Microbial Challenge Assay
Optima™ Steamer;
Brettanomyces dekkera.

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Efficacy of the Steamericas^{tm} Optima^{TM} Steamer for cleaning 3 surfaces challenged with *Brettanomyces dekkera*

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EXECUTIVE SUMMARY

3 test surfaces (plastic, glass and stainless steel) were inoculated with *Brettanomyces dekkera*. After initial protocol development, the surfaces were treated with the Optima^{TM} Steam cleaner. The results indicate that the Optima^{TM} Steamer effectively eliminates or kills viable *Brattanomyces dekkera* from plastic glass and stainless steel.

INTRODUCTION:

The Optima™ Steamer produced by SteAmericas™ Inc. is a portable steam-cleaning unit. The Optima™ Steam Cleaner superheats water, generating steam that is directed through the nozzle at the surface to be disinfected.

The purpose of this study is to determine if the Optima™ Steam cleaner can be used to disinfect 3 surfaces challenged with *Brettanomyces dekkera* (Brett). The surfaces selected were: 1. Plastic (Polystyrene), 2. Metal (Restaurant grade Stainless Steel), and 3. Glass (16 oz. Glass Tumblers).

MATERIALS AND METHODS

Microbes:

Brattanomyces dekkera (Dekkera bruxellensis ATCC catalog number 52905*)* was purchased from: American Type Culture Collection (ATCC)
10801 University Boulevard, Manassas, VA 20110, USA. Upon receiving, the bacterium was cultured according to supplier instructions. Initial cultures were separated into master stocks (both agar slants and frozen glycerol stocks). In addition, working stocks were streaked on the appropriate agar medium such that single colonies could be obtained. Prior to the start of an experiment, a single colony was grown for 3-7 days in Brett SD medium and used to produce a working stock. Working stocks used were late log phase with confirmed viability between 1 million and 10 million viable cells per milliliter.

Unless otherwise indicated, *Brettanomyces dekkera* was maintained in YM or Brett SD medium at 30°C with agitation.

Test Surfaces:

3 test surfaces were used:

1. Plastic (Polystyrene), Fisherbrand 60mm polystyrene Petri plates.
2. Metal (Restaurant grade Stainless Steel), "Metal By The Inch" Restaurant grade Stainless Steel, custom cut into 4inch by 4 inch square pieces. Edges slightly bent to fit into 150mm sterile petri dishes. 60mm circular inoculation target indicated with Sharpie pen.
3. Glass (16 oz. Glass Tumblers). "16 oz. Glass tumblers" Wal-Mart, Anaheim, CA. with inner surface diameter of approximately 55 mm.

Test surfaces were sterilized using a standard laboratory autoclave. After sterilization, test surfaces were challenged by addition of one milliliter of a late log phase *Brattanomyces dekkera* culture (approximately 10^6–10^7 CFU/ml). After surface inoculation, the culture was spread such that entire target area was covered. The test surface was then incubated for a minimum of one hour. Following incubation, excess culture media was removed by aspiration.

Test Armature:

A test armature was constructed from galvanized ¾ inch pipes, pipe clamps and standard scientific clamps. Different clamps were employed to hold the 3 different surfaces to be tested. When necessary, clamps were sterilized before use. The test armature was housed within the vented laboratory space next to the Optima™ Steamer. A large plastic tray and plastic backing were used to collect excess condensation and define the test area.

SANITIZATION PROCEDURES PROTOCOL DEVELOPMENT

Evaluation of Output Temperature:

A laboratory grade thermometer (Fisher Scientific) with temperature range -40°C to 120°C was clamped to the test armature. The boiler of the Optima^{TM} Steamer was pre-warmed for 15 minutes according the manufacturer instructions.* 3 operators conducted temperature studies. Operator one, wearing heat resistant gloves, face shield and laboratory coat held the steam gun, operator two wearing heat resistant gloves, face shield and laboratory coat held a metal ruler and Operator 3 held a stop watch. The 3 operators worked together to measure output temperature at different distances and durations.

* The boiler turned on and off intermittently during operation. It was later pointed out by the manufacturer that super-heated steam occurs only when there is continuous boiler operation, which occurs only after a different mode of operation than employed here.

1. **Preliminary Studies**

   Test groups treated with steam were treated using the following procedure, which was pre-determined in preliminary studies. The nozzle was held from 0-30 cm away from the plates for 5-30 seconds as described above.

2. After treatment, 5 mL of fresh YM liquid media is added to each plate and then the plates were sealed with parafilm and placed at 30C with rocking for 10 days.

3. After the incubation, 1 mL of liquid was removed and the OD 660 was measured using a spectrophotometer (BioMate 3 Thermo Spectronic) blanked with naïve YM medium.

A series of preliminary studies were performed in order to Optimize the variables of spray duration and distance. During these studies it was found that 30 seconds at distances ranging form 0-6 cm eliminated all microbes capable of growing in YM broth at 30°C. This finding was consistent with early findings for other microbes.

Protocol Definition:
Validation and additional optimization studies were conducted with at least 3 repeats at a nozzle distances ranging from 3-10 cm (typically 5 cm) and duration of 30 seconds. All other conditions were as described above. Several generations of validation studies were conducted by different operators. In some cases both Optical Density and plating efficiency were evaluated. Successful protocols and results are described below.

VALIDATION STUDIES AND FINAL STUDY PROTOCOLS

Procedure (Plastic)

Preparation of plastic surface prior to testing:

1. *Brettanomyces dekkera* was seeded in 150 mL of fresh Brett SD liquid media from a previous liquid culture and incubated at 30°C for 3-5 days.
2. On the day of testing, the stock culture was diluted from 1 to 10^{-7} and plated on LB plate to assess initial culture density.
3. A 1.5 mL of the stock culture was transferred to 150 mL of fresh YM media in sterile flasks (1:100 dilution) and mixed.
4. 6 mLs of the newly diluted culture was added to 6 cm petri plates and then incubated overnight.
5. After incubation, 90%+ of the liquid liquid culture was aspirated from plates and plates were immediately used for cleaning studies.

Cleaning Plates with the Optima™ Steamer

1. Plastic petri plates were cleaned as follows (developed during preliminary studies): The nozzle was held about 2-6 cm away from the plastic surface and cleaned for 30 seconds. The cleaning pattern was specifically: 10 seconds around the edge in the clock-wise direction, 5 seconds in horizontal zigzag pattern, 5 seconds in vertical zigzag pattern and the last 10 seconds around the edge in the counter clockwise direction.
2. After treatment, 6 mL of fresh YM liquid media was added to each plate and the plates were placed on rocker. In addition, on the day of testing, the stock culture was diluted from 1 to 10^{-7} and plated on Brett SD plates to confirm the initial culture density and viability.
3. After 1hr of mixing, 50 µL of the following dilutions from each plate was plated on YM plates: original and diluted 1:10 (in liquid ym).
4. YM plates were incubated at 30C temp for 14 days and resulting colonies were counted. After counting, CFU/ml was calculated.

Results (Plastic) Trial 1

Plate name	YM Original	1:10 Dilution
C1	TNTC	TNTC
C2	TNTC	TNTC
C3	TNTC	TNTC
C4	TNTC	TNTC
C5	TNTC	TNTC
C6	TNTC	TNTC
SA1	0	0
SA2	0	0
SA3	0	0
SA4	*1	0
SA5	0	0
SA6	0	0

C = control, SA = SteAmerica Optima™ cleaning as described, TNTC = Too numerous to count, 0= no colonies present, *1 Filamentous mold.

Results (Plastic) Trial 2

Name	Result
C1	TNTC
C2	TNTC
C3	TNTC
C4	TNTC
C5	TNTC
C5	TNTC
SA1	0
SA2	0
SA3	0
Sa4	0
SA5	0
SA6	0

C = control, SA = SteAmerica Optima™ cleaning as described, TNTC = Too numerous to count, 0= no colonies present

Conclusion (plastic)

This protocol effectively removed or all of the killed *Brettanomyces dekkera* that was on the plastic surface.

Procedure (Glass)

Preparation of the glass surface prior to testing:

6. *Brettanomyces dekkera* was seeded in 150 mL of fresh Brett SD liquid media from a culture and incubated at 30°C for 3-5 days.
7. On the day of testing, the stock culture was diluted from 1:100 in YM and plated on Brett SD plate to assess initial culture density.
8. 5 mL of the new diluted culture was added to sterile glass tumblers (16oz Walmart Brand) (Previously covered with tinfoil and sterilized in an Napco 900 Autoclave for 35 minutes, according to manufactures suggestions).
9. After an overnight incubation, 90% + of the liquid culture was aspirated from glasses and immediately used for cleaning studies.

Cleaning with the Optima™ Steamer

5. Inoculated glass tumblers cleaned as follows (developed during preliminary studies): The nozzle was held about 5cm away from bottom of the glass. The surfaces were cleaned for 30 seconds. The cleaning pattern was as follows: 10 seconds around the edge in the clock-wise direction, 5 seconds in horizontal zigzag pattern, 5 seconds in vertical zigzag pattern and the last 10 seconds around the edge in the counter clockwise direction. In some cases the exact pattern differed due to interference by a jet of steam emitted from the glass.
6. After treatment, 6 mL of fresh LB liquid media was added to each glass and the glasses were covered with tinfoil and then saran wrap to prevent evaporation.
7. The glasses were incubated at 30°C for 14 days. Following the 14-day incubation, the plating efficiency was determined (as described above).

Results (Glass) Trial 1

Condition	Plating Efficiency
C1	TNTC
C2	TNTC
C3	TNTC
C4	TNTC
C5	TNTC
SA1	0
SA2	0
SA3	0
SA4	0
SA5	0

C = control, SA = SteAmerica Optima™ Steamer as described (BAM), TNTC = Too numerous to count, 0= no colonies present

Results (Glass) Trial 2

Condition	Plating

	Efficiency
C1	TNTC
C2	TNTC
C3	TNTC
C4	TNTC
SA1	0
SA2	0
SA3	0
SA4	0

C = control, SA = SteAmerica Optima™ Steameras described (BAM), TNTC = Too numerous to count, 0= no colonies present

Conclusion (Glass)

Under this set of conditions, the Optima™ Steam cleaning effectively removed or killed all of the challenge organisms.

Procedure (Stainless Steel)

Preparation of the stainless steel surface prior to testing:

1. A 60 mm diameter circle was drawn in the center of each stainless steel plate. Plates were then covered in tinfoil and sterilized in the autoclave for 35 minutes following manufactures instructions.

2. *Brettanomyces dekkera* was seeded in 150 mL of fresh Brett SD liquid media from a previous liquid culture and incubated at 30°C for 8 days.

3. 1 mL of the culture was added to the center of the plate (within circle area).

4. After a 1-hour incubation, the liquid culture was aspirated from surface and plates were immediately used for cleaning studies.

Cleaning Plates with the Optima™ Steamer

5. Stainless steel plates were cleaned in the following manner (developed during preliminary studies): The nozzle was held about 2-6 cm away from the surface and cleaned for 30 seconds. The cleaning pattern was as follows: 10 seconds around the edge in the clock-wise direction, 5 seconds in horizontal zigzag pattern, 5 seconds in vertical zigzag pattern and the last 10 seconds around the edge in the counter clockwise direction. An additional 30 seconds of cleaning was used to clean the area around the test circle and an additional 30 seconds of cleaning was used to clean the back of the plates. Preliminary studies indicated that this extra cleaning was necessary to eliminate all microbial growth.
6. After treatment, plates were place in 150 cm sterile petri dishes and 40 mL of fresh YM liquid media was added to each plate and the plates were placed on rocker overnight.
7. Plates were put into plastic "zip-lock" bags and incubated for 14 days at 30C. Following incubation, 50 ul was aspirtated form test media and plated on 60mm YM agar test plates.

Results (Stainless)

Results (Stainless) Trial 1

Condition	Result
C1	TNTC
C2	TNTC
C3	TNTC
C4	TNTC
C5	TNTC
C6	TNTC
SA1	TNTC
SA2	0
SA3	0
SA4	TNTC
SA5	1*
SA6	1*

C = control, SA = SteAmerica Optima™ Steameras described (BAM), TNTC = Too numerous to count, 0= no colonies present, *1 Filamentous mold.

Results (Stainless) Trial 2

Condition	Result
C1	TNTC
C2	TNTC
C3	TNTC
C4	TNTC
C5	TNTC
C6	TNTC
SA1	0
SA2	0
SA3	0
SA4	0
SA5	TNTC
SA6	0

C = control, SA = SteAmerica Optima™ Steameras described (BAM), TNTC = Too numerous to count (same as start), 0= no colonies present

Results (Stainless) Trial 3

Condition	Result Trial 3
C1	TNTC
C2	TNTC
C3	TNTC

C4	TNTC
C5	TNTC
C6	TNTC
SA1	0
SA2	TNTC
SA3	TNTC
SA4	0
SA5	0
SA6	TNTC

C = control, SA = SteAmerica Optima™ cleaning as described, TNTC = Too numerous to count, (same as start), 0= no colonies present

Conclusion (Stainless)

Under this set of conditions, the Optima™ Steam cleaning effectively removed or killed the majority of the challenge organisms.

Summary of Results and Conclusion

Under this set of conditions, the Optima™ Steam cleaning effectively removed or killed all of the challenge organism from plastic, glass and mostly from the stainless steel surface.