP	4	44	2	5	Faramarzi et al., 2012
Maize	41	68	7	7	Methu et al., 2001

Table 1 presents the proportions of fiber content among 8 most cultivated crops in the ESA region. Bambara groundnut and amaranth are not included as their production data is unavailable. From the table, the crops with more than 35% of fiber content include sorghum, finger millet, faba bean and maize, which are the most potential materials for biocomposite. The fiber content in this study was determined by crude fiber, also known as Weende cellulose. It contains true cellulose and insoluble lignin resulted from an acid hydrolysis followed by an alkaline one (Feedipedia, n.d.). Of which, the higher content of cellulose the more tensile strength, so those crops with 50% or more cellulose content are most preferred, namely finger millet, faba bean and maize. Sorghum containing nearly 40% of cellulose is relatively acceptable. Teff and cowpea are considered as medium materials for composite due to their fiber contents. On the other hand, OFSP should be least preferred as its fiber content is below medium level, 4% only. There is no data about cellulose and lignin percentages in banana's crude fiber, plus its fiber is quite low as well.

The use of biomass residues as reinforcement agents in polymeric composites not only increases the tensile strength of the material, but also promotes its sustainability thanks to fibers' combustibility and biodegradability, which has attracted great interest in recent decades (Jawaid et al., 2017, p. 4; Šercer et al., 2009, p. 722). Yet, the ash content i.e., a rough estimation of mineral matters in the side stream holds a great drawback for the sustainability of the materials, as it can wear down the machine during the process (Rosa & Lenz, 2013, p. 442). Therefore, using residues with low ash percentage i.e., less than 20 wt.% (weight percent) for biocomposite can be more profitable as well as sustainable in the long term (Werther et al., 2000, p. 5). From Table 1, all crops contain ash at an acceptable level, suggesting a wide range of side stream selections. Overall, the residues from listed crops in ESA all have satisfactory characteristics for biocomposite manufacturing, except for banana whose cellulose and lignin composition are undetermined.

Despite the profound quantity in Africa as well as its well-qualified lignocellulosic content, maize should not be considered for this study because of its Mesoamerica indigenous history i.e., the crop was first domesticated in America regions and is widely cultivated worldwide (Watkins et al., 2012). Hence, maize is not a representative cereal from Africa. In addition, due to the global use of maize, it has already been studied more actively than the other crops from the above list. This leads to sorghum, finger millet and faba bean becoming potential candidates for biocomposite production, among which sorghum is selected to be studied in the thesis.

3. OVERVIEW OF MOLD AND ITS IMPACTS ON QUALITY OF AGRICULTURAL SIDE STREAM

Considerable amounts of byproducts from agriculture in Africa are still underutilized for recycling, resulting in them being disposed in dumpsites or incinerated. Regarding the manufacturing of biocomposites, the biomass residues are mostly gathered directly from the field where they have accumulated after the harvest. However, the raw materials which have been exposed to the external environment for several days to weeks before the collecting will be damaged by various factors such as rainwater, dust, strong ultraviolet (UV) light from the sun or insects, etc. Among those, biological contamination by microorganisms is a serious issue as they can degrade the biomass composition, affecting the quality of biocomposites. In addition, the infection of microorganisms on residues can spread rapidly when the crop waste is left in the same place. (Kerdraon et al., 2019, p. 246). Common microbial contaminants are bacteria, and fungi, including molds and yeasts (Bunn, 2002, p. 307). Among these, molds usually show the most conspicuous symptoms in plants. In this section, a detailed study of molds and their related effects on crop residues is demonstrated.

3.1 Mold morphology

Mold is a fungus that exists in the form of multicellular thread-like filaments, which are known as hyphae (McGinnis & Tyring, 1996). Hyphae develop by apical expansion i.e., they constantly grow through their extreme tips and branch into a complex and expanding patchwork which is called a mycelium, from which hyphae seek out and absorb nutrients (Walker & White, 2011, p. 2). Figure 2 describes the anatomy of mold in general. The network of several mycelia contains vegetative parts of fungi, growing on their surface and carrying conidia which are often known as spores (Sergei, 2019). Mold spores, which are responsible for the reproduction of filamentous fungi, are diverse in size, shape or color and can germinate other spores in an appropriate environment, resulting in millions of spores that appear as color-stained (e.g., grey, white, blue) fuzzy areas on the external surfaces of the host (McGinnis & Tyring, 1996; Sergei, 2019).

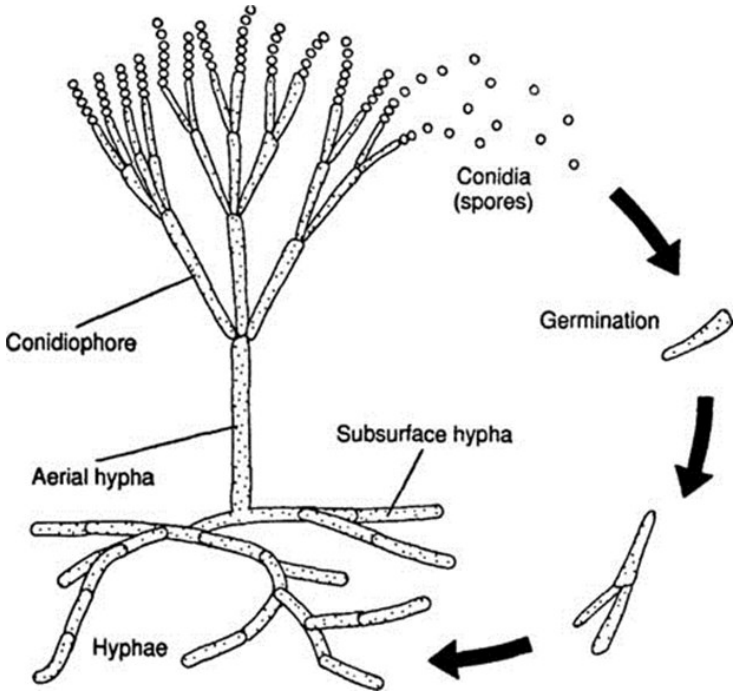

Figure 2. *Structure of filamentous fungi (Medical Laboratories, n.d.)*

The majority of molds multiply via the dissemination of conidiospores, which are borne externally from the cells and directly dispersed from the tips or sides of the hyphae, landing on the nutritious environment to continuously develop (Figure 2) (Clark et al., 2018). This method of reproduction is fast and easily widespread, which ensures the continuation of existence, as mold spores have considerably tough resistance to severe conditions e.g., freezing, heating or even to some hazardous chemicals (Sergei, 2019).

3.2 Growth conditions

The most common spreading pathway of molds is distributing spores through the flow of air e.g., wind or being carried by a living host, flora or fauna (Clark et al., 2018). Averagely, one cubic meter of air sample carries around 10 000 spores while in certain cases, the amount can go up to 2 million spores per cubic meter (Sergei, 2019). When traveling into the atmosphere, mold spores can attach to both living and non-living subjects, settling on non-porous surfaces or penetrating through porous materials (Adan & Samson, 2011, p. 21). Mold spores grow by translocating nutrients from surroundings e.g., air, and soil or they can biodegrade the cell walls and suck out nutrients from their host body to increase their colonization and penetration (Clark et al., 2018). Therefore, theoretically, molds can propagate endlessly if they are provided with sufficient supplements as well as an appropriate environment, which makes them have no predetermined maximum longevity or amount (Walker & White, 2011, p. 3).

The majority of fungal species (spp.) requires moist, sugary and slightly acidic conditions with relatively high temperature and a certain proportion of oxygen to thrive (Clark et al., 2018; Walker & White, 2011, p. 26). The temperature range for molds to exist is quite various in reality, but most will prosper at approximately 25 °C (McGinnis & Tyring, 1996). Despite their rare appearance, low-temperature psychrophilic and high-temperature thermophilic fungi do exist in nature as well as in artificial habitats, thanks to the transient ability to surroundings of fungal cells, according to Walker and White (2011, p. 26). Particularly, a great number of mold spp. go into inactivation mode when encountering extremely cold situations i.e., they still survive but do not continue reproducing. Conversely, fungi would not adjust immediately their internal temperature to their surroundings when facing a heat shock, so the cells experience heat damage and gradually decrease viability to some extent. Yet, their thermotolerance also increases with duration of elevated temperature exposure, which assists fungi survival. (Walker & White, 2011, p. 26.) These adapting mechanisms explain why simple physical treatments such as drying or freezing would not eliminate the molds completely.

Water plays a crucial role in mold metabolism, and is considered the most significant factor for these fungi to develop (Adan & Samson, 2011, p. 22). Specifically, fungal cells would suffer damage to their physiology if the water availability to molds is reduced, which is known as water stress (Walker & White, 2011, pp. 26-27). The sources of water for mold can vary in different forms e.g., water drops, vapors, mist and so on. The water availability for mold growth present in a certain area is usually estimated through the relative humidity (RH) level, which establishes the fundamental prevention of mold spreading (Walker & White, 2011, p. 26).

In terms of pH, most molds can thrive between pH 4 and 6, but some spp. can endure to a further extent ranging from pH 3 to 8 (Clark et al., 2018). It has been reported that organic acids such as acetic and lactic acids have more effective inhibition on mold growth than mineral acids like hydrochloric or phosphoric acids since organic ones can decrease intracellular pH via mold translocation across the plasma membranes (Walker & White, 2011, p. 27). These findings have paved the way for the development of organic preservatives in the food industry recently, which are also potentially applied in mold inhibition for the biocomposite and biopackaging sectors.

3.3 Mold impacts on biomass residues

Molds, or fungi in general, obtain energy and nutrients from the organic matters on the host/surface they inhabit by utilizing heterotrophy, which is different from the photosynthesis mechanism of vegetation (Clark et al., 2018). To be specific, molds tend to do not fix carbon dioxide or nitrogen from the atmosphere like some bacteria, but rather they consume complex organic compounds. In addition, molds are mostly saprophyte, meaning that they reside on dead organisms and derive supplements from decaying organic matters, in this case are the insoluble compounds of agricultural residues including starch, cellulose and lignin of dead plants (Daniel, 2016, p. 135). In spite of applying similar mechanism as animals, molds perform their nutrient absorption in completely reverse order, digestion prior to ingestion: hyphae secrete exoenzymes (hydrolytic enzymes) to degrade complicated biopolymers into more absorbable glucose molecules, which would be transported back to the mycelium by hyphae (Clark et al., 2018). Consequently, the fiber contents of crop residues will be damaged if they are infected with molds.

In addition to the cellular degradation, some plant fungal pathogens can also produce mycotoxins as known as the toxic second metabolites that are generated when molds experience stress such as lack of nutrients, moisture or oxygen (Wareing, n.d.). Mycotoxins are mainly consisted of five significant groups, namely aflatoxins, fumonisins, ochratoxin A, zearalenone and deoxynivalenol (Kpodo & Bankole, 2008, p. 103). *Aspergillus* spp., *Penicillium* spp., *Alternaria* spp. & *Fusarium* spp. are regarded as the most common mycotoxin manufacturers within the fungal classification, in terms of plant infection and damage both in the field and during storage with poor conditions (Wareing, n.d.). What is more, typical African crops are susceptible to especially above-mentioned fungal genera (Siame & Nawa, 2008). Therefore, the biomass pretreatment should not only eliminate the mold appearing in external surfaces but also mitigate/decrease mycotoxin concentration if present to the required safety level. Mycotoxins have been reported to have adverse effect on both human and animal health via ingestion (the most dangerous route), inhalation and dermal exposure (Liu et al., 2022, p. 1). Thus, if the moldy residues were not properly treated and mycotoxins were potentially contained in the materials, the biocomposite would also probably transfer the substances into domestic products and cause harm to the consumers.

3.4 Mold detection methods

It is likely that various different genera of molds inhabiting on biomass residues, and since each genus has its own tolerance to environmental modifications, it is indeed essential that the most prevalent mold type appearing on the residues can be identified. Only after the determination of fungi is confirmed should the implementation of pretreatment strategies is conducted. Many fungal pathogens cause similar symptoms in the host while they require separate treatment methods, thus it is significant to apply proper detection techniques. The determination of molds also includes counting their population on the residues as well, which assists in the analysis of removal efficacy for pretreatment methods later on. In this section, a brief overview of mold identification methods would be presented, in which these methods are classified into two main categories i.e., traditional methods and modern technologies.

3.4.1 Conventional methods for mold detection

The fundamental foundations for conventional fungal pathogen investigations are principally established from morphological, microbiological and biochemical determinations (Ray et al., 2017, p. 709). Respectively, to investigate the mold culture on postharvest crop residues, the traditional methods that can be utilized are visual examination, culturing and plating isolation, and isozyme analysis (Narayanasamy, 2011, p. 137).

Visible contamination of mold on external surface of the samples can be investigated to some extent with visual examination, by indicating and comprehending the disease symptoms such as spots, blights, tumors, galls, etc. (Ray et al., 2017, p. 710). For instance, the white spots appear on sorghum stalks in Figure 3. Under circumstances of latent infections or indistinct symptoms, the fungal pathogens have to be analyzed by isolation of the pathogen in suitable culture media, coupled with the study of morphological properties under the light microscope (Narayanasamy, 2011, p. 131).

Although the visual examination method has been increasingly innovated thanks to standardized guidelines for assessment as well as open access to an extensive database, the implementation is not practical and applicable in every situation, as it requires an expert on microbiology (Ray et al., 2017, p. 710). In addition, the results from this method are mostly based on individual experience, so the reliability and objectiveness of the data remain uncertain.

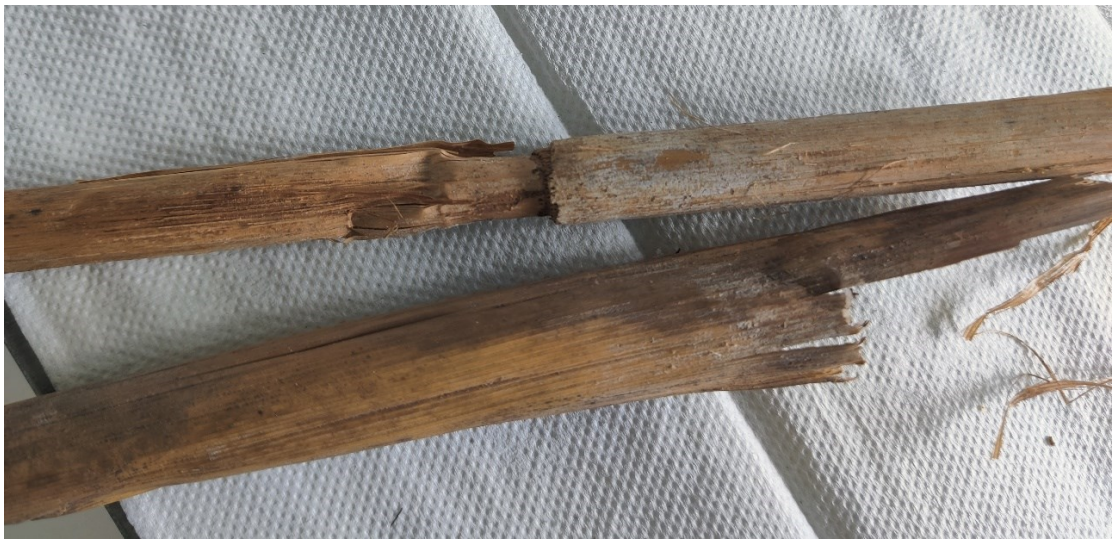

Figure 3. *White-molded sorghum stalks*

Isozymes or isoenzymes are the numerous molecular forms of similar single enzymes, which usually have identical enzymatic activity (Micales & Bonde, 1995, p. 116). The different nucleotide slight differences of each enzyme in isozymes are the amino acid substitutions, as a result of the arrangement of the deoxyribonucleic acid (DNA), the chemical present at the cell's center, that codes for the protein (Narayanasamy, 2011, p. 28). Isoenzymes with great alterations in size or shape, or distinct net charge can be separated by electrophoresis (Micales & Bonde, 1995, p. 116). Accordingly, around one-third of the amino acid substitutions would modify the net charge of a protein significantly, thus only amino acids are electrophoretically detectable, while the rest of the substitutions would neutralize the effect. Thus, isozyme analysis provides a relatively precise and comprehensible determination of genetic variation within a large population. However, there are limitations in isozyme application in fungal pathogen detection, which are chiefly attributed to the low level of polymorphism i.e., the condition of appearing in various forms available in different fungal taxa analyzed (Ray et al. 2017, p. 710).

Culturing and plating is regarded as the most simple detection method for microorganism study in general, and remains widely applied at both laboratory and industrial scales despite its old age (Ray et al., 2017, p. 710). In particular, the technique's operational principles involve the isolation of mold samples and nurturing them in an artificial media, along with visual and microscopic observation. The microscopic analysis mainly evaluates the mold's morphological characteristics such as its spore morphology, sporulation varieties, production and properties of sporulating systems generating asexual and sexual spore forms, in which the optimal temperature for mold development is the most favored indicator of fungal identification (Narayanasamy, 2011, p. 137). Plate counting

determines mold population based on the assumption that the microorganisms are homogenously distributed within the host i.e., the cells are assumed to be The method is considerably affordable and requires no advanced technology, yet the operational duration could be time-consuming as the mold could take a few days to weeks to grow in large colonies for the microscopic analysis, plus the accuracy of the results heavily depends on the microbiologist's expertise (McCartney et al., 2003, p. 129).

Among those three traditional methods, culturing and plating is most favored as it is simple to perform, requires inexpensive equipment and materials while provides reliable results e.g. the results of mold enumeration in mozzarella cheese using different culturing media were highly equivalent (Spangenberg & Ingham, 2000), suggesting that this method was quite consistent utilizing whichever medium.

3.4.2 Current advances in detection technology

Conventional methods for microorganism identification are not accurate and reliable in most of cases, and their assessment significantly depends on skills and experiences of microbiologists which could be not impartial in certain specifics, leading to regrettable errors. Thus, more novel techniques have been under development and innovated to enhance the accuracy rate in recent years. The advanced technologies are divided into two main groups, direct and indirect methods respectively (Ray et al., 2017, p. 710). The direct methods classify mold spp. based on their typical properties such as structure, morphology, physiology, reproduction, etc. For indirect methods, Ray et al. (2017, p. 712) presented that the majority utilizes plant stress profiling, gaseous metabolites profiling and plant metabolites profiling fungal diagnosis. Specifically, the plant diseases are not detected by studying the pathogen's direct characteristics, but instead their impact on physiological plant reactions.

Regarding direct method group, it is then classified into another two major sub-groups which are immunology-based methods utilizing antibodies or antibody alternatives and polymerase-chain-reaction-based (PCR-based) methods using nucleic acid probes (Ray et al., 2017, p. 710). Antibodies are the most critical reagent in immunological assays, hence it is primarily required that the most suitable reagent must be selected prior to conducting the identification tests (Peruski & Peruski, 2003, p. 506). The antisera include fundamental and heavy chain antibodies, polyclonal antibodies, monoclonal antibodies and phage-displayed recombinant antibodies, which have been constantly developed to increase the sensitivity and accuracy of immunological reactions (Ray et al., 2017, p. 710). Although the immunology-based methods are much quicker, more simple and

more cost-effective than PCR-based processes, the reactions of antisera are less sensitive and specific.

According to Ray et al. (2017, p. 711), PCR technology is regarded as the most reliable detection method for fungal pathogens so far due to its high precision as well as sensitivity. The PCR tests have been reported to obtain the fastest results as well. They explained that the examination is fundamentally based on the detection of target nucleic acids, which by far more sensitive compared with other microbiological and serological fundamental techniques in identification of fungi. Yet, PCR systems are complex and expensive, which is not practically applied in most of industries.

Indirect determination technologies include spectroscopic and imaging detections that works on symptoms of stress-based diseases, and indications of volatile organic compounds (VOC) or biomarkers which mark biological conditions in particular. As the method name suggests, radiation such as infrared (IR) is utilized in spectroscopic techniques since microorganisms contain distinctive chemical compounds within their cell membranes and cell walls and these components would illustrate different IR absorption spectra (Wang et al., 2018, p. 257). Spectroscopy is quite simple to perform, plus the operational cost is quite reasonable. Nonetheless, in certain cases, some different molds can have similar IR spectra because their compositions are just slightly different (Wang et al., 2018, p. 257). Thus, spectroscopy is usually conducted in combination with other detection methods to assure the accuracy.

The fungal pathogens can be traced from VOCs when plants release them into their surroundings, from which information related to the host physiological response to the diseases would be retrieved (Ray et al., 2017, p. 712). The most common technology used in this case for both qualitative and quantitative analysis of volatile metabolites is gas chromatography coupled with mass spectroscopy (GC–MS). The method requires to gathering samples of pre-collected VOCs before the data analysis, which could be time-consuming. What is more, distinguishing between VOCs for specific infections and the volatile biomarkers indicating environmental or nutrient stress still remains challenging (Ray et al., 2017, p. 712).

A summary of different approaches from each modern category that have been performed in the industry is presented in Appendix A. There are several advanced methods yet considering the thesis scale which requires high precision along with affordable operational cost, simple procedures and easy access to the apparatus, conventional methods, specifically visual examination and plating isolation will be the most suitable process to implement within the thesis scale.

4. BIOMASS RESIDUE PRETREATMENT

Lignocellulosic biomass has a complex composition containing cellulose, hemicellulose, lignin, pectin and other mineral matters (Khalil et al., 2013, p. 674). Hence, the yields and efficiency of other biorefinery processes will be hindered if the raw materials are proceeded without proper pretreatment (Batista Meneses et al., 2020). The common pretreatment methods for agricultural waste have quite a wide range, from physical, chemical, physiochemical to biological techniques, or the various combinations among these processes (Isikgor & Remzi Becer, 2015, p. 4500). Nevertheless, most of the research related to biomass pretreatment focuses on the depolymerization of the lignocellulosic components that extends to enhancement of biomass degradability, with little mention of microbial contaminant removal rate (Batista Meneses et al., 2020, pp. 547-548). Thus, in this chapter, the thesis will center on investigating the mold removal efficiency of current pretreatment methods.

4.1 Physical/Mechanical pretreatment methods

Decontamination of mold by physical techniques includes sorting and separation, scrubbing/brushing, heating, sonication and irradiation. Regarding sorting and separation, as its name suggests, this technique is in fact filtering the moldy samples from batches and isolating them for disposal. However, molds are micro fungi appearing on highly small-scale, which can cause difficulty in sorting effectively. Furthermore, to promote sustainability, mold contamination should be eliminated from the biomass residues rather than discarding the molded samples. Therefore, sorting and separation will not be studied in the thesis.

4.1.1 Scrubbing

The mold spores can still spread in the air and attach to the subject even after the molds are dead or inactivated (Palaty, 2010, p. 11). Thus, their physical remains must be removed from the host to prevent the dispersal of mold spores and fragments. Scrubbing or brushing the molds away is the simplest process of physical removal, however, the method is not applicable to all situations. To be specific, the physical clean-up of mold considerably depends on the type of affected materials, which is determined by the porosity. That is, a material can be porous, semi-porous or non-porous. The scrubbing would only be effective with non-porous materials such as ceramic tiles or glasses. In

the case of porous matters like the biomass residues, it requires more extensive treatment that can penetrate inside the subjects and remove mold fragments more thoroughly (Palaty, 2010, p. 11). Scrubbing alone, however, can spread the spores into surrounding air as they are really light, leading to worse contamination (Robbins & Morrell, 2006, p. 9). Generally, scrubbing is usually combined with water or bleaching/oxidizing detergent for more effective results. Once the molds are wetted, they will attach to the brush/cloth used for scrubbing better, preventing the emission. The bleaching chemicals such as chlorine bleach (as known as sodium hypochlorite) can break down the fungi and scrubbing will remove their remains from the surface of the contaminated areas (Lee, 2009, p. 2). A vacuum with a High Efficiency Particulate Air filter may be required to collect the leftover spores in some serious cases (Lee, 2009, p. 2). After the scrubbing, it is essential that the materials must be dried to prevent moisture, which is the ideal growing condition for mold.

Overall, this technique is not effective on biomass residues unless it is combined with chemical and thermal treatments. In addition, most steps are manual work, which is time-consuming and inefficient. Hence, it is not recommended to apply this method in this study.

4.1.2 Heating

Thermal pretreatment for biomass residues has been applied widely with the aim of vaporizing the water content as well as killing microbes. Based on the research of heat resistance threshold for some mold spp. in food contamination by Rico-Munoz et al. (2015, p. 2), most of examined molds cannot tolerate if the temperature goes above 100 °C and the duration varies from a few minutes to some hours. Apart from the mold colonies, the presence of mycotoxins in the samples is possible as the residues have been left untreated for quite a long period. Mycotoxins are, on the other hand, tougher with high temperatures i.e., aflatoxin B1, deoxynivalenol, zearalenone, fumonisin B1 require 237, 175, 220, and 150 °C to decompose respectively, which indicates that traditional thermal treatment has issues in eliminating mycotoxins if they are present in the side stream (Liu et al., 2022, p. 3). Theoretically, the temperature can be increased to that level or even higher at 300 °C maximum since that is when hemicellulose and cellulose will start to decompose; lignin, on the other hand, degrades at a much higher temperature i.e., 700 °C (Burhenne et al., 2013, p. 178). However, it is not practical to perform the thermal process at such high temperatures as it would require a large amount of energy, considering the African conditions (Pankaj et al., 2018). Plus, such high temperatures

will certainly destroy the materials fast. Thermal treatment combined with other techniques e.g. with chemicals can result in higher efficiency without raising to such high heat, as the reaction rate can be elevated. For instance, it was reported that the time to kill 95% of Rhizopus stolonifera infecting nectarines with 10% ethanol at 50 °C was around 46 seconds, much less than ca. 400 seconds with water alone at the same temperature (Margosan et al., 1997, p. 1406).

4.1.3 Sonication

Sonication is the technology that utilizes ultrasound (US) vibrating through a fluid to generate the pressure variations (Batista Meneses et al., 2020, p. 553). In the research of ultrasonic application on food pasteurization by Evelyn & Silva (2020), high-power US is defined as acoustic sound waves of low frequency ranging from 20 to 100 kHz with a typical sound intensity of 10-1000 W/cm^2, which have the ability to disrupt the vegetative cells of microbial pathogens in food. In Figure 4, the working mechanism of the sonication technique on microorganisms is presented. During sonication in an aqueous medium, cavitation which refers to the formation, growth, and explosion of micro gas bubbles occurs (Piyasena et al., 2003, p. 208). The shock waves resulting from bubble collapse then break down the structures of cell walls and membranes of microorganisms, leading to microbial inactivation. Cavitation can also separate water vapor, generating free radicals H^+ and OH^-. These radicals can either disrupt the DNA of microbial cells or react to form hydrogen peroxide which has antimicrobial effects. (Dolas et al., 2019, p. 2.)

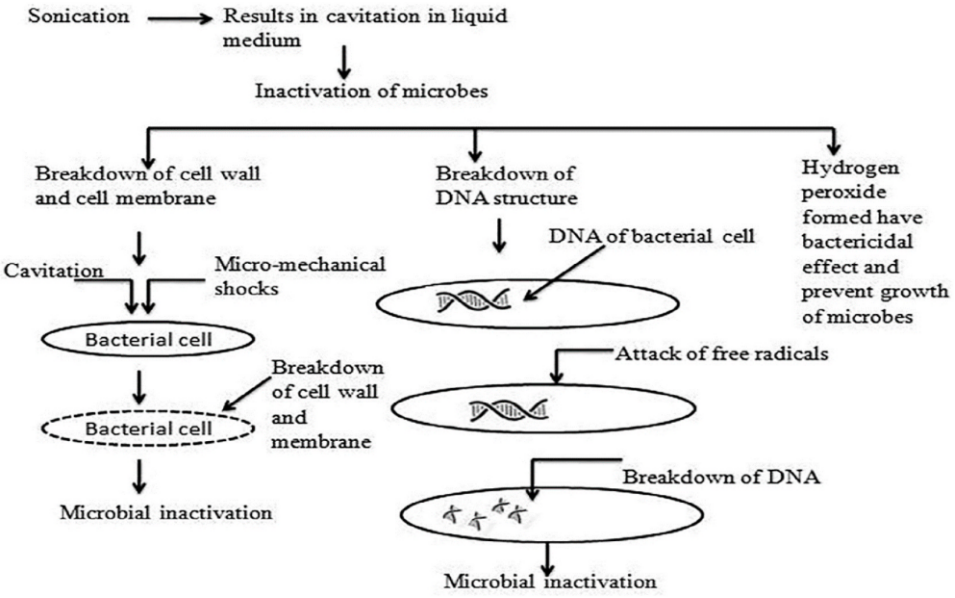

Figure 4. *US mechanism on microbial inactivation (Dolas et al., 2019). Reprinted with permission from Elsevier*

US technology has been applied in some food processing operations such as enzyme inactivation, microbial deactivation, and drying, to name but a few (Evelyn & Silva, 2020, p. 402). In addition, US technique requires lower temperature i.e., ranges from 50 °C to 90 °C depending on microorganisms (J. Li et al., 2019, pp. 115–118; Tremarin et al., 2019, pp. 160–162) to implement than conventional thermal treatment, which can reduce energy consumption. A study by Song et al. (2018) has experimented sonication on side stream from wheat straw and the results showed that the residues that underwent US vibration had higher quality e.g. stronger durability than those did not, implying that sonication can also improve the quality of biomass residues. Yet, the involvement of water in sonication might be a drawback considering water scarcity in Africa, plus the materials need drying after the treatment which can increase the operational expense.

4.1.4 Irradiation

Irradiation as mold removal is the technology which utilizes radiated energy sources such as radio waves, microwave, IR, UV, X-rays, and γ-rays (gamma rays) to remove the microscopic substances in contaminated samples, according to Calado et al. (2014). There have been several studies of irradiation impacts on microbial reduction and elimination in food and livestock feeds to decrease the mycotoxin concentration since the 1950s (Webb et al., 1959). For example, disinfection using gamma-irradiation with doses of 1-2 kGy (unit of ionizing radiation dose) was able to remove *Aspergillus* from infected corn kernels (Cuero et al., 1986); A dose of 6 kGy was reported to eliminate the concentration of aflatoxin B_1 (AFB_1) and total aflatoxins in chilies by 1-2 logs (Iqbal et al., 2013). Irradiation has been proved to safely remove contaminants such as bacteria, fungi, and pesticides from food without alternating its nutritional components as well as physical and chemical characteristics, in most cases (Calado et al., 2014, p. 1049). Therefore, irradiation can have potential for sterilization of molded biomass residues. UV-A and UV-B (wavelengths of 320-400 nm and 280-320 nm respectively) are solar radiation that can occur naturally and cause no significant impacts on the crop residues, but rather interrupt the structure of various fungal pathogens (Raviv & Antignus2, 2004, p. 219). This suggests that African households can possibly pretreat the residues by drying them under the sunlight which is available all year round.

However, to eliminate microorganisms more effectively, stronger radiation such as UV-C (wavelength of 200-280 nm) or γ-rays is required, which has not been applied broadly due to its complex and expensive operational equipment. Some irradiators need maintenance monthly as the produced isotopes would continuously decay, leading to concurrent loss of radioactive energy (Calado et al., 2014, p. 1049). Furthermore, as strong

irradiation (X-rays and gamma-rays) accelerates the decomposition of organic matters, it is possible that the method will also affect the structure of lignocellulosic fibers besides the microorganisms (Y. Li et al., 2016, p. 2568). Therefore, it is not practical to apply irradiation in treating biomass residues at the domestic scale.

4.2 Chemical pretreatment methods

The core principle of chemical pretreatment methods is utilizing the chemical reaction of solvents on fungal cell walls. In some articles, the mechanical methods also mention washing and solvent extraction (Liu et al., 2022, p. 2) as one option, but this technique should be discussed under the chemical pretreatment section due to the involvement of chemicals as removal reagents. A variety of organic solvents (in pure form or diluted solutions) including alcohols, esters, formaldehyde, phenols, etc. (Rodríguez et al., 2018, pp. 35-36) have been reported to show disinfecting activity against microbial contamination in food or building surfaces (Kuyukina et al., 2014, p. 23). Yet, their antifungal efficacy on biomass residues has hardly been contemplated, resulting in little information to demonstrate for this section.

4.2.1 Acid pretreatment

Acid pretreatment of biomass is one of the earliest methods to be developed and commonly applied today, with various types of mineral acids such as sulfuric acid (H_2SO_4) and hydrochloric acid (HCl) being the most utilized at the industrial scale (Batista Meneses et al., 2020, p. 550). In the food industry, most studies have shown that food pretreated with acids decays less than that is not during storage, caused by pathogenic microorganisms (fungi and bacteria) (Alp & Bulantekin, 2021). The inactivation of microorganisms in biomass can be implemented with organic acids such as ascorbic acids (DiPersio et al., 2004; Yoon et al., 2004), citric acids (Pao & Petracek, 1997; Yoon et al., 2004), lactic acids (Uyttendaele et al., 2004), acetic acids (Yu et al., 2001), and fatty acids (Pohl et al., 2011). These acids in their hydrophobic undissociated form can infiltrate into the cell membrane of microorganisms by neutralizing their electrochemical potential to increase their permeability. From then, they reduce the pH level in the fungal cytoplasm (the molecular fluid that fills the cell) and cease metabolic activities (Dalié et al., 2010, p. 374).

Besides those, hydrogen peroxide or H_2O_2 is also currently used as a biocide in many industries including food sterilization thanks to its disinfecting capacity while its decompositions are non-toxic substances (oxygen and water) (Linley et al., 2012, p. 1589). The

generated hydroxyl radicals H^+ and OH^- can work as oxidizing agents and react with lipids, proteins, and DNA, which accounts for fungicidal effects of H_2O_2 (Linley et al., 2012, p. 1590). Thus, H_2O_2 can be applied as a fungicide for crop residues. On the other hand, acids that have been utilized in combination with hydrothermal pretreatment for biofuel process (and possibly other biorefinery products) are reported to release harmful byproducts such as furfural, 5-(Hydroxymethyl)-2-furaldehyde (HMF), acids (acetic, formic, levulinic and fungal), or aliphatic and phenolic groups when they start to degrade sugars and lignin (Rasmussen et al., 2014; Zeng et al., 2014). This indicates a challenge in selecting proper acid types with right dosage so that they can eliminate the fungal pathogens efficiently while minimizing the degradation of biomass properties.

4.2.2 Alkaline pretreatment

The application of alkaline in biomass pretreatment is quite similar to acid pretreatment i.e., immersion coupled with drying, yet alkaline reagents have been shown to be less aggressive than acid solutions while still obtaining equivalent results (Kim et al., 2016). The high pH environment that alkaline chemicals create imposes profound impacts on fungal cells e.g., difficulties in the nutrient acquisition or decrease in essential elements like iron, copper (Fernandes et al., 2017, p. 1). The most widely used alkaline reagents in biomass pretreatment are sodium hydroxide (NaOH), sodium carbonate (Na_2CO_3), ammonia (NH_3), and calcium hydroxide [$Ca(OH)_2$] (Batista Meneses et al., 2020, p. 551). Of which, sodium hydroxide and calcium hydroxide have been added to many commercial bleaches thanks to their effective deactivation against mold. The antimicrobial mechanism of NaOH or $Ca(OH)_2$ is quite similar to H_2O_2: hydroxyl ions are released in an aqueous environment and oxidize various biomolecules, leading to the destruction of the cytoplasmic membrane, proteins and DNA of microbial cells (Siqueira & Lopes, 1999, p. 362).

The use of most alkaline reagents is reported to be ecofriendly as there are no toxic byproducts released to the environment after the pretreatment. What is more, alkaline pretreatment is more efficient in delignification of crop residues without tremendously altering the cellulosic structures, compared with acid pretreatment (Rabemanolontsoa & Saka, 2016). Hence, alkaline usage should be preferred over acid when considering the quality of biomass materials for biocomposite process.

4.2.3 Gas treatment

The gas treatment presented in this thesis is the combination of both gaseous acidic and basic reagents along with other chemicals utilized in fungal control. The gas treatment has also been developed for pathogenic microbial control in the food industry with various chemical experiments, namely chlorine dioxide (ClO_2) (H. Lee et al., 2020), ammonia (Montesinos-Herrero et al., 2011), hydrogen peroxide (Linley et al., 2012) and ozone (O_3) (Gabler et al., 2010). These chemicals are strong oxidants i.e., they can damage the cell walls, and penetrate and oxidize essential molecular components including membrane and DNA. The protein synthesis is then interrupted while the metabolism of microbial cells is stopped, resulting in the reduction of microorganisms (H. Lee et al., 2020, p. 159).

The use of vaporized H_2O_2 as a sterilant for food has been more favored than the liquid form because the gaseous form leaves no residual traces on the treated samples and with similar volume, vaporized H_2O_2 can disinfect more effectively while damaging less aggressively to the materials and devices, in comparison with exposure to the solutions (Linley et al., 2012). Ozone is similar as its degradation is oxygen, leaving no residues on disinfected objects (Gabler et al., 2010, p. 86). Additionally, the use of gas treatment can reduce the drying step afterward, which also decreases the workload, energy consumption as well as operational expense. Another benefit of utilizing gas treatment e.g., hydrogen peroxide, ozone over other aqueous chemicals is that they break down into non-toxic byproducts to both humans and the environment, and can be collected with a ventilation system provided that the fumigation is implemented in a confined space (Linley et al., 2012, p. 1594).

4.2.4 Ionic liquids pretreatment

Ionic liquids (ILs) are consisted of organic heterocyclic cations and several anions existing in liquid form at extremely low temperatures i.e., approximately below 373 K (Batista Meneses et al., 2020, p. 551; Kohli, 2018, p. 621). Many ILs have been proved to have antimicrobial activity, especially in paper preservation (Dimitrić et al., 2019), and disinfection of medical facilities (Kohli, 2018, p. 644), to name but a few. The working principle of ILs is that the cation and anion pairings as well as the length of alkyl chains can be altered when ILs react with contaminants, from which the toxicity along with other features of ILs can be tuned and removed (Kohli, 2018, p. 644). Specifically, ILs contain large organic cations e.g., imidazolium or pyridinium and alkyl chain substituents that can modify the hydrophobicity of molecules (Docherty & Kulpa, 2005, p. 185). These compounds have been observed and nominated for toxicity testing against a wide range

of fungi and bacteria by United States National Toxicology Program (Docherty & Kulpa, 2005, p. 186).

In addition, ILs have been examined to have high recyclability and biodegradability rate, coupled with low volatility that reduces risk to humans and surroundings (Dimitrić et al., 2019). The research also stated that their high viscosity can hinder the penetration of pathogenic microorganisms into materials, plus their adjustable miscibility with water or less harmful solvents assists in their removal from the treated samples more easily. However, despite these advantages of ILs, they have not been utilized as fungicides for biomass residues, resulting in few references for the study. Additionally, it requires the facility to recycle ILs, leading to extra operational costs.

4.3 Physicochemical pretreatment methods

Physicochemical pretreatment methods can be simple understood as the combination of physical and chemical procedures, with the aim of reducing the disadvantages of both fields while enhancing operational efficiency.

4.3.1 Steam explosion

In steam explosion (SE) pretreatment, the biomass residues undergo a high-pressure steam at a high temperature (around 200°C) in a really short time i.e., 2-8 minutes, from which the steam penetrates into the interior of the materials and fill their tissue pores (Martin-Sampedro et al. 2011, p. 8761). The steam is gradually saturated within the pores, extending and then exploding once the pressure is removed, which leads to the decomposition of fibrils and other lignocellulosic contents (Brodeur et al., 2011, p. 8). The same mechanism also affects microbial cells, indicating its antimicrobial activity. For instance, SE has been reported to enhance the aerobic stability of biomass by inhibiting the growth of *C. humilis* (Jiang et al., 2020).

Based on Arrhenius equation which describes the dependence of reaction rate on temperature, the rate constant of chemical degradation is less affected by temperature than that of microbial deactivation. The reason can be that chemical degradation has lower activation energy than microbial deactivation does. (Zhao et al., 2015, p. 548.) Hence, it is possible that any treatment process that utilizes high temperatures in a short duration like SE can eliminate pathogenic fungi without lowering the quality of residue components. Additionally, the short treatment time can help accelerate the efficiency of the whole process in general by saving time and expense for other procedures. Yet, SE has

only been applied at the industrial level since it requires a complex and expensive system (Brodeur et al., 2011, p. 9).

On the other hand, autoclave works based on moist heat sterilization which has a similar antimicrobial principle as SE, except that the temperature in the autoclave is heated lower, at 121 °C (Kleis & Sachs, 2000, p. 1180). With a lower temperature to operate, the autoclave is expected to consume less power and water than SE, indicating a more affordable replacement. The autoclave is also widely commercialized in various industries e.g. medical and food sterilization, so there are more available models of autoclave to compare and select than SE.

4.3.2 Ammonia fiber expansion (AFEX™)

The ammonia fiber expansion (AFEX) can be simply understood as the enhanced ammonia pretreatment for biomass residues. Specifically, during this process, anhydrous or gaseous liquid ammonia is directed to the raw materials under moderate pressure (around 100 to 400 psi) and at mild to high temperatures (70 to 200°C) prior to releasing the pressure rapidly (Bals et al., 2010, p. 2). The antimicrobial activity of ammonia has been already discussed above; in addition, the decrystallisation of cellulose, the removal and polymerization of lignin along with the hydrolysis of hemicellulose haven been accelerated in AFEX pretreatment, resulting in higher yields of products from biorefinery processes, compared with when raw materials are not pretreated. Another advantage of AFEX is that the ammonia gas can be reused if a recycling system is equipped with the main mechanism, which would be beneficial for the reagent consumption, implementational cost as well as waste emission. (Batista Meneses et al., 2020, p. 554.) However, the safety issue when handling NH_3 coupled with complicated equipment makes AFEX impractical for the locals to implement the pretreatment on their own. The cost of an additional system to retrieve used NH_3 is also of concern.

4.3.3 Supercritical carbon dioxide explosion

Supercritical carbon dioxide (CO_2) is a more promising technique than both SE and AFEX, as it requires lower pressure and lower expense to operate due to the lower price of CO_2 than ammonia (Zheng et al., 1995, p. 846). To be specific, its critical temperature is 31 °C and its critical pressure is 1071 psi (Gu et al., 2013, p. 1738). In addition, the process is considered environmental-friendly since CO_2 as the solvent is non-toxic and recyclable through photosynthesis once released into the surroundings. The pretreat-

ment has been demonstrated to break down the structures of lignocellulosic components, improving the afterward hydrolysis (Batista Meneses et al., 2020, p. 554). Nonetheless, in terms of mold removal or antimicrobial activity in general, supercritical CO_2 is utilized in the extraction of natural fungicides. Particularly, CO_2 is used as a solvent for the supercritical fluid extraction of several herbal spp. such as yarrow, chamomile, dandelion, catnip (Schoss et al., 2022, p. 2), and ginger (Mesomo et al., 2013), to name but a few. Therefore, supercritical CO_2 is not a direct elimination of mold for crop residues, but rather implemented in combination with other methods. The technology can be combined with biological pretreatment methods (which are described in subchapter 4.4) in order to generate biocides for the fungi on biomass residues. However, its installation, as well as operation, can be complicated and costly, which is not appropriate for conditions in Africa generally.

4.3.4 Nonthermal plasma

Nonthermal plasma (NTP) technology has been inspected for its ability in microorganism inactivation for food preparation, biofilm degradation, or especially in healthcare such as cancer treatment, initiation of apoptosis, inactivation of prion and other biomolecules, and so on (Scholtz et al., 2015, p. 1108). Hence, NTP emerges as an auspicious method for decontaminating both infected surfaces and internal structures. The application of NTP in bacterial inactivation had been conducted around 1988 with the experiment of suspension on *Saccharomyces cerevisiae* and *Bacillus natto* by pulsed high voltage (Mizuno & Hori), yet the work of Laroussi in 1996 should be regarded as the first experiment of NTP as a strong sterilization agent. In terms of filamentous fungi, there has been little investigation of their reaction with NTP, compared with bacteria, which makes it difficult for the research to learn about NTP's fungicidal activity.

According to Scholtz et al. (2015, p. 1111), the fungicidal inactivation of NTP seems to have a more inefficient effect than bactericidal inactivation, under similar conditions, which could be due to the more complex structure of fungi. In addition, different fungal species have different reactions with NTP concerning exposure duration as well as plasma sources. The statement still concluded that NTP has the ability to disinfect mold, yet for the application of biomass pretreatment, several trials must be implemented to adopt the appropriate plasma conditions.

4.4 Biological pretreatment methods

In the food industry, the use of natural preservatives and antimicrobials has been increasingly developed in recent years due to the concerns of adverse health effects along with environmental pollution. Natural resources for antimicrobials include plant extracts, essential oils, organic acids and bacteriocins – the toxins produced by bacteria, which are more preferred alternatives than conventional food sterilization since these methods cause little to no loss of organoleptic characteristics (the properties that affect or are related to the senses i.e., taste, odor of substances) of food and prevent the spoilage in a much safer way (Negi, 2012, p. 7). The usage of bacteriocins would not be contemplated in the thesis because there might have been other microorganisms residing on the crop residues after the application, which is an unfavorable issue.

The applicability of organic acids in biomass pretreatment has been discussed in the previous section (subchapter 4.2.1). The other natural antimicrobials have not been widely examined in this field, but considering various references from food decontamination research, it is possible that the application of plant extracts or essential oils can be implemented for the project target, the moldy crop residues. In fact, there have been bio-based mold disinfectants commercially introduced and consumed, such as Thymox containing thymol (thyme oil extracts) that has the ability to eliminate parasites and germs (Thymox, n.d.).

The antimicrobial activity of different plant extracts varies regarding their residual components, which is listed in Appendix B (Negi, 2012). The main decontaminating mechanism of plant extracts can be owing to the combined impacts of the adsorption of polyphenols to microbial membranes which cause damage to the cell membranes thus leaking the cellular contents, and the formation of hydrogen peroxides from polyphenols that have strong inactivation against microorganisms (Akagawa et al., 2003; Ikigai et al., 1993; Otake et al., 1991). The extraction of plant antimicrobials, however, is sophisticated and contains several stages, which can cause excessive expenses and workload.

Essential oils (EOs) or volatile oils are defined as aromatic oily solutions extracted from plant components such as flowers, buds, leaves, seeds, fruits, and so on through distillation (Negi, 2012, p. 8). EOs contain several compounds that have fungicidal effects, namely terpenes, alcohols, acetones, phenols, organic acids, aldehydes, and esters (Burt 2004). Furthermore, EOs are also effective disinfectants regarding microorganisms since the antimicrobial properties of various chemical compounds from EOs do not specifically target one certain type but can cause damage to several cells. Some common EOs used in food decontamination are classified in Appendix C. Similar to plant extracts,

the biomass pretreatment process with EOs could be complicated and expensive due to the extra steps of EOs extraction and drying after the immersion. In addition, there has been little data that specifies which EOs or plant extracts can be applied for molds on crop residues, which leads to several trials to determine.

4.5 Selection criteria for pretreatment methods

The thesis aims at inspecting the fungicidal effect of the pretreatment methods from 4 categories (subchapters 4.1-4.4) above. There has been little data related to this issue, especially on agricultural residues. Hence, the thesis selects the most suitable methods and implements experiments to study their removal efficiency, according to the following criteria: applicability, operational cost, and environmental impacts. Table 2 demonstrates the pros and cons of each pretreatment method considering the former criteria.

Table 2. *Assessment of current pretreatment for biomass residues*

Pretreatment methods	Applicability	Affordability	Sustainability
Physical	Most are widely implemented at industrial scales Sonication & irradiation have not been applied much compared to heating Irradiation requires skilled experts & advanced equipment	Heating is cheaper than sonication & irradiation, but less effective Equipment for sonication & irradiation is expensive Irradiation requires regular maintenance => increase cost	Heating consumes energy Sonication & irradiation: lower energy consumption than heating, no toxic emissions
Chemical	Mostly applied in biomass pretreatment & food industry concerning mold removal capacity, except for ILs & organic solvents Largest amount of available research for references (H_2O_2, O_3) Simple operational equipment Safety instruction for chemical handling is accessible	Most chemicals are largely available at affordable prices/can be generated with simple & cheap process Recycling system can be expensive, except for H_2O_2	Conventional chemicals such as sulfuric acid are aggressive, yet others like H_2O_2, O_3 release harmless by-products Used gas can be collected and recycled, but not applicable to all oxidants
Physiochemical	Widely applied in biomass pretreatment but little data concerning fungal removal efficiency Requires modern and sophisticated technology => technical expertise required	More expensive than chemical/physical alone while the better effectiveness is not confirmed yet Supercritical CO_2 is considered more profitable than SE & AFEX	Most of them are green techniques as they release no harmful by-products to the environment SE might consume much energy for the high pressure & temperature to operate
Biological	Applied in food decontamination but little data regarding to decontamination of biomass Extraction might not be applicable to all African regions due to the availability of plant spp. Still requires more research	The price of plant spp. might be cheap but the extraction process could be expensive Involves several stages from extracting, and dipping to drying, which might increase the expense	Natural biocides are eco-friendly, yet the exploitation of plant extracts and EOs is a considerable issue

These requirements concern the practical implementation in Africa, including domestic and industrial scale i.e., the process should be simple and safe so that even the residents and farmers can handle it. The materials or facilities for the pretreatment should be accessible as well. Thus, the removal of molds from crop residues can be operated soon after the gathering. The expense to implement such a method should not be too pricy, considering the fact that most African countries are developing with low average income, especially in rural areas. Green methods with minimal waste generation after the pretreatment are preferred as they are expected to contribute to sustainable development in Africa.

From the assessment above, it is clear that chemical pretreatment has the most benefits in all three criteria. Most of the chemical techniques follow a straightforward working principle: submerging the contaminated samples into chemicals and drying (if needed) afterward. Therefore, among all above-mentioned methods, the study will focus on the chemical application on biomass residues, particularly affordable chemicals like H_2O_2.

In addition, autoclave in the physicochemical group shows great potential as well: the simplest form of autoclave equipment is a pressure cooker, which has been used widely at the household scale, implying that autoclave can be applicable for both farmers and biocomposite manufacturers. Hence, the autoclave will also be examined further in the experimental phase.

On the other hand, physical pretreatment requires the installment of complex equipment plus the power to run those systems, which does not meet the demand for low cost and sustainability. Methods in the biological category are also neglected as the extraction of natural biocides is expensive and time-consuming, and the research of suitable substances exceeds the thesis scope.

5. MATERIALS AND METHODS

Due to limited research regarding the mold removal efficiency of each method, the experiment was conducted in two phases: preliminary/screening testing and statistical analysis. Specifically, in the preparatory stage, several chemical pretreatment methods including different chemicals at different concentrations along with autoclave were applied to contaminated samples for one test each. The removal efficiency, which was regarded as antifungal efficacy in this experiment, was calculated based on the logarithmic removal measurement. From then, a selection of methods whose antifungal efficacy reached 3-$\log_{10}$ reduction (99.9% removal) or above, in addition to their practicality in the African context, was obtained and a repetition testing was implemented on these methods for the analytical phase i.e., each procedure was repeated in several parallel samples and their means were calculated.

5.1 Residue preparation

Sorghum was selected for this experiment as it was the first to show contamination of mold during the harvest and afterward. The batch of sorghum residue was delivered from Uganda, containing leaves and stems. The raw materials were collected on the fields, dried at 105.0 °C overnight and then every 0.5 kg was packed in sealed plastic bags for transportation. Once the package arrived in Finland, the residues were dried a second time at the same temperature and duration with the aim of eliminating all insects possibly residing on the samples. After that, the materials were packed and stored in the freezer at 5.0 ± 1.0 °C, which deactivated the growth of microorganisms.

Figure 5. *Sorghum residues from Uganda with molded stains*

After the second drying, there were still traces of mold appearing on the surface of residues i.e., brown and white stains that can be seen in Figure 5 and the materials emitted an unpleasant and musty odor, which was typical smells from molds (US EPA, 2021).

5.2 Mold characterization

The culturing and plating method was utilized for mold detection and enumeration in the thesis. The method is intended for application in routine analysis that determines the quantitative assessment of mold presence in food and feed (Nordic Committee on Food Analysis, 2005, p. 1), which was in sorghum leaves and stems in the thesis. The population of mold was detected based on colony-forming units (CFU) which indicate spores, conidia or hyphal fragments growing on a medium and assembling into various colonies (Nordic Committee on Food Analysis, 2005, p. 1). Since the colonies of mold can accelerate to the point of being too numerous to count, further dilution of the samples can help increase the accuracy of enumeration (3M Petrifilm^{TM}, 2020, p. 3). The minimum approvable counting range has been considered as 10 colonies by ISO standard 7218 (2013, p. 32).

The number of replicate plates has been recommended as singular plating with at least 2 successive dilutions to enhance the accuracy of enumeration, stated in ISO standard 7218 (2013, p. 38). Therefore, the experiment started with a 10-fold dilution series with 8 dilutions i.e., the original sample was diluted to 10^{-8}. To prepare the serial dilutions of all samples including control (contaminated residues) and treated ones, 5 gr of residues were mixed with 100 ml of diluent, which was peptone saline (PS) (peptone 1 g/L; sodium chloride 8.5 g/L; pH = 7.0) in this study. The mixture was then homogenized in a paddle blender or as known as the brand name Stomacher for about 2 minutes. Through pressure and constant motion, the microorganisms containing molds were extracted from the solid samples and circulated into the solution intact. The mixture was filtered after that to remove solid particles and 5 ml of the solution was transferred into a tube, working as the original tube for the serial dilution. Specifically, 0.5 mL was taken from 5 mL of the control sample and transferred into 4.5 mL of diluent (can be either distilled water or PS) to create a 1:10 ratio. The second tube was mixed thoroughly to ensure the solvent was homogenized, then 0.5 mL from it was directed into the next tube. A similar work was repeated to the last tube.

Once the dilution series was completed, 1 mL of sample suspension from each tube was transferred onto the center of the 3M Petrifilm^{TM} Yeast and Mold count plate. The inoculum was then gently distributed over the circular area with a spreader placed on top of

the film. The plates were left undisturbed for about 1 minute so that the gel could be formed (Figure 6). After that, the plates were marked, stacked in an upright position, and transferred into ventilated plastic bags, which were incubated at 25.0 ± 1.0 °C for 7 days. Generally, most colonies stop to grow further after one week, while not all types of fungi will appear in 2-3 days. Hence, a 7-day incubation is regarded as a standardized duration (Nordic Committee on Food Analysis, 2005, p. 2).

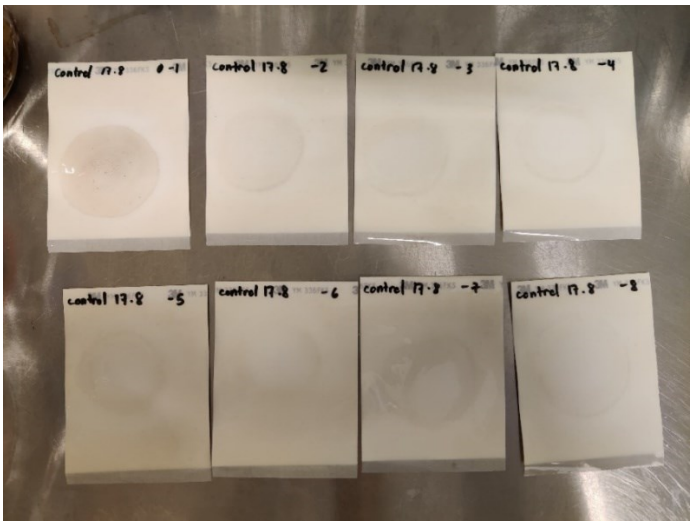

Figure 6. *Mold culturing with 3M Petrifilm™ count plates*

For the identification of mold spp. on the materials, the mold was cultured in potato dextrose agar (PDA) on Petri dishes (Figure 7), which was more suitable for the microscopic examination than with Petrifilm™ count plates. Antibiotics were added to the agar so that bacteria could not grow on the plates and affect the accuracy of mold enumeration. The dilution series was conducted similarly, yet only 0.1 ml of suspension from each tube was transferred onto the media and gently spread with a sterile bent rod so that the solution could be absorbed completely into the agar. The PDA plates were also incubated at 25.0 ± 1.0 °C for 7 days.

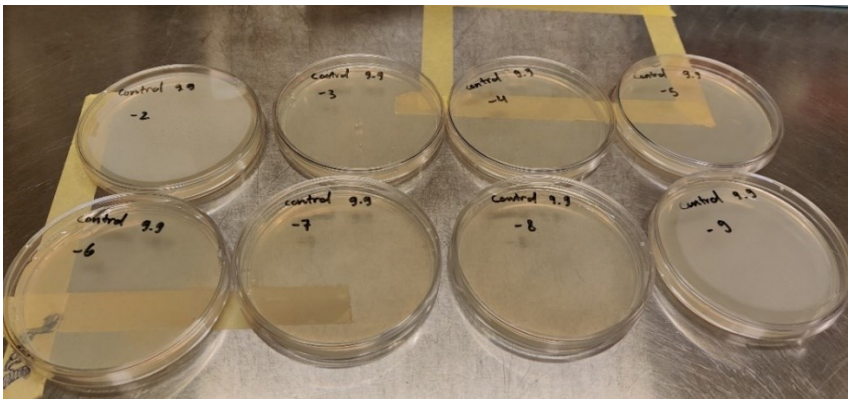

Figure 7. *Mold culturing with PDA petri dishes*

After 1 week, the colonies of mold were counted and mold spp. were determined. The counting should be done on at least 1 plate containing 10 colonies at minimum. The number N of mold in the original sample (CFU/mL or CFU/g) was then calculated as a weighted mean of 2 successive dilutions according to formula (1) where $\sum C$ is the total colonies counted from 2 successive diluted plates in which either one showing at least 10 colonies, V is the volume of inoculum placed on the plate (mL), and d is the dilution factor corresponding to the first dilution retained (ISO:7218, 2013, p. 30).

$$N = \frac{\sum C}{V * 1{,}1 * d} \quad (1)$$

The minimum precision range for mold counting is 10 colonies per dish, thus the result accuracy decreases when the total colonies decrease below 10. Nonetheless, the precision might vary depending on the purpose of the test, leading to different methods of calculation for low-amount cases. Particularly, if the total number of colonies are less than 10, the estimated number N_E of microorganisms per gram is given according to formula (2). (ISO:7218, 2013, p. 32.)

$$N_E = \frac{C}{V * d} \quad (2)$$

On the other hand, if there is no presence of microorganisms even on the first diluted plate, the result should be reported as: “less than $\frac{1}{V*d}$ microorganisms per gram” (ISO:7218, 2013, p. 33).

For mold identification, the PDA plates after incubation underwent visual and microscopic examination. For visual analysis, the mold spp. was investigated through their macromorphological characteristics such as the basis of the colony’s diameter, elevation, margins, color, and texture of colony in both sides of the plate. With microscopy, the micromorphological features i.e. hyphae shape, conidial structure, spore shape, spore size, etc. were inspected. (Samson et al., 2019.)

5.3 Preliminary testing

For screening purposes, each method was applied to one sample during this phase. Hydrogen peroxide and ethanol were more contemplated than other substances thanks to their great availability at an affordable price and simple preparation. Ozone was also first considered for the thesis; however, it was omitted as ozone could cause potential health issues through inhalation i.e., ozone is a strong oxidant that irritates human

breathing and is related to cancer (US EPA, 2022). Hence, the African locals might be affected if they were accidentally exposed to ozone during the pretreatment.

5.3.1 Hydrogen peroxide (aqueous) pretreatment

Regarding hydrogen peroxide pretreatment in liquid form, 5 g of residues were immersed into 100 mL of 3% H_2O_2 in different duration i.e., 20, 40, 60 and 120 minutes- while with 5% H_2O_2, the experiment was conducted in 10, 20, 40 and 60 minutes. The solutions were prepared with H_2O_2 30% (VWR Chemicals, France) and were adjusted based on the dilution formula. The selection of H_2O_2 concentration was based on several studies of its application on food disinfection/sterilization such as Smilanick et al. (1995), Martin and Maris (2012), Cerioni et al. (2013) and Meng et al. (2019).

It was noticed that there were a lot of bubbles formed which can be seen in Figure 8, right after the residues were added to the removal reagent. This indicated that hydrogen peroxide reacted with the materials quite quickly.

Figure 8. *5% H_2O_2 reaction with molded residues*

After the soaking, the solutions were filtered with filter bags and the solid residues were placed on aluminum plates under the fume hood for H_2O_2 to vaporize at room temperature, 21.0 ± 1.0°C. The atmosphere inside the hood was monitored with an H_2O_2 sensor. Once the concentration showed 0 ppm which indicated that there was no trace of hydrogen peroxide left, the residues were transferred and heated in the oven at 60.0 °C until they were completely dried.

5.3.2 Hydrogen peroxide (aqueous) in combination with acids pretreatment

According to Martin and Maris (2012), the fungicidal efficacy of hydrogen peroxide can be enhanced in combination with acids. Among 17 mineral and organic acids that have been tested, formic acid (HCOOH) and acetic acid (CH_3COOH) remained synergetic with H_2O_2 against 4 and 2 strains of common fungi in food spoilage respectively (Martin & Maris, 2012, p. 1458). Thus, these two acids were also applied in the experiment.

The ratios of acid/hydrogen peroxide were adjusted based on minimal fungicidal concentration (MFC), which is the minimum concentration of reagents at which mold will be eliminated and cannot continue reproducing (Martin & Maris, 2012, p. 1453). Specifically, previous research discovered that the MFC for $HCOOH/H_2O_2$ was 0.31/1.56 (%/%) and that of CH_3COOH/H_2O_2 was 2.5/0.78 (%/%). It was expected that the molds residing on the sorghum residues would have higher tolerance with external factors than the molds cultured in laboratory conditions, hence, the MFCs in this case were also assumed to be higher. The concentration ratios were then modified into 1/5 (%/%) for $HCOOH/H_2O_2$ and 5/2 (%/%) for CH_3COOH/H_2O_2. These concentration values were also easier to prepare with higher accuracy.

The acids used in this experiment were formic acid 99-100% (VWR Chemicals, France) and acetic acid ≥99.7% (Sigma-Aldrich, Germany). The residues (5 g) were then dipped into each solution and soaked for 15 minutes. Unlike the case of pretreatment with H_2O_2 alone, the reaction between acid/H_2O_2 and the residues was quite slow, with fewer bubbles formed which can be seen in Figure 9.

Figure 9. *Acid/H_2O_2 reactions with molded samples*

Once the dipping was finished, the treated samples underwent similar processes as in the H_2O_2 pretreatment above.

5.3.3 Ethanol pretreatment

Several studies concerning the use of ethanol (EtOH) in controlling the postharvest molds in fruits, such as Margosan et al. (1997) and Smilanick et al. (1995), were utilized as the basis for determining the concentration and temperature of EtOH, and treatment duration in the experiment. Specifically, 5 g of raw materials were treated in 100 mL of 35% and 50% EtOH at room temperature, ca. 21.0 °C and at 50.0 ± 1.0°C. The solvents were prepared from ethanol ETAX Aa ≥ 99.5% (Altia Oyj, Finland) based on the dilution formula.

With respect to 50.0 °C EtOH, the solutions were heated on a hot plate. Once the solvents reached the desired temperature, the residues were then poured into the chemical and constantly stirred. The temperature was regulated with a thermometer. The pretreatment was conducted for 10 minutes for each ethanol concentration. On the other hand, the experiments with ethanol at room temperature were quite simple i.e., the materials were soaked into EtOH solvents for also 10 minutes. In all cases, ethanol reacted with contaminants on the raw materials relatively slowly and a little bubble was released, which can be seen in Figure 10.

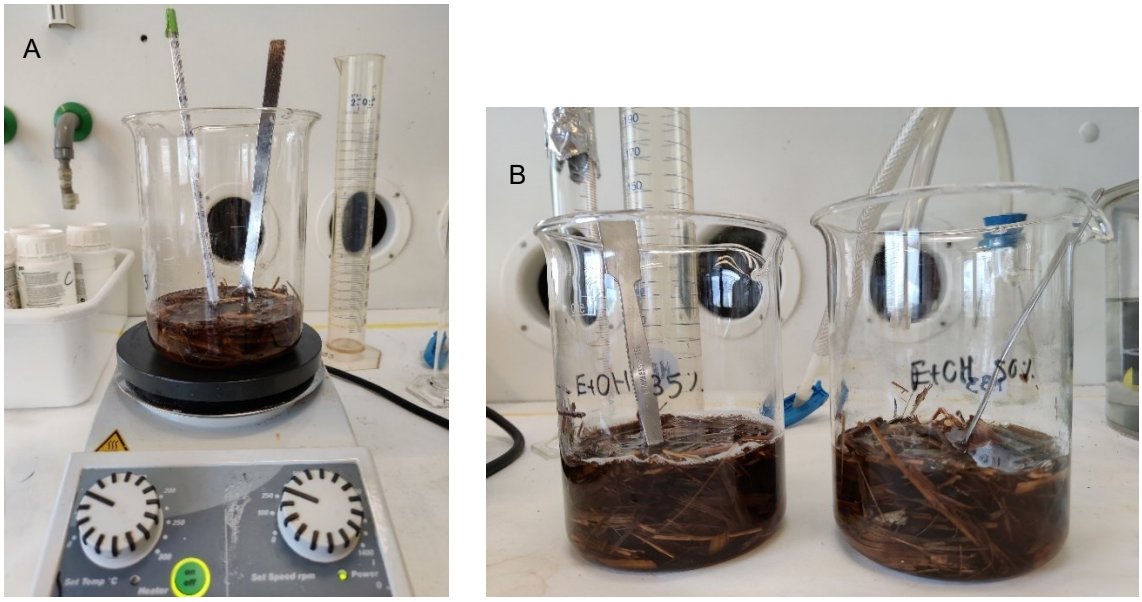

Figure 10. *(A) Heated EtOH pretreatment, and (B) EtOH pretreatment at room temperature*

After that, all samples were progressed similarly to above-mentioned experiments, including chemical vaporization and drying steps.

5.3.4 Hydrogen peroxide vapor pretreatment

Hydrogen peroxide gas was generated by the Cleamix VCS-100Cr equipment in this experiment. The generator was connected with a fume hood through Cleamix HC-hose connector set i.e., two tubes attached to the vapor output and air intake gates of the device, which is displayed simply in Figure 11 A. From then, H_2O_2 vapor was directed into the hood, wherein a bag of the contaminated sample was placed and treated. 35% H_2O_2 (Merck KGaA, Germany) was utilized for the fumigation. The H_2O_2 gas concentration within the chamber was monitored with a Vaisala HPP 272 hydrogen peroxide sensor, connected via sensor port (no.11 in Figure 11 B) and the data were delivered and presented on a touch screen.

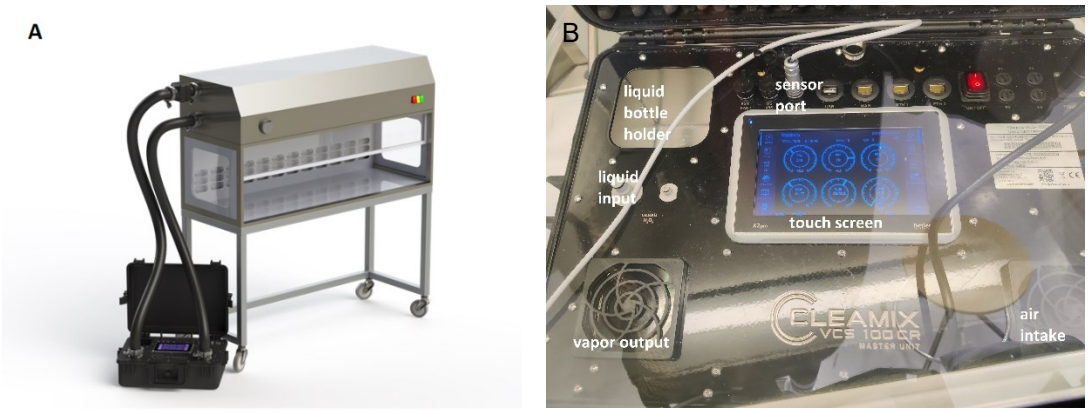

Figure 11. *(A) H_2O_2 fumigation setup, and (B) structure of Cleamix VCS-100Cr (Cleamix Oy, 2020)*

During the fumigation, 50 g of sorghum side stream was treated for 1 hour with an H_2O_2 concentration of approximately 200 ppm, which consumed around 5 mL of 35% H_2O_2 solution. After the treatment, the bag remained inside the chamber and was ventilated overnight, so that the leftover gas would have time to decompose. The data was recorded every 5 minutes and the average concentration c_A can be calculated through its integral, and the time difference between the starting point t_{start} and finishing point t_{finish}, which is presented in equation (3).

$$c_A = \frac{1}{(t_{finish} - t_{start})} * \int_{t_{start}}^{t_{finish}} f(x)dx \tag{3}$$

Since hydrogen peroxide gas decomposed into hydrogen and water vapor, the residues required no further steps such as washing or drying after the pretreatment. Once the

H_2O_2 concentration within the chamber decreased to a safety level i.e., 1 ppm or less, the sample was taken out and prepared for mold incubation.

5.3.5 Autoclave

For the autoclave pretreatment on contaminated samples in this experiment, a Getinge HS Lab Steam Sterilizer was utilized. The sample was experimented with two situations: a small amount ca. 5 g was placed into a beaker and an 0.5 kg sample bag. The main aim was to investigate if the increase in mass could affect the heated steam reaching the core of the sample. Both cases were conducted within one programmed cycle i.e., steam-sterilizing at 121.0 °C for 20 minutes. The volume of a laboratory-scale autoclave is approximately 600 L with a usable space of 660 x 670 x 1300 mm (width x height x depth) (Getinge, n.d.). It was estimated that with these dimensions, 2 to 3 bags containing 500 g of side stream could be fit into the chamber.

Figure 12. *(A) Getinge HS Lab Steam Sterilizer, and (B) the sample bag placed inside the sterilizer*

The autoclave pretreatment required no washing or drying step afterward since there was no involvement of chemicals. Once the cycle was completed, the samples were taken out for mold determination, which was the same as in above-mentioned methods.

5.4 Statistical analysis

From preliminary testing, the most promising methods were selected and repeated in pentaplicate i.e., each method was implemented in 5 replicates. The mold removal efficiency or in other words, antifungal efficacy E of each pretreatment was determined through the logarithmic reduction between the number of mold in the control sample N_o (CFU/g) and the number of mold in the treated sample N_t (CFU/g), which is demonstrated in equation (4). All the data are expressed as mean values ± standard deviation.

$$E = \log_{10} \frac{N_o}{N_t} \tag{4}$$

The $\log_{10}$ or simply log reduction is simply understood as a 10-fold decrease in terms of microorganism population, mold in this case. This measure is a more precise quantity to illustrate the level of mold elimination than percentage reduction since the number of mold colonies can go up to above millions. Particularly, if there was 1 million CFU per 1 g of the residues, a 99% removal will equal a 2-log reduction and approximately 10,000 CFU remaining, which is considered too low according to European standards e.g., EN 13697/+A1 (2019) and EN 17272 (2020) required 3-log and 4-log reduction or above (equal to 99.9 and 99.99% respectively) of microorganisms in medical, veterinary, food, industrial, domestic, and institutional areas. Therefore, the thesis focused on those methods that either reached the desired removal or possibly obtained with small modifications during the screening phase.

Apart from the estimated removal efficiency from the experiment, each method was evaluated in terms of three main aspects, namely affordability, applicability and sustainability. Particularly, every method is scrutinized if its technology/mechanism is easily assembled in Africa, especially in areas close to or within farmers' residences. The installation coupled with operation expenses should be as low as possible so that the overall cost will not accelerate and the biocomposite production can make profits. The pretreatment is also a part of a contribution to Africa's sustainable development; thus its procedure must impose no hazardous impacts on nature. From then, the most effective and pragmatic pretreatment was selected for Africa in practice.

6. RESULTS AND DISCUSSION

6.1 Mold species identification

The identification was also implemented on 5 PDA-plates culturing mold from control samples. From 7-day-incubated plates in Figure 13 A, the dark brown or black colonies relatively dominated the medium. The growth rate of most of these colonies after incubation was averagely larger than 30 mm in diameter, which indicated that they could be *Aspergillus* or *Cladosporium* spp. (Samson et al., 2019, pp. 32, 46). This was possible as both species are common with worldwide distribution spreading on a variety of substrates and mediums, including atmosphere as well as cereal grains, beans, peas, etc. in Africa (Samson et al., 2019, p. 45).

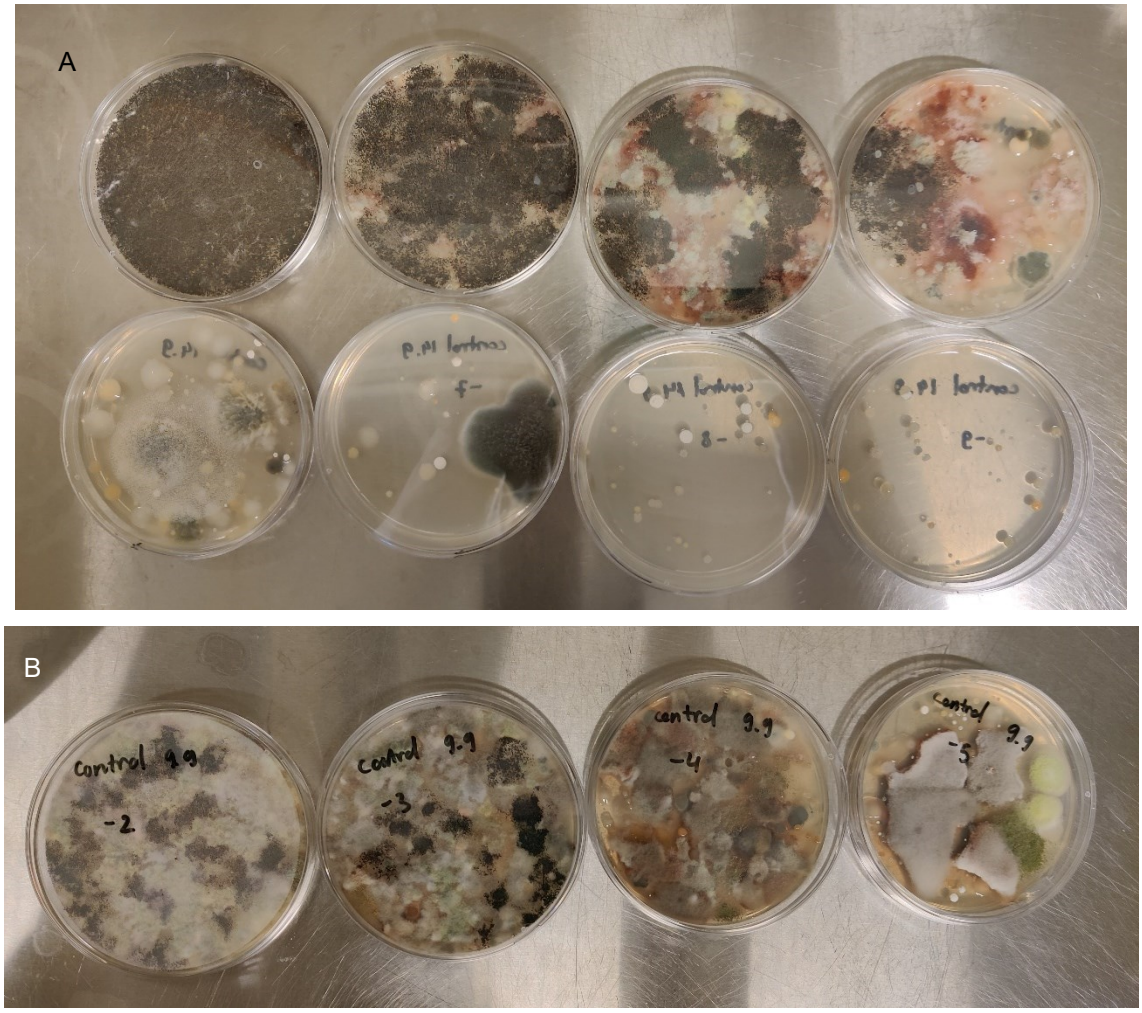

Figure 13. *(A) Control sample after 7-day incubation and (B) a duplicate of control sample*

Fusarium spp. were considered to appear on the residues as well, as their colonies on PDA-plates could be cream white, yellowish, brownish, pink, or reddish. The fuzzy green and white colonies might specify *Penicillium* spp. These colonies also formed a denser layer than the others (approximately 0.5 and 0.3 mm respectively), which might reflect the tendency of *Penicillium* spores growing i.e., the conidia can arise straightly from the agar or from erect hyphae (Samson et al., 2019, p. 57). More fuzzy fungi can be detected more clearly in another replicate (Figure 13 B).

The investigation of possible fungi spp. on sorghum samples was enhanced with the analysis of micromorphological features with microscopy. All of the fungi mentioned so far were confirmed to be present, plus the occurrence of *Sarocladium*, *Monascus* and *Trichoderma* spp. was also identified and captured in Figure 14.

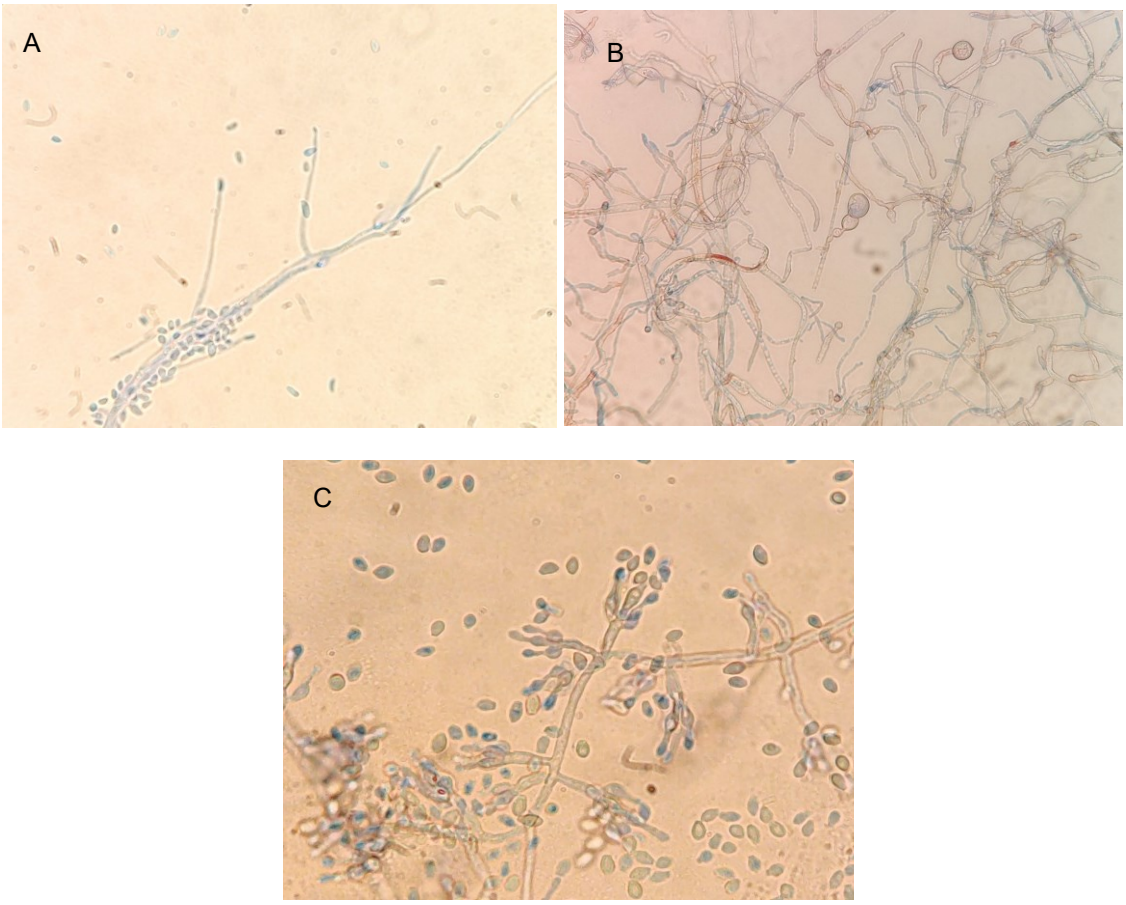

Figure 14. *Micromorphology of (A) Sarocladium, (B) Monascus and (C) Trichoderma*

It is possible that there were other molds that had not been determined or were unknown to the present knowledge of microbiology. This could be due to the fact that the distribution of mold on sorghum samples was uneven i.e., there were areas of mold occupying more densely than others while with the plating and culturing method, only some small portions of the whole sample bag were examined at laboratory scale. However, out of 5

tested replicates, 4 samples showed the appearance of *Penicillium*, *Aspergillus* and *Fusarium* spp. whereas *Cladosporium* and *Trichoderma* spp. were detected in 3 samples. This might suggest that these mold spp. dominate within the residues.

Table 3 below summarizes the biodeterioration and pathology of these fungi i.e., how they affect the biomass residues as well as potential toxicity to humans. Among which, *Penicillium*, *Aspergillus* and *Fusarium* spp. should be most concerned in terms of health issues once the side stream is turned into biocomposite for biopackaging, as they produce mycotoxins.

Table 3. *Potential impacts of detected molds on the residues and human (Samson et al., 2019)*

Mold spp.	Biodeterioration and pathology
Aspergillus	Deteriorate cereals, oilseed, nuts; produce rot in crops (maize, peanuts, etc.); produce mycotoxins which are toxic to mammals
Cladosporium	Decay cereal grains, fruits; deteriorate cellulosic materials; human pathogen on skin and nail
Fusarium	Pathogenic to many plants; cause keratitis (an eye disease) in human; toxic to animals when feeding contaminated food; cause root rot, head blights in cereals; produce mycotoxins
Penicillium	Cause blue mold in cereals, blue eye rot in maize, rot in citrus fruits and apples, pears, plums, etc.; deteriorate plant stems; produce mycotoxins
Trichoderma	Can produce cellulases and other enzymes that decompose cellulose; emerging opportunistic human pathogen

Mold spores were still present even after drying at 105.0 °C two times which has been observed during residue preparation (subchapter 5.1), indicating that some molds infecting the sorghum samples possibly had resistance to high temperature. Additionally, the heating or boiling activities in fact only eliminated the living fungi at that moment while the spores remained and germinated after cooling, thus molds kept reproducing within the residues and might even spread through the airway (Andersson et al., 1995, p. 145). The RH in the African environment was also indeed high, varying between 70 and nearly

100%, coupled with high porosity of the materials i.e., water vapor could easily penetrate inside that resulting in moist conditions for molds to grow. The freezing could only inhibit the growth of existing molds. Thus, the pretreatment required stronger techniques to also eliminate the spores e.g., the involvement of oxidants or bleaching agents, or the steam power at high pressure.

The average concentration of mold in the original sample was $2.39 \times 10^7 \pm 2.92 \times 10^6$ CFU/g, calculated from 5 replicas. The value was then used to estimate the log reduction of all experiments in both the screening and replication phases.

6.2 Removal efficiency from preliminary testing

From the first screening (Table 4), EtOH pretreatment shows the most promising removal of the mold with all 4 experiments obtaining 4-log reduction and above. With 50% EtOH at 50°C, 6-log reduction was even achieved, which was equal to 99.9999% of mold removal. In other words, with approximately 2.4×10^7 CFU/g in the original sample, there were only about 20 CFU/g left in the treated one.

Table 4. *Antifungal efficacy of each method from first preliminary test*

Method	Application	Treatment duration (min)	Temperature (°C)	Mold concentration (CFU/g)	*E*
3% H_2O_2	Liquid	20	21	1.45E+04	3.22
3% H_2O_2	Liquid	40	21	6.36E+05	1.57
3% H_2O_2	Liquid	60	21	5.64E+04	2.63
3% H_2O_2	Liquid	120	21	4.91E+04	2.69
H_2O_2 + formic acid	Liquid	5	21	1.60E+05	2.17
H_2O_2 + acetic acid	Liquid	5	21	5.27E+04	2.66
35% H_2O_2	Vapor	60	25	1.89E+07	0.49
35% EtOH	Liquid	10	21	1.09E+03	4.34
35% EtOH	Liquid	10	50	2.36E+03	4.00
50% EtOH	Liquid	10	21	1.45E+02	5.22
50% EtOH	Liquid	10	50	2.00E+01	6.08
Autoclave		20	120	<200	ND

Abbreviation: ND, means that cannot be determined

The antifungal efficacy seemed to be enhanced when ethanol was heated. At 35% concentration, the improvement was not greatly different whereas, at 50% concentration, the removal efficiency of the heated sample was 1-log higher than the one at room temperature i.e. 21.0 ± 1.0 °C. The results are quite aligned with one research by Smilanick et al. (1995), in which the concentration of green mold in lemons was approximately 27% under treatment of 40% EtOH at 32.0 °C and reduced to less than 5% once the temperature rose to 50.0 °C. Yet, the reference experiment shows one conflict with the thesis regarding the impact of EtOH concentration on removal efficiency. Particularly, the authors stated that there was no significant improvement in mold control when the EtOH control exceeded 10% at between 44.0 and 50.0 °C (Smilanick et al., 1995, p. 745), whereas in this experiment, the removal efficiency of 50% EtOH was 2-log higher than that of 35% EtOH at the same heat, 50.0 °C.

On the other hand, the pretreatment with hydrogen peroxide was not that effective as there was only the sample with a treatment duration of 20 minutes obtained the required removal efficiency, ca. 3-log reduction. Previous research has shown that a 3% H_2O_2 solution was ineffective (0 to 1-log reduction) against 18 fungal strains in food after 10-min contact time at 20 °C (Martin & Maris, 2012, p. 1451). According to the authors, the poor fungicidal effects might come from low chemical concentration coupled with short contact duration. However, in this case, the samples with longer pretreatment time had no higher removal efficiency than the shortest with the same H_2O_2 concentration: while the time was doubled, the log reduction was reduced by 50%; 60-minute and 120-minute samples were more satisfactory than the 40-minute one, yet they both had weaker effect than that of 20-minute. Plus, there was no significant gap between them, despite the time difference. This could be resulted from the high moisture content of the original samples that led to condensation, causing H_2O_2 concentration to decrease quickly. In other studies, such as those of Meng et al. (2019) or Cerioni et al. (2013), the fungicidal activity of H_2O_2 towards fruit decay (citrus fruits) was found at 2% within 2-minute treatment at 25 °C, much lower concentration and shorter contact time than those of above experiments. These significant variations can stem from differences in biomass structure and mold spp., particularly the sorghum side stream has been detected to contain at least 7 spp. and their distribution on the residue surface is possibly ununiform. By any means, it is radical to increase the H_2O_2 concentration for sorghum samples in the following screening.

The pretreatment with hydrogen peroxide vapor was the least effective, which could not even reach to 1-log reduction. The first guess for the reason behind this situation was

due to insufficient concentration of hydrogen peroxide. Therefore, for the second screening, the target concentration of H_2O_2 vapor inside the chamber was raised to 600 ppm, while the diffusion time remained the same. The second concentration value was based on the recommendation by Rasin (2021), at which the suggested range was 80 to 600 ppm. The upper limit value was selected concerning the diversity of molds presenting on sorghum residues as well as their great tolerance to surroundings. One advantage when using Cleamix VCS-100Cr decontamination units was that they consumed only around 30-50% of aqueous hydrogen peroxide solution to produce vapor that could eliminate most of the microorganisms at low concentrations and room temperature (Cleamix Oy, 2020, p. 2). Moreover, H_2O_2 gas left no residues on the treated sample and decomposed into hydrogen and water, so the samples did not need washing or drying after the pretreatment. Yet, compared with other methods, the antifungal efficacy obtained was not time-efficient: the fumigation took around 1 hour to operate but the removal efficiency could not reach 1-log reduction.

In autoclaved sample, there was no colony formed on all culture plates after the incubation, even on the first diluted one, leading to the undetermined value for log N_o/N_t. It was also noticed that the musty odor of molds disappeared. Consequently, it could be assumed that with mold concentration less than 200 CFU/g, the log reduction could reach 5 or more, equivalent to 99.999% and above. In fact, autoclave has been commercially applied in various industries to sterilize such as medical or laboratory equipment and results in the total elimination of microorganisms.

For the second screening, aqueous H_2O_2 concentration was raised to 5% while the contact times were reduced to 10, 20, 40 and 60 minutes. The main aim of this modification was to inspect whether higher concentration with shorter duration could achieve better antifungal efficacy than the previous test. In the cases with the addition of organic acids, the first screening produced quite satisfactory results when the contact time was solely 5 minutes. Hence, the concentrations of acid/H_2O_2 remained and the duration was doubled. Pretreatment methods with ethanol and autoclave did not need any change at that moment.

From Table 5, it can be seen that with 5% hydrogen peroxide, all samples with different contact times had reached above the required log reduction. The highest fungicidal effect, approximately 5-log reduction, was attained within 20 minutes of pretreatment. The effect started to decline when the contact time was prolonged, 4.38 for 40-minute and 4.22 for 60-minute samples. Similar results with the first screening had enhanced the above-mentioned explanation: the high moisture content of untreated residues might

cause H_2O_2 concentration to degrade rapidly, thus extended contact time had no considerable impact on removal efficiency. Based on two screenings, 20 minutes were assumed as starting point for the chemical to decay i.e., a decrease in molecules of H_2O_2 to react with the materials.

Table 5. *Antifungal efficacy of methods with modifications*

Method	Application	Treatment duration (min)	Temperature (°C)	Mold concentration (CFU/g)	*E*
5% H_2O_2	Liquid	10	21	1.20E+03	4.30
5% H_2O_2	Liquid	20	21	2.00E+02	5.08
5% H_2O_2	Liquid	40	21	1.00E+03	4.38
5% H_2O_2	Liquid	60	21	1.45E+03	4.22
H_2O_2 + formic acid	Liquid	10	21	2.00E+02	5.08
H_2O_2 + acetic acid	Liquid	10	21	2.00E+02	5.08
35% H_2O_2	Vapor	240	25	3.33E+04	2.86

As expected, the data for hydrogen peroxide in combination with organic acids was quite consistent concerning two pretests. Particularly, when the contact time was doubled, the log reductions in both cases also increased to nearly 2 times more than those of the first screening i.e., for the formic acid/H_2O_2 sample, it was raised from 2.17 to 5.08 while in terms of acetic acid/H_2O_2, 2.66 went up to 5.08. It was noticed that the concentration of H_2O_2 in combination with acetic acid was also 5%, and the fungicidal effect of the compound was similar to the use of H_2O_2 only within 20 minutes. This might indicate that with the addition of acetic acid, the treatment duration could be shortened, resulting in more beneficial impacts on general operational time and expense.

It was noted that the original sample was quite problematic for the fumigation to be performed efficiently with 600 ppm of H_2O_2. To be specific, the high moisture content of the residues had caused condensation of the vapor, resulting in H_2O_2 concentration dropping rapidly, which is presented in Figure 15. The RH inside the sample bag was measured, which went up to 70% or more. Therefore, the treatment was extended to 4 hours while the amount of sample remained at 50 g. In addition, once the H_2O_2 concentration dropped to below 200 ppm, the gas generator was stopped while the ventilator suctioned

out all vapors. When the gas was emptied, the generator was started again. This process was repeated whenever the concentration was below 200 ppm, which was to regulate and maintain the H_2O_2 concentration at around 600 ppm as long as possible, optimizing the consumption of chemical solution. This activity also caused the fluctuation in chemical consumption e.g. during the first 45-minute period, 3 mL of solvent was used which 1 mL was consumed within 30 minutes. Once the chamber was emptied and the fumigation started all over again, the H_2O_2 consumption went up to 4 mL within 1.5 minutes and the vapor concentration accelerated from ca. 100 ppm to nearly 500 ppm in around 5 minutes. The highest peak the concentration could obtain was 635 ppm after 17 mL of H_2O_2 had been consumed, but it dropped really quickly i.e., by half within 4.5 minutes.

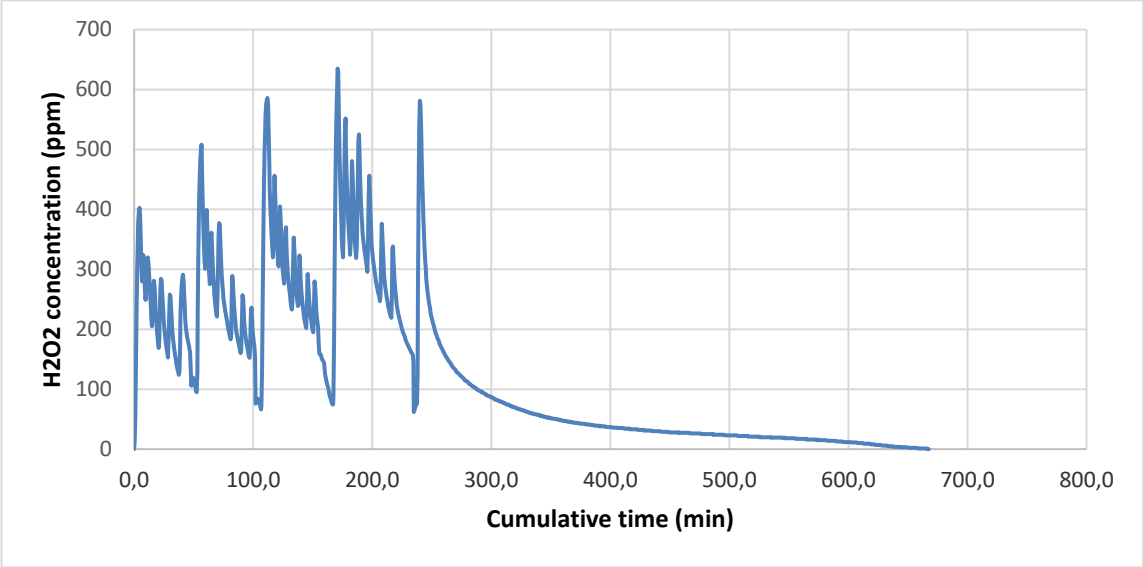

Figure 15. *Changes in H_2O_2 concentration during 4-hour gas treatment with 50 g of the original sample*

Applying equation (3), the average concentration of the chemical was ca. 126 ppm, which was much lower than the target concentration and that of performance in the first screening, ca. 200 ppm. This implied that further experiments might require extra heating/drying of residues prior to fumigation to reduce the moisture content as much as possible and elevate the efficiency. Otherwise, if the vapor pretreatment was to be utilized in reality, it was essential that the pretreatment must be implemented immediately after the gathering and drying of the biomass residues so that the materials had little time exposed to the surrounding atmosphere and absorbed moisture. Despite the prolonged treatment time, hydrogen peroxide vapor could still not accomplish the minimum removal efficiency i.e., 3-log reduction. Besides the concern of lengthy executing time yet poor fungicidal efficacy, the installment along with the expense of Cleamix VCS-100Cr decontamination units or similar facilities could be a severe issue for local farmers in Africa. What is more,

the energy consumed to operate the fumigation system in such extensive hours was considerably huge and costly. Therefore, it was concluded that the gas treatment with H_2O_2 was not suitable for samples used in the thesis and no further experiments should be progressed.

Although ethanol demonstrated potential antifungal efficacy towards sorghum side stream, the high concentrations (35 and 50%) experimented within the laboratory might be troublesome when applied on a larger scale. Particularly, the high composition of ethanol in the decontaminant implied that a great number of chemicals must be consumed when pretreating millions of tonnes of residues in reality. The regulation of EtOH temperature might also be tiring for the farmers to do manually with a such huge amount of side stream; otherwise, they needed to invest in technology to ensure efficiency, which resulted in higher operational expenses. Moreover, prolonged ethanol vapor inhalation might lead to intoxication, and any health issues related to the consumers should be avoided. Hence, EtOH was also excluded from further research in the thesis.

Because of the inconsistent distribution of molds on the original materials, the samples used for different methods might also contain distinct mold concentrations before the pretreatment. As a consequence, the data from the pretest was not concrete enough to conclude the removal efficiency of each method, which led to the repetition testing. It can be seen from Table 5 that it was not necessary to continue with long-time immersion with 5% H_2O_2, but rather concentrate on short-term processes. Additionally, it was crucial that the amount of control sample be increased for the experiment with autoclave since, with larger volume and mass, the steam might require a longer time to pass every corner of the residues.

6.3 Removal efficiency from replication

Five methods were selected for the repetition tests, namely 5% H_2O_2 with contact times of 10 minutes and 20 minutes, H_2O_2 in combination with organic acids (acetic and formic acids) and autoclave. Another 5 minutes were added to the treatment with acid/H_2O_2 to examine if these methods could reach higher removal, or the time factor did not pose any impact on the overall results. All the data are expressed as means ± standard deviation and summarized in Table 6.

The result shows that the value of the fungicidal effect in the case with 20-minute 5% H_2O_2 did not vary significantly among all replicates, taking the screening result into account as well. Specifically, the average log reduction was 4.66 ± 0.39 whose standard deviation was considered small. This indicates that the difference between each replicate

and mean value was not highly substantial i.e., the log reduction varied from ca. 4.3 to 5.0, equivalent to 99.99 and 99.999% respectively which is within the satisfactory range for removal efficiency.

Table 6. *Antifungal efficacy (log ± sd) of different treatments with H_2O_2 and autoclave*

Method	Application	Treatment duration (min)	Temperature (°C)	Mold concentration (CFU/g)	*E*
5% H_2O_2	Liquid	10	21	<200	ND
5% H_2O_2	Liquid	20	21	702 ± 320	4.66 ± 0.39
H_2O_2 + formic acid	Liquid	15	21	200 ± 0.00*	5.08 ± 0.00
H_2O_2 + acetic acid	Liquid	15	21	<200	ND
Autoclave		20	120	<200	ND

*The value of the only sample with mold was selected to be present in Table 6 in order to make a comparison with other results, which explained why the standard deviation for this method was 0.00.

On the other hand, the parallel samples of 5% H_2O_2 pretreatment in 10 minutes resulted in quite impressive antifungal efficacy: Of all 5 replicates, there was no presence of molds reproducing on any PDA-plate. Consequently, with the mold concentration in treated samples less than 200 CFU/g average, the log removal could not be determined specifically but it could be assumed that the fungicidal effect might fall between 5 to 6-log reduction, which was beyond the minimum limit according to European standards i.e. 3-log removal. However, the result relatively conflicted with that of the pretest. Particularly, the trial showed that in 10 minutes of 5% H_2O_2 immersion, the reduction achieved was about 4-log, which was less than 10-100 times compared with the repetition test. One reason could be that the sample used for the preliminary test might have been infected with contaminants from surroundings such as from the atmosphere or accidentally in contact with unsterilized apparatus. Uneven dispersal of molds was also considered related to the inconsistent data i.e. the residue parts for a trial test might consist of a larger amount of mold than the ones used during replication, thus affecting the reliability of the results.

In the case of hydrogen peroxide added formic acid pretreatment, there was only 1 out of 5 samples displayed mold colony, thus the other plates were assumed to carry less

than 1 colony which means that the concentration of mold was averagely less than 200 CFU/g. While the contact time was added 5 more minutes, the result shows no significant difference. The replicate with mold was similar to the pretest, which might indicate that a longer duration was unnecessary. On the other hand, other samples could not be specified with concrete numbers, so the removal efficiency can fall into 2 presumptions: it could reach up to 6-log reduction as known as 99.9999% or it would still be around 5-log reduction which was less effective 10 times than 6-log. In either circumstance, it would still exceed the requirement for removal limit.

In terms of hydrogen peroxide in combination with acetic acid, the result indicates that their removal mechanism might be slightly more effective than with formic acid. Particularly, no plates of all 5 parallel samples contained mold presence, implying that the average mold concentration was probably less than 200 CFU/g and the fungicidal effect varied between 5 and 6-log reduction, but relatively higher than that of formic acid/H_2O_2 solution because 1 colony of mold had been detected in its replication. Nevertheless, the little incidence of mold on PDA plates could also be interpreted that the samples being affected by a small amount of mold coming from the external environment e.g., the atmosphere. Thus, the samples might not carry any microorganism in the beginning, or in other words, the pretreatment with formic acid/H_2O_2 possibly obtained nearly absolute removal efficiency, just the same as with acetic acid. The difference between samples with two acids was not crucially visible, so further selection needs to be followed by other requirements besides the antifungal efficacy, e.g. price, availability, safety issues, etc.

From the results of autoclaved samples, the removal of mold spores from the residues was not influenced by the change in quantity, even though it was increased by 100 times i.e., from 5 to 500g. In fact, as long as the sample container was fit inside the chamber, the removal can work to its maximum efficiency. This was probably due to the high porosity of the materials which assisted the steam to penetrate deep into the core. Despite the lack of solid data to demonstrate the fungicidal effect, the autoclave can be concluded that the most efficient method for eliminating molds from the biomass side stream. The removal efficiency can be regarded as absolute i.e., 99.9999% or up to 100% which has been proved by the prevalent application in various industries, from the medical to the food sector nowadays.

6.4 Assessment of pretreatment methods

In summary, after two screenings, most of the methods had been selected for replication as their removal efficiency reached the required limit, except for the vapor pretreatment

with hydrogen peroxide. Meanwhile, ethanol, in either concentration or temperature, had indeed shown a satisfactory impact on the mold colonies within the residues but it was not chosen for repetition test due to several factors, namely complicated implementation (monitor of temperature), considerable consumption of solvent along with possible intoxication to the farmers. Further modifications on ethanol pretreatment can be studied to solve these issues, such as testing with smaller ethanol concentrations or designing a system with good ventilation to prevent health hazards. However, these studies require extra time and budget, which is out of the scope of the thesis, so no further research on ethanol application on the sorghum side stream was implemented at that moment.

Table 7. *Evaluation of pretreatment methods for final selection*

Method	Removal efficiency	Applicability	Affordability	Sustainability
5% H_2O_2 in 10 min	≥ 5*	Easy access to the chemical Simple preparation, setup, and implementation Wastewater can be collected by local waste management companies since H_2O_2 concentration is not too high	Low-priced chemical and common equipment	H_2O_2 decomposition is eco-friendly Low energy consumption (drying step can utilize sunlight) Consumption of water might be problematic and unethical in rural areas => requires medium at village or higher level to assist
5% H_2O_2 in 20 min	≥ 4	Same as above		Longer contact time might require longer heating => higher power needed than previous method H_2O_2 waste is eco-friendly Issue with water consumption (same as above)
H_2O_2 + formic acid	≥ 5	Both chemicals are easily accessible Simple setup and implementation. Yet, the preparation of solvent is slightly more complex than with H_2O_2 alone Wastewater might require further study before disposal	Affordable price for chemicals and equipment Additional price for research of wastewater management (if needed)	H_2O_2 and formic acid are eco-friendly Low energy for drying; sunlight can be applied Water consumption needs further study and modification if required
H_2O_2 + acetic acid	≥ 5*	Same as with formic acid		
Autoclave	≥ 5*	Large capacity sterilizers are available at institutional/industrial scale High pressure cooker can act as a small-scale autoclave for domestical application	Equipment, energy, and water price can cause burden on the overall budget	Energy and water consumption of autoclave can be an issue, otherwise no chemical needed => no harmful emission Use of pressure cooker is not efficient due to small capacity => time, energy and water consumption

*The log reduction value is assumed based on the interpretation of replication results

For the replication, all selected methods exceeded the mandatory limit for the level of mold elimination i.e., the average log reduction reached above 4 or equal to more than 99.99% of removal efficiency. Therefore, the most prominent method is determined based on the following criteria apart from the fungicidal effect, considering African backgrounds: applicability, affordability, and sustainability, which have been described in Table 7.

Theoretically, with 5% H_2O_2 immersion in 10 minutes, the pretreatment will produce the most efficient result in all aspects concerned. However, the experimental antifungal efficacy is not completely precise which requires other methods to determine the number of molds from the residues, possibly with a technology that can detect less than 1 colony per plate or 200 CFU/g. On the other hand, the most consistent result is from pretreatment with 5% H_2O_2 only in 20 minutes, although its log reduction is the lowest among the 5 replication tests. According to Martin and Maris (2012), H_2O_2 used in this thesis was found fungicidal at 5%, which falls within the MFC range for H_2O_2 i.e. 0.39 to 12.5%. Considering the similarities in contact time and temperature (10 minutes and 20 °C in the experiment of Martin and Maris), the reliability of data obtained in the thesis is enhanced, proving that 5% H_2O_2 dipping for 20 minutes is possibly efficient as agricultural side stream pretreatment.

Thanks to the low concentration of hydrogen peroxide used, it is expected that the wastewater from H_2O_2 methods can be disposed of into the municipal sewage system. Yet, considering the living conditions in rural areas where the farmers have limited access to waterbodies, a medium at the community level is essential to assist them with residue pretreatment and wastewater collection. On the other hand, H_2O_2 leaves nothing once reacting with water, so the wastewater is assumed to only contain the dead biomass from the fungi. Thus, the wastewater can be directly disposed into the soil or work as fertilizer for plant irrigation. This assumption, however, needs further analysis to verify. Additionally, this method includes quite simple preparation of solvent and the overall operation can be handled even by the farmers who have little knowledge of chemistry or microbiology. The water consumption for dilution might be a problem, but wastewater is possibly treated and recycled for several uses. Overall, the decontamination method with H_2O_2 only is the most suitable process for agricultural side stream pretreatment in Africa, regarding the experimental results and assessment.

7. CONCLUSIONS

In this thesis, based on a summary of possible technologies for removing mold from agricultural side stream, a list of the most promising processes for African locals has been selected for experimental analysis. From then, throughout preliminary and replication examinations, a simple yet effective pretreatment method for residues has been successfully investigated, while fulfilling certain criteria concerning African conditions.

According to the pretest, steam sterilization with autoclave showed the greatest elimination as there was no presence of mold after 7-day incubation, assuming out of all experiments, this method can achieve closet to 100% of removal efficiency. Followed by that, ethanol with 50% of concentration at 50 °C could reach 6-log reduction which is equal to 99.9999%. However, ethanol had been excluded after first screening due to large consumption of chemical and possible intoxication. On the other hand, all experiments with H_2O_2 needed modification as only one result showed the removal efficiency of 3-log.

In either case of 3 or 5% H_2O_2, the contact time of side stream with H_2O_2 from 40 minutes onwards caused no enhancement on the overall antifungal efficacy i.e., the log reduction of 40-minute sample was 4.38 while that of 60 minutes was slightly decreased to 4.22. Instead, with 20 minutes or less, the removal efficiency that could be obtained was up to 5-log or 99.999%. With the addition of organic acids, the results were slightly equivalent while the contact time was 5 minutes shorter. On the contrary, the vapor pretreatment with H_2O_2 could not be implemented with planned concentration i.e. 600 ppm due to the high moisture content of residues, thus the removal efficiency did not meet the requirement and the method was removed from replication.

Based on the repetition results, the pretreatment with 5% H_2O_2 only in 20 minutes and with autoclave show the most consistent (with screening results) and reliable data. Yet, the operation of H_2O_2 immersion is considered less complex and pricy than that of steam sterilizer at domestic scale thanks to low-cost chemicals and apparatus. Additionally, H_2O_2 leaves no harmful residues on the side stream and its wastewater can be possibly released into the surroundings directly or collected to municipal wastewater treatment plants via a third party. Overall, concerning all aspects, this method is selected to be implemented in practice. In the following phase, its effect on side stream quality such as its durability, tensile strength, etc. will be studied. In addition, the wastewater composition is also inspected whether it is safe to be directly disposed into the environment, based on international as well as African environmental regulations.

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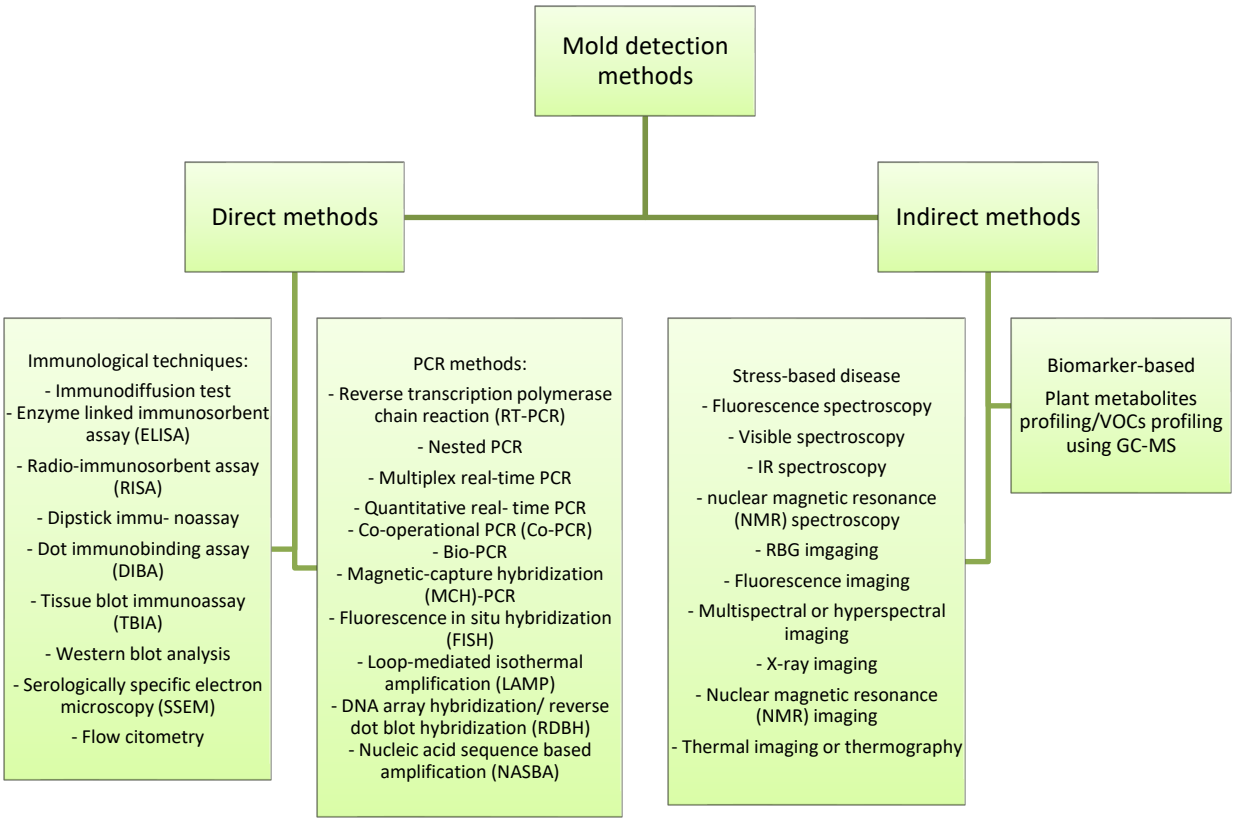
APPENDIX A: AVAILABLE ADVANCED METHODS FOR MOLD DETECTION (ADAPTED FROM RAY ET AL. 2017 WITH PERMISSION FROM ELSEVIER)



APPENDIX B: ANTIMICROBIAL PHYTOCHEMICALS OF SEVERAL PLANT EXTRACTS (NEGI 2012)

Family	Latin name	English Name	Part (s) used	Phytochemicals
Araceae	*Amorphophallus campanulatus*	Olkachu	Root	Amblyone
Aristolochia-ceae	*Aristolochia bracte-ata*	Aristolochia	Root	Aristolochic acid
Bignoniaceae	*Jacaranda mimosifo-lia*	Jakaranda	fLowers/ leaves	Flavones, flavonoids iridoids, triterpenes
Bixaceae	*B. orellana*	Achiote	Leaves/ seeds	Alkaloids, tannins, flavonoids, terpenoids, phenolics, glycosides
	B. pilosa	Hairy beggar ticks	Leaves/ flower	α-pinene, β-pinene, β-myrcene, limonene, oci- meme, terpineol, elixine, cubebene, cary-opyllene
Combreta-ceae	*Terminalia citrine*	Citrine	Fruit	Tannins
Elaeagnaceae	*Hippophae rham-noides* L.	myrobalan Seabuckthorn	Berries	Flavonoids, tannins, triterpenes
			Leaves	Isorhamnetin, quercetin
			Seeds	Phenolics
Guttiferae	*Garcinia* spp., *G. in-dica, G. combogia G. guttifera, G. man-gostana G.subellip-tica; G. diocia*	Kokum	Fruit	Garcinol, α-mangostin, xanthones and xan-thone derivatives, xanthochymol rubraxan-thone
Lamiaceae	*Mentha* spp., *M. piperata, M. longifolia*	Pudina	Leaves	Flavonoids, menthol, terpenoids
	Plectranthus spp. Plectranthus am-boinicus (Lour) Spreng (syn *Coleus ambonicus* Lour or *Coleus aromaticus* Benth)	Indian Borage	leaves	Phenolics, flavonoids, catachol, saponins, steroids, terpenoids, tannins, anthraquinone
	Marrubium vulgare L.	Horehound	Whole plant	Caryophyllene oxide, trans-caryophyllene, mar-ruboside, α-sitosterol, vulgarol, lupeol, marru-biin
Lauraceae	*Cinnamomum* spp.	Cinnamon	Bark/ Leaves/ Root bark/ Fruit	Cinnamaldehyde, flavan-3-ols, *p*-coumaric acid, Eugenol, Camphor *α*-pinene, *β*-pinene, *β*-caryophyllene, γ-cadinene
Liliaceae	*Allium cepa* L.	Onion	Bulb	Saponins, kampferol, ferulic acid, *β*-sitosterol, myritic acid, prostaglandins, quercetin, alkenyl cysteine sulfoxide
	Allium sativum L.	Garlic	Bulb	Allicin, diallylisothiocyanate, allicin, dial-lyl disulfide, diallyl trisulfide, alkenyl cysteine sulfoxide, ajoene, methyl allyl thiosulfinate
Lythraceae	*Lythrum salicaria* L.	Purple loosestrife	Herb	Flavanoids, phenolics,tannins, phthalates, sterols, terpenes, vescalagin, oleanolic acid, ursolic acid
Meliaceae	*Azadirachta indica* A.	Neem	Bark	Azadirachtin, margosic acid, nimbin, nimbinin, nimbidin, nimbolide, gedunin, gallic acid, epi-catechin, catechin, mahmoodin, margolone, margolonone,
Moraceae	*Ficus religiosa* L.	Pipal	Leaves	Bergaptol and bergapten
Myrtaceae	*Syzgium aromaticum* L.	Clove	Bud	Eugenol, eugeniin, acetyl eugenol, quercetic acid, gallic acid, vanillin
	Eugenia sp	Cherry of the	Fruit	Catechin and epicatechin
	Plumbago zeylanica	Rio Grande White	Root	

Plumbaginaceae	L.	leadwort		Plumbagin, seselin, 5-methoxyseselin, suberosin, xanthyletin, ß-sitosterol
Poaceae	*Avena sativa* L.	Oat	Seed	Phenolic acids Class I chitinases
Polygonaceae	*Polygonum hydropiper* L.	Smart weed	Whole plant	Flavones and flavonoid glycosides, sesquiterpene acid, viscosumic acid
Punicaceae	*Punica granatum* L.	Pomegranate	Rind	Anthocyanins, ellagic acid, gallotannins, gallic acid, Punicalagin, ellagitannins
			Husk	Punicalagin and its isomers, punicalin, ellagic acid
			Seeds Heartwood Leaves	Punicic acid Ellagitannins and gallotannins Tannins (gallo and ellagitannins)
Ranunculaceae	*Nigella sativa* L.	Black cumin	Seeds	Glucoside, melanthin, saponin, thymoquinone
Rosaceae	*Filipendula vulgaris* Moench (syn. *Filipendula hexapetala* Gilib. ex Maxim.)	Dropwort	Leaves	Tannins, phenolic acids, flavonoids
	Rubus chamaemorus L.	Cloudberry	Leaves	Flavonoids, ellagic acid, tannins, gallic acid and their derivatives
Rutaceae	*Citrus reticulata*	Blanco Coorg mandarins	Rind	Polymethoxylated flavones
	Citrus paradisi	Grapefruit	Rind	Coumarins, flavanones, methoxyflavones
Sapindaceae	*Sapindus* sp.	Soapnut	Fruit	Acetylated triterpenes, saponins
Vitaceae	*Vitis vinifera* L.	Grape	Fruits	Catechin, epicatechin, rutin, Myricetin, *trans*-resveratrol, gallic acid, caffeic acid, ferulic acid, ellagic acid, quercetin
Zingiberaceae	*Curcuma longa* L.	Turmeric	Rhizome	Curcumin, turmerone ar-turmerone, curlone, trans-β-farnesene, α-Zingiberene, β-bisabolene

APPENDIX C: COMMON EXTRACT OILS (EO) THAT EXHIBIT ANTIMICROBIAL ACTIVITIES (BURT 2004)

Common name of EO	Latin name of plant source	Major components	Approximate % Composition
Cilantro	Coriandrum sativum (Immature leaves)	Linalool	26%
		E-2-decanal	20%
Coriander	Coriandrum sativum (seeds)	Linalool	70%
		E-2-decanal	–
Cinnamon	Cinnamomum zeylandicum	Trans-cinnamaldehyde	65%
Oregano	Origanum vulgare	Carvacrol	Trace-80%
		Thymol	Trace-64%
		g-Terpinene	2 – 52%
		p-Cymene	Trace-52%
Rosemary	Rosmarinus officinalis	a-pinene	2 – 25%
		Bornyl acetate	0 – 17%
		Camphor	2 – 14%
		1,8-cineole	3 – 89%
Sage	Salvia officinalis L.	Camphor	6 – 15%
		a-Pinene	4 – 5%
		h-pinene	2 – 10%
		1,8-cineole	6 – 14%
		a-tujone	20 – 42%
Clove (bud)	Syzygium aromaticum	Eugenol	75 – 85%
		Eugenyl acetate	8 – 15%
Thyme	Thymus vulgaris	Thymol	10 – 64%
		Carvacrol	2 – 11%
		g-Terpinene	2 – 31%
		p-Cymene	10 – 56%