Interim Report

Module-A. Disinfection efficacy of OptiMaser Microwave System.

Objective-2. Optimization of Rice Husk disinfection by OptiMaser.
Work Package- WP2.1-WP2.3

Introduction

Several types of mouse bedding are commercially available. Factors that are considered when selecting laboratory mouse bedding include animal health and comfort, cost, effects on personnel, and bioactive properties. Corncob bedding is economical and facilitates low intricate ammonia. However, neither mice nor rats prefer corncob over aspen, and corncob influences outcomes in certain types of research. Corncob contains tetrahydrofurandiols, linoleic acid derivatives with estrogenic properties, which disrupt mating behaviour in rats and confound some neuroendocrine studies. In addition, the Mycotoxin Zearalenone has been found in commercial corncob and leads to delayed vaginal opening in mice.

Rice hulls are the outer husk of rice that is removed during processing. Despite some industrial uses as biofuel and livestock litter, rice hulls are a relatively low-value side product of the rice industry. Rice hulls are used as bedding for pet rodents and in laboratory animal facilities in India. Rice husk provides several benefits as animal house bedding such as:

- Insect resistant.
- Do not pack, therefore, do not require as much litter depth as other types of litter material – cover larger areas for same volume.
- Easy to spread, cake very little, and clean out in half the normal time.
- Aerate and mulch poultry litter, and will tend to move to the top as poultry droppings are added, making litter condition ideal and aiding in the maintenance of health.
- Cushion livestock against otherwise hard surfaces which results in less foot and leg troubles, and in poultry less breast blisters and more “A” grade poultry.
- Available throughout the year. Supply is not affected by seasonal factors. Consistent quality.
- Improved thermal insulation.

Rice husk is a by-product of the rice milling process and comprise 20% of the weight of the rice crop. The major components of rice husk are organic materials such as hemicellulose, cellulose and lignin totalling about 75 – 90% and the remaining ash content of 17 – 20%. For disposal rice husk cannot be incinerated as the ash, mainly consists of >90% silica and some metallic impurities and could be an environmental hazard. Animal house rice hulls could be ideal for composting and improving soil structure.

It is imperative to decontaminate animal house bedding before disposal. The animal house manure may contain high concern endocrine-disrupting compounds (EDCs) such as steroid hormones, rodents, such as mice and rats, can leave droppings that can spread bacteria, contaminate food, and cause allergic reactions. Microwave technology provides an efficient and quick solution to the animal bedding decontamination.

Material and Methods

A. Microorganisms used in the study

- Bacteria
    - *Staphylococcus aureus*
    - *Escherichia coli*
- Spores (*Bacillus subtilis*)
- Fungi
    - *Candida albicans*
    - *Candida glabrata*

B. Sample preparation

1. Used animal house rice husk bedding was procured from CSIR-IITR rodent animal house.
2. Husk was packed into autoclavable biohazard plastic bags.
3. Water was added to these bags in 1:1 ratio (1kg Husk/ 1lit water)
4. Bacterial culture were grown in LB broth.
5. Fungal culture were grown in YPD (Yeast Extract-1%, Dextrose-2% and Peptone- 2%).
6. *Bacillus subtilis* sporulation was performed in sporulation media (SM). Composition of SM used was- Nutrient broth- 0.8%, KCl- 0.1%, $MgSO_4 \cdot 7H_2O$- 0.012%, pH adjusted to 7.6 with 1 M NaOH, volume adjusted to 1 litre with ddH_20. Autoclaved and allowed to cool to 50°C. Just prior to use, following sterile solutions were added: $Ca(NO_3)_2$- 1mM, $MnCl_2$- 0.01mM, $FeSO_4$-0.001mM. 0.5 ml of an overnight grown *B subtilis* culture was inoculated in liquid DSM media in Erlenmeyer flasks. Flasks were kept at 37 °C in an orbital shaker at 200 rpm for 7 days. Generation of spores was monitored microscopically. After 7 days of incubation > 90% of spore population was observed.
7. All cultures were maintained at 37°C.
8. Viable cell density of at least 10^{10} cells was maintained for bacteria and fungus.

C. Methodology

1. 1g husk was taken in sterile 10 ml screw capped glass tubes and 1ml of microbial culture (10^{10} cells) was added to it. The microbial load was determined by total aerobic plate count or spectrophotometrically at 600 nm wavelength.
2. Tubes were deeply embedded into the rice husk bags and the bags were sealed with autoclave resistant rubber bands.
3. Bags were arranged in two rows within the OptiMaser bucket namely top and bottom.
4. Following OptiMaser treatment 100µl of this culture was plated in triplicates on suitable medium as untreated control.
5. For calculating the disinfection efficacy (Log reduction) total aerobic plate count procedure was be followed.
6. For determining the rice husk disinfection efficacy, each organism was subjected to OptiMaser treatment within rice husk matrix at 100°C for two different time points (10 min and 30 min) (**Fig.1**).
7. For statistical significance disinfection efficacy of OptiMaser for each time point was determined using three biological replicates for each organism.
8. After OptiMaser treatment serial dilutions were prepared from each sample (up to 10^{-5}) and 100 µl sample of 10^{-1}, 10^{-3}, and 10^{-5} dilutions were plated in triplicates on suitable medium and incubated at 37°C for 48 h followed by calculation of log reduction (**Fig.1**).
9. As per 2016 guidelines of Biomedical waste management, *Bacillus atrophaeus* spores (10^4 spores) should be used as biological indicator for monitoring sterilization process. We used self-contained vials from gke for confirming the disinfection process in rice husk load.
10. Following OptiMaser cycles were used for this analysis:

    Sprinkle time- 5 sec

    Filament time- 90 sec

    Microwave on time- 300- sec

    Microwave Off time- 120 sec

    Temp hold time- 6**00- 1800** sec

    Cooling mode time- 400 sec

    Water draining time- 0

    Temp. Thresh hold- **100** °C

    Printing numbers- 6

Results

Disinfection efficacy of Bacteria:

1. OptiMaser bacterial disinfection efficacy in Rice Husk matrix was tested using representative gram negative (*Escherichia coli* K12) and gram positive bacteria (*Staphylococcus aureus*).
2. Bacteria was exposed to OptiMaser at 100°C for 10 and 30 min respectively.
3. It was observed that at 10 min OptiMaser treatment time the disinfection of bacteria was poor and only 4 log reduction was achieved in top row. The log reduction was very poor in the bottom row where only 2 log reduction was achieved for both gram negative and gram positive bacteria (Fig. 2).
4. After 30 min of OptiMaser exposure time a 10 log reduction was achieved in both top and bottom row for both gram negative and gram positive bacteria (Fig. 3).
5. Total aerobic plate count method was used for determine disinfection efficacy

Disinfection efficacy of Fungi:

1. OptiMaser Fungal disinfection efficacy in Rice Husk matrix was tested using representative pathogenic fungi *Candida albicans* and *Candida glabrata*.
2. It was observed that at 10 min OptiMaser treatment time the disinfection of Fungi was average with 6 log reduction in top row. However the log reduction was below average in the bottom row where only 4 log reduction was achieved for both *C. albicans* and *C. glabrata* (Fig. 2).
3. After 30 min of OptiMaser exposure time a 10 log reduction was achieved in both top and bottom row for both *C. albicans* and *C. glabrata* (Fig. 3).
4. Total aerobic plate count method was used for determine disinfection efficacy

Disinfection efficacy of Spores:

1. OptiMaser heat resistant bacteria spore disinfection efficacy in Rice Husk matrix was tested using representative *Bacillus subtilis* spores.
2. It was observed that at 10 min OptiMaser treatment time the disinfection of bacterial spores was very poor with only 2 log reduction in top row. However the spores were not killed in the bottom row (Fig. 2).

3. After 30 min of OptiMaser exposure time a 9 log reduction of bacterial spores was achieved in the top row whereas 4 log reduction was observed in the bottom row (Fig. 3).
4. Total aerobic plate count method was used for determine disinfection efficacy.
5. OptiMaser exposure at 100°C for 30 min was good enough to kill 10^6 *B. atrophaeus* spores, as observed by no change in colour of the growth media after 48hrs of incubation at 37°C. The colour of growth medium of untreated vial turned yellow within 8hrs of incubation at 37°C.

Conclusions

At least 6 log disinfection efficacy of representative bacterial and fungal culture in Rice Husk matrix was achieved via OptiMaser treatment at 100°C with a hold time of 30 min. A disinfection efficacy of 9 log reduction was achieved for heat resistant bacterial spores in the top row. Acceptable disinfection efficacy of bacterial spores (4 log reduction) was observed in the bottom row. Self-contained biological indicator having 10^6 *B. atrophaeus* spores was used for monitoring disinfection process. The spore vial was subjected to 30min OptiMaser exposure at 100°C along with rice husk load. No growth was recorded in the spore vial after 48hrs incubation at 37°C, suggesting successful disinfection process.

A

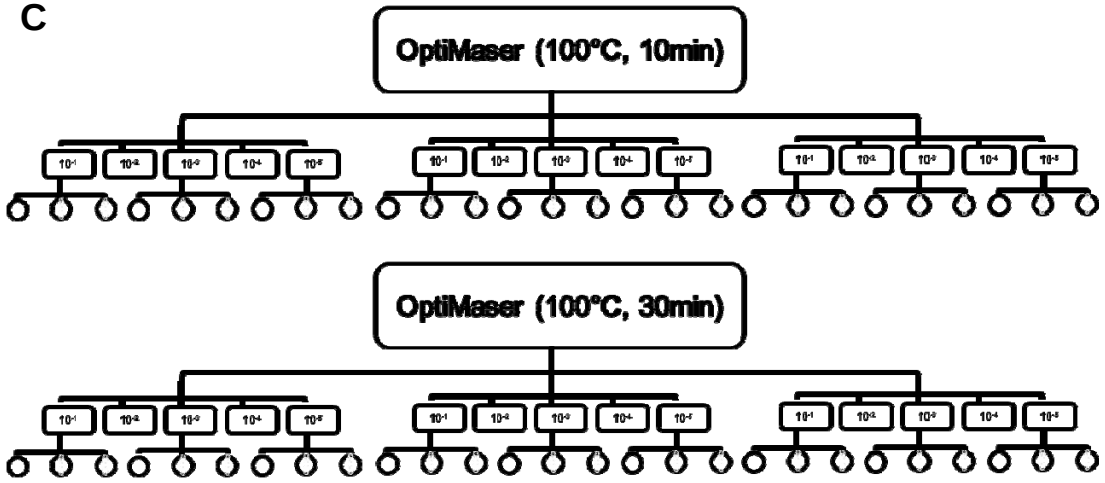
Microbes Tested

- Bacteria: *Staphylococcus aureus*
  Escherichia coli
- Spores: *Bacillus subtilis*
- Fungi: *Candida albicans*
  Candida glabrata

B

S.No	Temperature (°C)	Temp. Hold time (min)
1	100	10
		30

C



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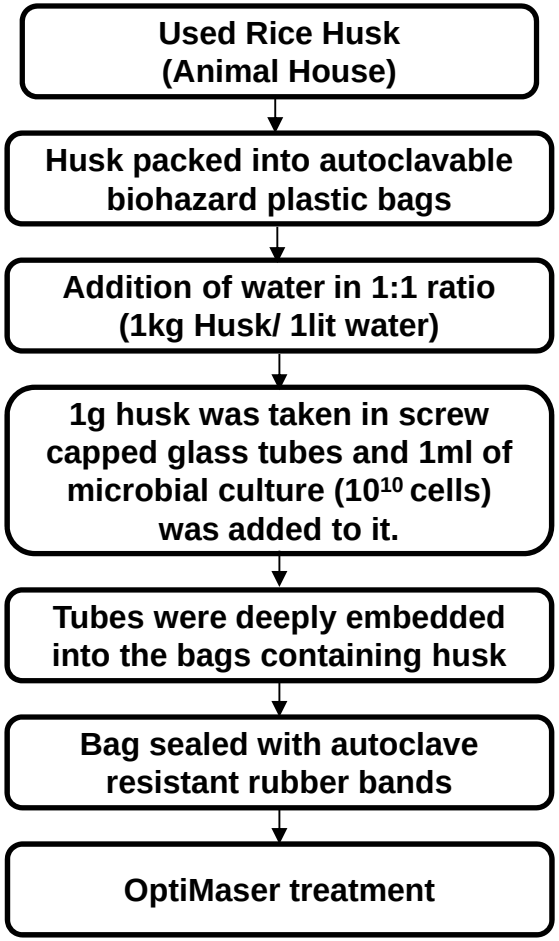
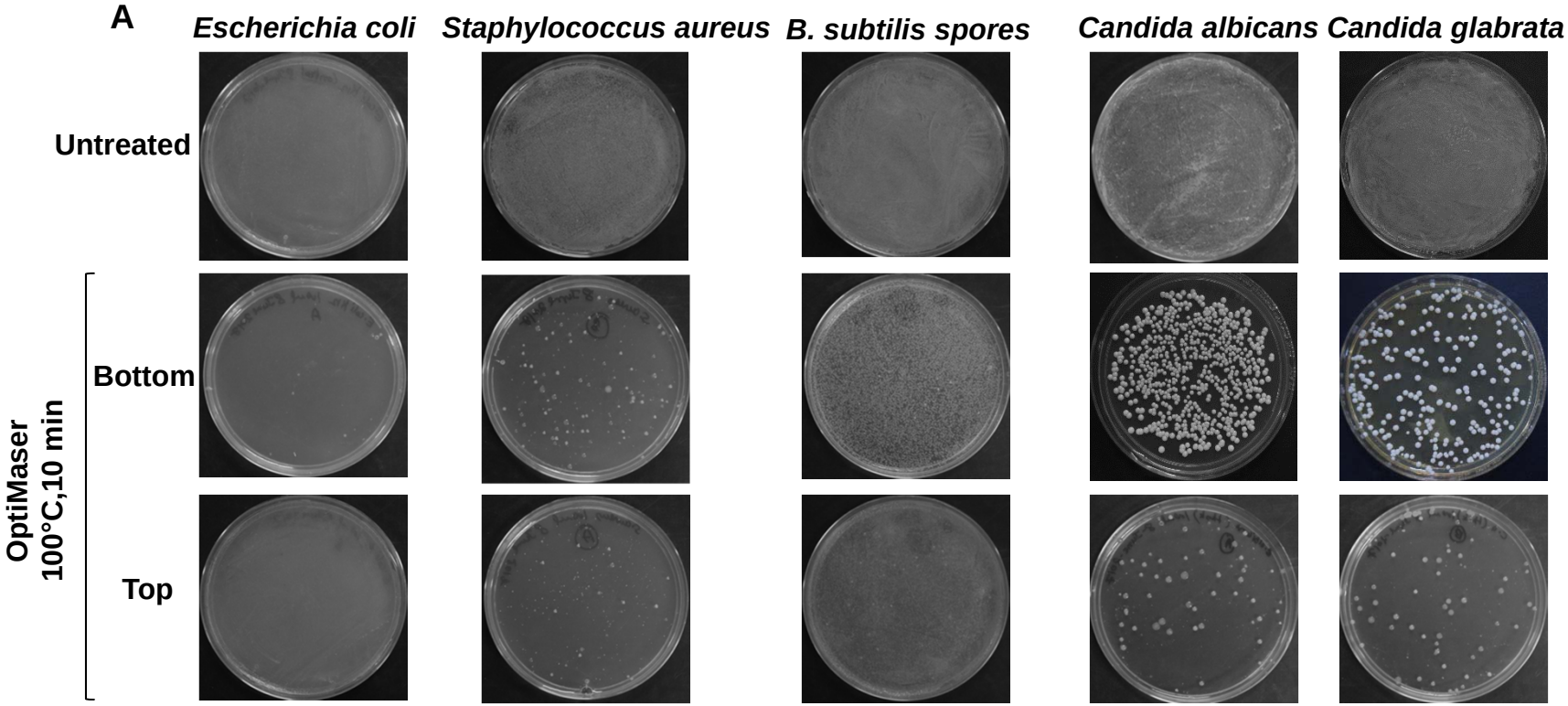


Figure 1: **Plan of study for determining the rice husk disinfection efficacy of OptiMaser-30 (2.45 GHz and 1.5 kW). (A)** Disinfection efficacy of OptiMaser was determined in rice husk matrix using representative bacteria, fungi and spores as indicated in the figure. (**B**) Due to complex matrix microbe disinfection efficacy was calculated at 100°C (two hold times). (**C**) For statistical significance three biological replicates of each samples were exposed to OptiMaser, following microwave exposure, samples were serially diluted and 10^{-1}, 10^{-3} and 10^{-5} dilutions were plated in triplicates on LB agar plates for total aerobic plate. **(D**) Brief methodology. Bacteria was grown in LB media at 37°C and 10^{10} cells were exposed to OptiMaser. Fungus was grown in YPD (Yeast Extract-1%, Dextrose-2%, Peptone- 2%) broth at 37°C and 10^{10} cells were exposed to OptiMaser. *Bacillus subtilis* spores were generated via magnesium sulphate method.

A



B

Temp. hold mode	Max . Temp. achieve	Total run time	Log reduction					
			Bacteria		Fungi		Spores	
			Top	Bottom	Top	Bottom	Top	Bottom
100°C for 10 min	100°C	49 min	4	2	6	4	2	-

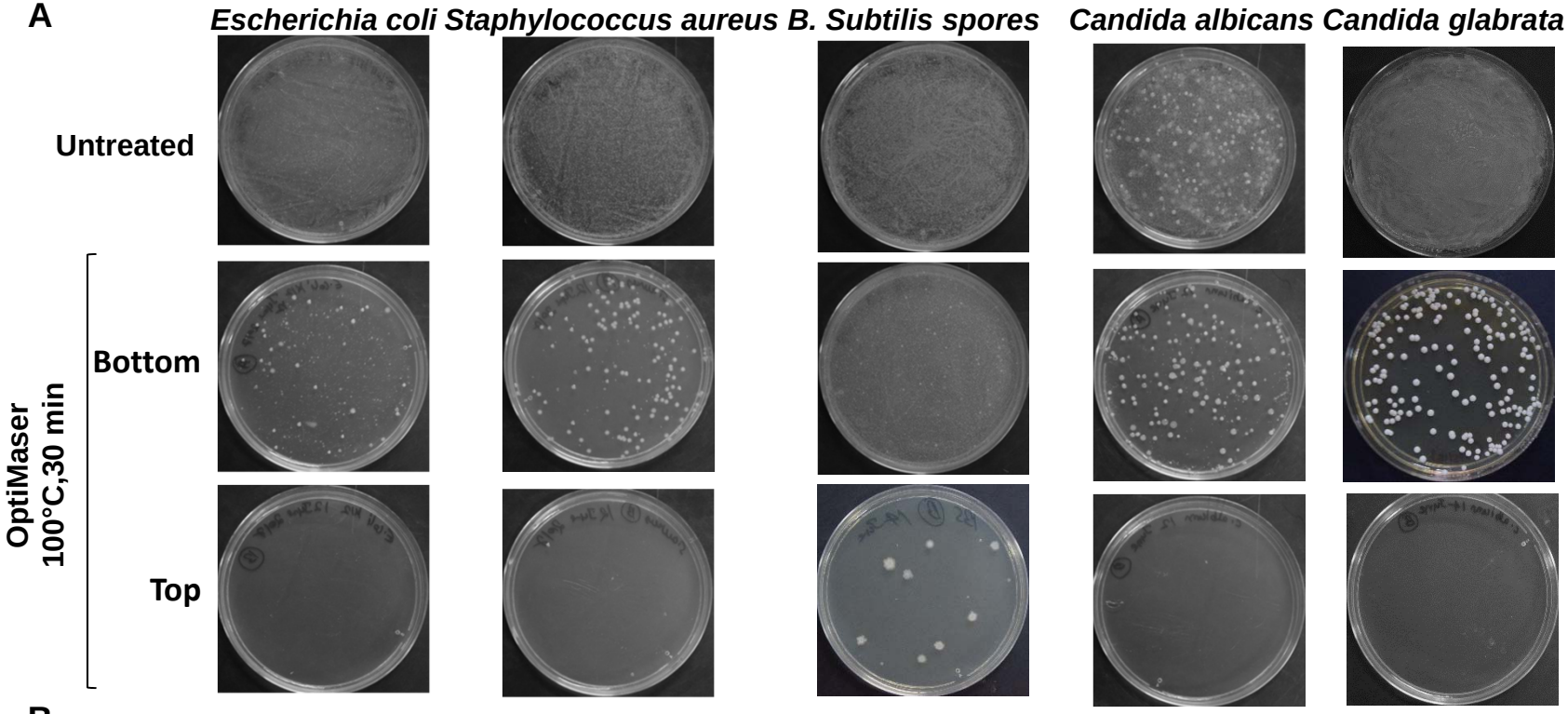
C

Log Reduction	% Reduction of bacteria
2	99
4	99.99
6	99.9999

D

Figure 2. Rice Husk Disinfection efficacy of OptiMaser at 100°C. (A) Representative plates for total aerobic plate count. (B) OptiMaser parameter at different hold times at 100°C and Log reduction in bacteria and Fungi upon OptiMaser treatment. (C) Relation between log reduction and % reduction. (D) Arrangement of husk bags in OptiMaser bucket. Bags were arranged in two rows namely top and bottom.

A



B

Temp. hold mode	Max . Temp. achieve	Total run time	Log reduction					
			Bacteria		Fungi		Spores	
100°C for 30 min	100°C	61 min	Top	Bottom	Top	Bottom	Top	Bottom
			10	5	10	6	9	4

C

Log Reduction	% Reduction of bacteria
4	99.99
5	99.999
9	99.9999999
10	99.99999999

D

Figure 3. Rice Husk Disinfection efficacy of OptiMaser at 100°C. (A) Representative plates for total aerobic plate count. (B) OptiMaser parameter at different hold times at 100°C and Log reduction in bacteria and Fungi upon OptiMaser treatment. (C) Relation between log reduction and % reduction. Bags were arranged in two rows namely top and bottom. D) Disinfection of 10^6 *B. atrophaeus* spores upon OptiMaser exposure at 100°C for 30min along with rice husk load. No change in colour of the medium after 48hrs of incubation at 37°C suggest complete disinfection. C, Untreated control; T, Treated vial.