Quality Assurance & Control Systems Ltd.,1 Antigonis St., 144 51, Metamorfosi, Greece
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STUDY REPORT
SUSPENSION TEST
ACCORDING TO EN 1650

Chemical disinfectants and antiseptics – Quantitative suspension test for the evaluation of fungicidal or yeasticidal activity of chemical disinfectants and antiseptics used in food, industrial, domestic and institutional areas
Test method and requirements (Phase 2 step 1)

TEST SUBSTANCE IDENTIFICATION

CERTIFICATE ID	:	2016-4035 / 16 23 00059 / EN 1650
PRODUCT NAME	:	ALCOMEDSEPT
PRODUCT TYPE	:	DISINFECTANT
ACTIVE SUBSTANCES AND THEIR CONCENTRATIONS	:	Ethanol
APPEARENCE OF THE PRODUCT	:	LIQUID
STORAGE CONDITIONS	:	ROOM TEMPERATURE, DARKNESS
LOT	:	L153712
METHOD	:	EN 1650
CONTACT TIME	:	15 minutes
DILUTION	:	AS IS
DILUENT RECOMENDED BY THE MANUFACTURER	:	POTABLE WATER
PRODUCT SUPPLIER	:	Σ. ΚΟΥΖΟΥΝΑΣ ΚΑΙ ΣΙΑ ΕΕ ALCOFARM MEDICAL
PRODUCT MANUFACTURER	:	Σ. ΚΟΥΖΟΥΝΑΣ ΚΑΙ ΣΙΑ ΕΕ ALCOFARM MEDICAL
RECEIPT DATE	:	6/5/2016
STUDY PERIOD	:	30/5/2016 - 4/6/2016
LAB ID	:	16 23 00059

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OBJECTIVE

The objective of this study was to demonstrate the fungicidal activity of the test material under the requirements of European Standard EN 1650 (at least 4 log reduction).

TEST SYSTEMS

Candida albicans	: ATCC 10231	-	LOT	4434841
Aspergillus brasiliensis	: ATCC 16404	-	LOT	3923205

TEST METHOD

European Standard EN 1650 Chemical disinfectants and antiseptics. Evaluation of fungicidal activity.

CONSERVATION

Candida albicans has been kept frozen; before its use it was transplanted on slants of Malt Extract Agar and kept in a refrigerator at 4°C ± 2°C.

Aspergillus brasiliensis has been kept in a refrigerator at 4°C ± 2°C on Malt Extract Agar.

PREPARATION OF THE YEAST SUSPENSION

Candida albicans strain has been twice consecutively transplanted on Malt Extract Agar slants and incubated at 30°C ± 1°C for 42-48 hours. Within two hours from the beginning of the test, the final culture has been suspended in a diluent using glass beads, and the suspension has been diluted to a count between 1.5 x 10^7 and 5.0 x 10^7 cfu/ml.

To make the count of test mycotic suspensions, dilutions 10^{-5} 10^{-6} in diluent have been prepared. A double count by inclusion in agar has been done. Dishes have been incubated at 30°C ± 1°C for 48 hours. At the end of the incubation period the number of cfu/ml has been determined and then the N value has been calculated.

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PREPARATION OF THE FUNGUS SPORES SUSPENSION

From maintenance slants using glass beads, adding 5ml of a sterile solution containing 0.05% of polysorbate 80, two dishes of Malt Extract Agar have been inoculated and incubated at 30°C ± 1°C for 5-7 days.

To each dish 10ml of a sterile solution with 0.05% of polysorbate 80 have been added a conidiospore suspension has been obtained.

After careful stirring, the suspension has been transferred in a sterile test tube and filtered to remove any existing mycelia.

Spores have been diluted to a concentration between 1.5×10^7 and 5.0×10^7 cfu/ml. To make the count of test mycotic suspensions, dilutions 10^{-5} and 10^{-6} in diluent have been prepared.

A double count by inclusion in agar has been done.

Dishes have been incubated at 30°C ± 1°C for 42-48 hours, at the end of this period uncountable dishes have been eliminated, the others have been incubated for additional 48 hours and, if necessary, for additional 20 -24 hours.

At the end of the incubation period the number of cfu/ml has been determined; then the N value has been calculated.

CULTURAL MEDIA AND REAGENTS

Malt Extract Agar (MEA)

Diluent

Casein Tryptone	1.0 g
NaCl	8.5 g
Distilled water q.s. to	1000 ml

Hard Water

Hard water consists of:

Solution A	6.00 ml
Solution B	8.00 ml
Sterile distilled water q.s. to	1000 ml

Solution A consists of:

Anhydrous $MgCl_2$	19.84 g
$CaCl_2$	46.24 g
Distilled water q.s. to	1000 ml
sterilized through a 0.45 µm filter	

Solution B consists of:

$NaHCO_3$	35.02 g
Distilled water q.s. to	1000 ml
sterilized through a 0.45 µm filter	

Interfering substance

The used interfering substance has been prepared with a concentration 10 times higher than the final concentration.

Bovine albumin	3 g SIGMA
Distilled water q.s. to	100 ml
sterilized through a 0.45 µm filter	

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EXPERIMENTAL DESIGN

Test temperature

The test has been conducted at 20°C ± 1°C.

Concentrations

The test substance has been not diluted.

Contact times

A contact time of 15 minutes has been used.

Interfering substances

A bovine albumin solution with final concentration of 0.3% has been used as interfering substance.

Neutralizer

The following neutralizer has been selected:

Lecithin	3 g
Polysorbate 80	30 g
Sodium Thiosulfate	5 g
L-histidine	1 g
Saponine	30 g
Tryptone-treated water q.s. to	1000 ml

ASSAY EXECUTION

Preliminary assay

A preliminary assay has been conducted prior to the execution of the assay. The assay sample, the mycotic suspensions and the interfering substance have been first stabilized at test temperature, while neutralizer and water have been stabilized at 20°C ± 1°C.

Count of the mycotic suspensions

Mycotic suspensions have been diluted to a concentration between 6×10^2 and 1.5×10^3 cfu/ml.

This suspension has been further diluted with decimal dilution and by inclusion in agar the number of colony -forming units of the suspension has been determined after incubation of *Candida albicans* dishes at 30°C ± 1°C for 48 hours; while *Aspergillus brasiliensis* dishes have been incubated at 30°C ± 1°C for 42-48 hours.

At the end of this period uncountable dishes have been eliminated, the others have been incubated for additional 48 hours and, if necessary, for additional 20-24 hours.

Then the Nv value has been calculated.

Preperation of test solution

The test substance has been diluted at the highest concentration tested during the assay.

Validation of experimental conditions

1ml of interfering substances and 1ml of mycotic suspension containing between 6×10^2 and 1.5×10^3 cfu/ml have been placed in a test tube.

The components have been left in contact for 2minutes at the temperature adopted during the assay and then 8ml of hard water have been added, left in contact for the longest period to be tested and at the temperature foreseen for the test.

At the end of the contact time, the mixture has been vortex -stirred and a double count has been performed by inclusion in agar.

Dishes of *Candida albicans* have been incubated at 30°C ± 1°C for 48 hours; dishes of *Aspergillus brasiliensis* have been incubated at 30°C ± 1°C for 42-48 hours. At the end of this period uncountable dishes have been eliminated, the others have been incubated for additional 48 hours and, if necessary, for additional 20-24 hours.

After the period the number of colony-forming units per ml of the mixture has been determined and the A value calculated.

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ASSAY EXECUTION (continued)

Validation of the neutralizer toxicity

For each test strain, 8ml neutralizer, 1ml of distilled water and 1ml mycotic suspension with concentration between 6 x 10^{2} and 1.5 x 10^{3} cfu have been mixed in a test pipe, left in contact at the temperature of 20°C ± 1°C for 15 minutes.

At the end of the contact time, the mixture has been vortex -stirred and a double count has been performed by inclusion in agar.

Dishes of *Candida albicans* have been incubated at 30°C ± 1°C for 48 hours; dishes of *Aspergillus brasiliensis* have been incubated at 30°C ± 1°C for ~ 48 hours, at the end of this period uncountable dishes have be eliminated, the others have been incubated for additional 48 hours and, if necessary, for additional 20-24 hours.

After the period the number of colony-forming units per ml of the mixture have been determined and the B value calculated.

Validation of the dilution neutralization

For each test strain, 1ml of interfering substances, 1ml of the diluent and 8 ml of the test substance at the highest tested concentration have be mixed in a test tube, left in contact at the temperature of 20°C ± 1°C, for the longest period foreseen by the test.

At the end of the contact time, 1ml the mixture has been transferred into a test tube containing 8ml neutralizer and left in contact for 2 minutes at 20°C ± 1°C, then 1ml mycotic suspension with concentration between 6 x 10^{2} and 1.5 x 10^{3} cfu/ml has been added, left in contact, at the temperature of 20°C ± 1°C, for 5 minutes.

At the end of the contact time, the mixture has been vortex -stirred and double count has been performed by inclusion in agar.

Dishes of *Candida albicans* have been incubated at 30°C ± 1°C for 48hours; dishes of *Aspergillus brasiliensis* have been incubated at 30°C ± 1°C for 48 hours, at the end of this period uncountable dishes have been eliminated, the others have been incubated for additional 48 hours and, if necessary, for additional 20-24 hours

After the period the number of colony-forming units per ml of the mixture has, been determined and the C value calculated.

Assay

The assay sample, the mycotic suspensions and the interfering substances have been first stabilized at test temperature, while neutralizer and water have been stabilized at 20°C ± 1°C.

The test substance has been prepared with a concentration of 1.25 times the requested test concentrations.

For each mycotic strain and for each concentration of the test substance a test pipe has been prepared containing 1ml of interfering substances and 1ml of mycotic suspension with a concentration between 1.5 x 10^{7} and 5.0 x 10^{7} cfu/ml at the temperature requested by the test.

After a contact period of 2 minutes, 8ml of test substance have been added and left in contact for the requested times and at the execution temperature of the test.

At the end of each contact interval, 1ml of the mixture has been transferred into a test tube containing 8ml of neutralizer and 1ml of distilled water. After 5 minutes of neutralization at 20 °C ± 1°C, the mixture has been vortex stirred and a double count has been performed by inclusion in Agar.

Dishes of Candida albicans have been incubated at 30°C ± 1°C for 48 hours; dishes of Aspergillus brasiliensis have been incubated at 30°C ± 1°C for 48 hours, at the end of this period uncountable dishes have been eliminated, the others have been incubated for additional 48 hours and, if necessary, for additional 20-24 hours.

After the period the number of colony-forming units per ml of the mixture has been determined and the Na value calculated.

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CALCULATION AND EXPRESSION OF THE RESULTS

Calculation of the mycotic count (cfu/ml)

For the final assay (Na) and for the preliminary assay (A, B, C e Nv) and for the test suspension (N), the calculation of mycotic count is performed in the following way:

$$cfu/ml = \frac{C}{n \times V \times d}$$

where:
C = total of colonies counted on both dishes number of counted dishes
n = number of counted dishes
V = volume used
d = dilution factor corresponding to the relevant dilution

The counting has been performed using the number of colonies counted on both dishes.

Only the dishes containing from 15 to 150 colonies have been used for the calculation of the results.

In the assay, where the number of cfu on every dish counted was <15, the number of cfu/ml should be recorded as $<1.5 \times 10^{2}$.

Where the number of cfu on every dish counted was >150, the number of cfu/ml should be recorded as $>1.5 \times 10^{3}$.

Calculation of vitality reduction

Vitality reduction has been calculated for each organism and test concentration using the following formula:

$$R = \frac{N \times 10^{-1}}{Na}$$

where:
R = vitality reduction
N = bacterial count of test suspension
Na = bacterial count of test mixture at the end of contact time

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ASSAY VALIDITY CRITERIA

Verify the following:

N: Must be included between 1.5×10^7 and 5.0×10^7.

Nv_0: Must be included between 30 and 160.

A,B,C: are equal to or greater than $0.5xNv_0$

where:

N = number of cfu/ml of the mycotic suspension

Nv_0 = number of cfu/ml in the mixture A, B and C at the beginning of contact time

A = number of cfu/ml of the solution in the control of experimental conditions

B= number of cfu/ml of the validation of neutralizer non toxicity

C= number of cfu/ml of the validation of dilution-neutralization

The test substance is considered fungicidal when for each mycotic strain at 20°C after 15 minutes of contact; it reduces vitality by at least 10^4.

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RESULTS

PRODUCT	:	ALCOMEDSEPT
DILUTION	:	AS IS
METHOD	:	EN 1650
STUDY PERIOD	:	30/5/2016 - 4/6/2016
LAB ID	:	16 23 00059

Preliminary assay

The N, A, Band C values of each mycotic strain comply with the validity criteria. The specific values are shown in Appendix No 1.

Test Microorganisms	N	Nv_0	A	B	C
Candida albicans ATCC 10231	10^{-6} : 40 -43 10^{-7} : 5-6 N = 4.3E+07	Vc = 87-96 Nv_0 = 9.2E+01	Vc = 79-83 A = 81	Vc = 84-90 B = 87	Vc = 65-60 C = 63
Aspergillus brasiliensis ATCC 16404	10^{-6} : 37-32 10^{-7} : 4-3 N = 3.5E+07	Vc = 101-92 Nv_0 = 9.7E+01	Vc = 105-103 A = 104	Vc = 100-92 B = 95	Vc = 55-62 C = 59

Nv_0 = Nv/10
N = Average number of mycetes (cfu/ml)
A = Average number of cfu/ml of the mixture mycetes + hard water + interfe substances
B = Average number of cfu/ml of the mixture mycetes + neutraliser
C = Average number of cfu/ml for the validation of neutraliser effectiveness
Nv = Average number of mycetes in the preliminary assay

Assay

Test Microorganisms	Contact Time	Vc	Na	**Vitality Reduction (R)**	**LOG Reduction**
Candida albicans ATCC 10231	15 minutes	10^{-1} : 0 10^{-2} : 0	< 1.5E+02	> 1.0E+04	> 4
Aspergillus brasiliensis ATCC 16404		10^{-1} : 0 10^{-2} : 0	< 1.5E+02	> 1.0E+04	> 4

Vc = number of mycetes per dish
Na = number of cfu/ml in the mixture mycetes + test substance in hard water + interfering substance
R = vitality reduction
N = Average number of mycetes (cfu/m l)
NC = Not countable

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CONCLUSION

TEST SUBSTANCE IDENTIFICATION

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ACTIVE SUBSTANCES AND THEIR CONCENTRATIONS	:	Ethanol
APPEARENCE OF THE PRODUCT	:	LIQUID
STORAGE CONDITIONS	:	ROOM TEMPERATURE, DARKNESS
LOT	:	L153712
METHOD	:	EN 1650
CONTACT TIME	:	15 minutes
DILUTION	:	AS IS
DILUENT RECOMENDED BY THE MANUFACTURER	:	POTABLE WATER
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On the basis of the results obtained in compliance with the assay validity criteria, the test substance **is** fungicidal against Candida albicans ATCC 10231 and **is** fungicidal against Aspergillus brasiliensis ATCC 16404 after 15 minutes of contact, in compliance with the provisions of EN 1650.

Signature Date: 4/6/2016

D. Melissos
Chemist MSc
Technical Manager

This document has been electronically signed by those names that appear on this report and are the authorized signatories.

The samples will be stored by the laboratory during 1 month from the end test date.The study report and raw data will be stored by the laboratory during 2 years.

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TEST SYSTEMS

Candida albicans	: ATCC	10231	-	LOT	4434841
Aspergillus brasiliensis	: ATCC	16404	-	LOT	3923205

METHODOLOGY ABSTRACT : A sample of the product is added to a test suspension of fungi (yeast cells or mould spores) in a solution of an interfering substance. The mixture is maintained at (20 ± 1) °C for 15 min ± 10 s (obligatory test conditions). At the end of this contact time, an aliquot is taken, and the fungicidal and/or the fungistatic activity in this portion is immediately neutralized or suppressed. The numbers of surviving fungi in each sample are determined and the reduction is calculated.

RESULT : On the basis of the results obtained in compliance with the assay validity criteria, the test substance **is** fungicidal against Candida albicans ATCC 10231 and **is** fungicidal against Aspergillus brasiliensis ATCC 16404 after 15 minutes of contact, in compliance with the provisions of EN 1650.

CONCLUSION : PASSES TEST

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