**Appendix C Method for determining the effective limit and maximum permissible content in paint/varnish (biotest)**

To determine the minimum quantity of the preservative preparation, the applicant shall conduct a test of the product’s fitness for use with at least 3 different concentrations of the preservative in accordance with the rules defined in the enclosed Annexes 4a-4c[[1]](#footnote-1). The applicant shall submit the results to the Federal Environmental Agency (Umweltbundesamt) Specialist Department III 1.4 for evaluation by BAM (Federal Institute for Materials Research and Testing) and the Federal Environmental Agency.

Annex 4a to the checklist:  
Carrying out a test of the product’s fitness for use using bacteria

The applicant shall carry out a test of the product’s fitness for use using the following method to determine the minimum quantity of the biocidal product[[2]](#footnote-2) [[3]](#footnote-3) required:

**A laboratory method for determining the concentration of a biocidal product that is required to combat bacteria in low-pollutant paints and varnishes according to DE-UZ 12a**

**1. Scope**

The method can be used to test how effective biocidal products are at preventing the growth and survival of damaging organisms in aqueous paints and varnishes.

**2. Health note**

National health regulations must be observed during the execution of the test. This also applies to EC Directive 200/54/EC “protection of workers from risks related to exposure to biological agents at work”. When handling paints/varnishes and biocides, the instructions in the corresponding safety data sheets and product information brochures for the safe use of the product must be observed.

**3. Instruments and nutrient media**

* Suitable sterile, screw cap containers (100 ml);
* Sterile measuring pipettes, nominal volume 1.0 ml and 5.0 ml;
* Sterile glass or plastic petri dishes, diameter 90 or 100 mm;
* Sterile diluent; e.g. distilled water (for agar) according to ISO 3696;
* Physiological saline (to rinse and dilute bacterial cultures);
* Scales;
* Pipette and 0.1 cm3 sterile tips;
* Bunsen burner;
* Incubator, thermostatically controlled (30°C +/2°C);
* Autoclave;
* Sterile inoculating loops or needles;
* Sterile spatulas;
* Sterile nutrient media for the corresponding micro-organisms, composition and production (see Annex 1);
* pH meter;
* Bacterial stock cultures;
* Test tubes;
* Test tube racks;
* Test tube shaker;
* Haemocytometer (depth 0.02 mm);
* Microscope.

**4. Test organisms**

The following bacteria should be used for the bacterial load test:

**Bacteria:**

*Alcaligenes faecalis* BAM 604 or DSM 6174 or ATCC 35655

*Escherichia coli* BAM 605 or DSM 787 or ATCC 11229

*Pseudomonas aeruginosa* BAM 60 or DSM 939 or ATCC 15442

*Pseudomonas putida* BAM 644 or DSM 291T or ATCC 12633

*Pseudomonas stutzeri* BAM 607 or DSM 5190T or ATCC17588

Other bacteria that continuously lead to infections can be additionally used in a separate inoculation suspension. It is necessary to carry out a molecular and biological characterisation of these organisms (which must be available as pure cultures) to clearly determine the bacterial strain being used. The organisms should be stored at a recognised culture collection.

**5. Method**

Inspect the materials received for testing before starting the test. To do this, use a sterile inoculating loop to spread the paint/varnish onto nutrient agar plates and check for any possible growth.

It is necessary to check both unaged samples and also artificially aged samples (see 5.2). The Pass/Fail criteria must be met in both cases (see 5.5).

**5.1** **Preparation of the inoculation suspension**

**5.1.1** Spread the test organisms taken from a continuous culture (e.g. cryo culture) onto nutrient agar plates and incubate them for 18 - 24 hours at 30 °C ± 2 °C\*.

**5.1.2** Use the bacteria from these plates to inoculate fresh nutrient agar plates (2nd subculture) and incubate them for 18 - 24 hours at 30 °C ± 2 °C\*. Only these cultures are used for the bacterial load test.

**5.1.3** Prepare separate suspensions for each bacterial strain by wetting the growing surface on the nutrient agar plates using a sterile diluent, e.g. physiological saline, and carefully wash off the bacterial growth using a sterile cotton swab.

**5.1.4** The number of organisms in each suspension is determined using a suitable haemocytometer (e.g. Thoma chamber, chamber depth 0.02 mm). The cell count of the individual bacteria suspensions should be

**1\*108 – 5 \*108 CFU/ml**

The prepared inoculation suspension must be used on the same day and should be kept in a refrigerator until used.

**5.1.5** In order to prepare a mixed suspension, combine and mix together identical volumes of each bacterial suspension. The cell count should also be

**1\*108 – 5 \*108 CFU/ml**

**5.1.6** Check the defined cell count for the mixed suspension by spreading an appropriately diluted mixed culture onto CASO agar and determining the number of colony-forming units.

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\* If the laboratory also uses its own test strains separately, the incubation time can be amended for these bacteria if necessary. The amended parameters used for testing the laboratory's own strains must be stated in the test report.

**5.2 Artificial ageing**

**5.2.1** Add the biocidal product to the paint/varnish at appropriate concentrations for the test series, vigorously mix the samples and store at room temperature for at least two days.

**5.2.2** Weigh out suitable portions (e.g. 50 g or 100 g) of the paint/varnish into sterile screw cap containers and close them tightly.

**5.2.3** Six non-preserved samples should be used as control samples. For this purpose, inoculate three samples with bacteria (positive control) and leave the remaining three samples uninoculated (negative control or retained sample).

**5.2.4** Store the samples for 4 weeks at 40 °C ± 2 °C or alternatively 18 weeks at 30 °C ± 2 °C.

**5.2.5** After the 4-week ageing process, carry out the bacterial load test as described in Paragraph 5.3.

**5.3 Bacterial load test**

**5.3.1** Inoculate each sample (except for the negative control) with the same volume of mixed suspension equivalent to 1.0 % of the sample weight. Mix the sample thoroughly using a sterile spatula and then screw the cap onto the container in such a way that oxygen can still enter the container.

**5.3.2** In order to determine the initial bacterial load for all samples, use an appropriate dilution series and spread the samples onto nutrient agar plates or use a pour plate method. Then incubate the plates at 30 °C ± 2°C for a maximum of 3 days\*. Use a suitable method to determine the number of colony-forming units.

**5.3.3** In order to check the vitality of the bacteria being used, spread and count the mixed suspension as described under Paragraph 5.3.2.

**5.3.4** Incubate all samples for 7 days at 30 °C ± 2°C.

**5.3.5** In order to determine the cell count in the samples, use an appropriate dilution series and spread the samples onto nutrient agar plates or use a pour plate method. Determine the colony-forming units after incubating the plates at 30°C ± 2°C for a maximum of 3 days\*.

**5.3.6** Repeat steps 5.3.1 to 5.3.5 at weekly intervals until at least 3 cycles have been completed. The test is concluded after a total incubation time of 6 weeks at 30 °C ± 2 °C. Determine the final number of colony-forming units.

**5.3.7** In order to determine the effectiveness of different biocidal concentrations depending on the incubation times, additional cell counts can be determined, e.g. 1 and 3 days after inoculation.

**5.4 Validity of the test**

The test is valid if the growth capacity of the inoculate can be verified on CASO agar in petri dishes (see 5.1.6).

**5.5 Pass/Fail criteria**

**5.5.1** The curative effect of the biocide in the paint/varnish at the added concentration

is verified if the cell count is ≤ 1000 CFU/ml at the end of the test.

**5.5.2** The effectiveness of a tested biocide cannot be verified if the cell count in the

biocide-free varnish after inoculation is < 1000 CFU/ml at the end of the test.

**5.6 Verification of a significant difference**

Use suitable statistical measures to verify any significant differences between the cell count in the samples treated with in-can preservatives and in the untreated samples.

**Annex 1**

**Nutrient medium**

**Nutrient agar CASO**

Typical composition (g/L)

Peptone from casein 15.0

Peptone from soybean 5.0

Sodium chloride 5.0

Agar 15.0

pH 7.3+/- 0.2

Preparation

Suspend 40 g of nutrient agar in 1 L of distilled water, stir until completely dissolved and then autoclave for 15 minutes at 121°C.

# Annex 4b to the checklist:

# Carrying out a test of the product’s fitness for use using yeasts

The applicant shall carry out a test of the product’s fitness for use using the following method to determine the minimum quantity of the biocidal product2 required:

A laboratory method for determining the concentration of a biocidal product that is required to combat yeasts in low-pollutant paints and varnishes according to DE-UZ 12a

**1. Scope**

The method can be used to test how effective biocidal products are at preventing the growth and survival of damaging organisms in aqueous paints and varnishes.

**2. Health note**

Before starting any test, ensure that national public health regulations and EC Directive 200/54/EC “protection of workers from risks related to exposure to biological agents at work” are being observed. When handling paints/varnishes and biocides, the recommendations in the corresponding safety data sheets and product information brochures for the safe use of the product must be observed.

**3. Instruments and nutrient media**

* Suitable sterile screw cap bottles (100ml);
* Sterile measuring pipettes, nominal volume 1.0 ml and 5.0 ml;
* Sterile glass or plastic petri dishes, diameter 90 or 100 mm;
* Sterile diluent; e.g. distilled water (for agar) according to ISO 3696;
* Physiological saline (to rinse and dilute

the yeast cultures);

* Scales;
* Pipette and 0.1 mlsterile tips;
* Incubator, thermostatically controlled (30 ± 2°C);
* Autoclave;
* Sterile inoculating loops or needles;
* Sterile spatulas;
* Sterile nutrient media for the corresponding micro-organisms,

composition and production (see Annex 1);

* Yeast stock cultures;
* Test tubes;
* Test tube racks;
* Test tube shaker;
* Haemocytometer (depth 0.1 mm);
* Microscope.

**4. Test organisms**

The following yeasts should be used for the yeast load test:

**Yeasts:**

*Candida boidinii* BAM 649

*Yarrowia lipolytica* BAM 641

*Candida valida* BAM 643

Other yeasts that continuously lead to infections can be additionally used in a separate inoculation suspension. It is necessary to carry out a molecular and biological characterisation of these organisms (which must be available as pure cultures) to clearly determine the yeast strain being used. The organisms should be stored at a recognised culture collection.

**5. Method**

Inspect the materials received for testing before starting the test. To do this, use a sterile inoculating loop to spread the paint/varnish onto nutrient agar plates and check for any possible growth.

It is necessary to check both unaged samples and also artificially aged samples (see 5.2). The Pass/Fail criteria must be met in both cases (see 5.5).

**5.1 Preparation of the inoculation suspension**

**5.1.1** Spread the test organisms taken from a continuous culture (e.g. cryo culture)

onto nutrient agar plates and incubate them for 24 - 48 hours at 30 °C ± 2 °C\*.

**5.1.2** Use the yeasts from these plates to inoculate fresh nutrient agar plates (2nd subculture) and incubate them for 24 - 48 hours at 30 °C ± 2 °C\*. Only these cultures are used for the yeast load test.

**5.1.3** Prepare separate suspensions for each yeast strain by wetting the growing surface on the nutrient agar plates using a sterile diluent, e.g. physiological saline, and carefully wash off the yeast growth

using a sterile cotton swab.

**5.1.4** The number of organisms in each suspension is determined using a suitable

haemocytometer (e.g. Thoma chamber, chamber depth 0.1 mm).The cell count of the individual yeast suspensions should be

**8\*107 – 2\*108 - CFU/ml**

The prepared inoculation suspension must be used on the same day and should be kept in a refrigerator until used.

**5.1.5** In order to prepare a mixed suspension, combine and mix together identical volumes of each yeast suspension. The cell count should also be

**8\*107 – 2\*108 - CFU/ml**

**5.1.6** Check the defined cell count for the mixed suspension by spreading and checking an appropriately diluted mixed culture.

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\*If the laboratory also uses its own test strains separately, the incubation time can be amended for these bacteria if necessary. The amended parameters used for testing the laboratory's own strains must be stated in the test report.

**5.2 Artificial ageing**

**5.2.1** Add the biocidal product to the paint/varnish at appropriate concentrations for the test series, vigorously mix the samples and store at room temperature for at least two days.

**5.2.2** Weigh out suitable portions (e.g. 50 g or 100 g) of the paint/varnish into sterile screw cap containers and close them tightly.

**5.2.3** Six non-preserved samples should be used as control samples. For this purpose, inoculate three samples with yeast (positive control) and leave the remaining three samples uninoculated (negative control or retained sample).

**5.2.4** Store the samples for 4 weeks at 40 °C ± 2 °C or alternatively 18 weeks at 30 °C ± 2 °C.

**5.2.5** After the 4-week ageing process, carry out the yeast load test as described in Paragraph 5.3.

**5.3 Yeast load test**

**5.3.1** Inoculate each sample (except for the negative control) with the same volume of mixed suspension equivalent to 1.0 % of the sample weight. Mix the sample thoroughly using a sterile spatula and then screw the cap onto the container in such a way that oxygen can still enter the container.

**5.3.2** In order to determine the initial yeast load for all samples, use an appropriate dilution series and spread the samples onto nutrient agar plates or use a pour plate method. Then incubate the plates at 30 °C ± 2°C for a period of 3 - 7 days. Use a suitable method to then determine the number of colony-forming units.

**5.3.3** In order to check the vitality of the yeasts being used, spread and count the mixed suspension as described under Paragraph 5.3.2.

**5.3.4**  Incubate all samples for 7 days at 30 °C ± 2°C.

**5.3.5** In order to determine the cell count in the samples, use an appropriate dilution series and spread the samples onto nutrient agar plates or use a pour plate method. Determine the colony-forming units after incubating the plates at 30°C ± 2°C for 3 - 7 days.

**5.3.6** Repeat steps 5.3.1 to 5.3.5 at weekly intervals until at least 3 cycles have been completed. The test is concluded after a total incubation time of 6 weeks at 30 °C ± 2 °C. Determine the final number of colony-forming units.

**5.3.7** In order to determine the effectiveness of different biocidal concentrations depending on the incubation times, additional cell counts can be determined, e.g. 1 and 3 days after inoculation.

**5.4 Validity of the test**

The test is valid if the growth capacity of the inoculate can be verified on PD agar in petri dishes (see 5.1.6).

**5.5 Pass/Fail criteria**

**5.5.1** The curative effect of the biocide in the paint/varnish at the added concentration

is verified if the cell count is ≤ 1000 CFU/ml at the end of the test.

**5.5.2** The effectiveness of a tested biocide cannot be verified if the cell count in

the biocide-free paint/varnish after inoculation is < 1000 CFU/ml at the end of

the test.

**5.6 Verification of a significant difference**

Use suitable statistical measures to verify any significant differences between the cell count in the samples treated with in-can preservatives and in the untreated samples.

**Annex 1**

**Nutrient medium**

**Nutrient agar**

Potato glucose agar is used to isolate, determine the number and identify yeasts and moulds.

Typical composition (g/L)

Potato infusion 4,0

Glucose 20.0

Agar 15.0

pH 5.6 +/- 0.2

Preparation

Suspend 39 g of nutrient agar in 1 L of distilled water, stir until completely dissolved and then autoclave for 15 minutes at 121°C. In order to suppress the undesired growth of bacteria, add the nutrient medium chloramphenicol to produce a final concentration of 30 µg/ml.

Alternatively, sabouraud agar or malt extract agar can also be used as a nutrient medium.

# Annex 4c

# Carrying out a biotest using moulds

The applicant shall carry out a test of the product’s fitness for use using the following method to determine the minimum quantity of the biocidal product2 required:

**A laboratory method for determining the required concentration of a biocidal product that is required to combat moulds in low-pollutant paints and varnishes according to DE-UZ 12a**

**1. Scope**

The method can be used to test how effective biocidal products are at preventing the growth and survival of damaging organisms in aqueous paints and varnishes.

**2. Health note**

National health regulations must be observed during the execution of the test. This also applies to EC Directive 200/54/EC “protection of workers from risks related to exposure to biological agents at work”.

When handling paints/varnishes and biocides, the instructions in the corresponding safety data sheets and product information brochures for the safe use of the product must be observed.

**3. Instruments and nutrient media**

* Suitable sterile, screw cap containers (100ml);
* Sterile pipettes, nominal volume 1.0 ml and 5.0 ml;
* Sterile pipette tips;
* Sterile centrifuge beakers (35 ml);
* Sterile suction filters, size 1;
* Sterile glass or plastic petri dishes, diameter 90 or 100 mm;
* Sterile diluent; e.g. distilled water or physiological saline;
* Sterile wetting agent solution;
* Scales;
* Incubator, thermostatically controlled (30 ± 2 °C);
* Autoclave;
* Drying cabinet;
* Sterile cotton swabs;
* Sterile inoculating loops or needles;
* Test tube racks;
* Test tubes;
* Test tube shaker;
* Centrifuge;
* Safety cabinet, class 2;
* Sterile nutrient media for the corresponding mould cultures, composition and production (see Annex 1);
* Mould stock cultures;
* Haemocytometer (depth 0.1 mm);
* Microscope.

**4. Test organisms**

The following moulds should be used for the mould load test:

**Moulds:**

*Aspergillus oryzae*  BAM 613 or NBRC 100959

*Paecilomyces variotii* BAM 19 or DSM 1961 or ATCC 18502

*Penicillium ochrochloron* BAM 25 or DSM 1945

Other moulds that continuously lead to infections can be additionally used in a separate inoculation suspension. It is necessary to carry out a molecular and biological characterisation of these organisms (which must be available as pure cultures) to clearly determine the strain being used. The organisms should be stored at a recognised culture collection.

**5. Method**

Inspect the materials received for testing before starting the test. To do this, use a sterile inoculating loop to spread the paint/varnish onto nutrient agar plates and check for any possible growth. It is necessary to check both unaged samples and also artificially aged samples (see 5.2). The Pass/Fail criteria must be met in both cases (see 5.5).

**5.1 Preparation of the inoculation suspension**

**5.1.1** Inoculate nutrient agar plates with the test moulds approx. 14 days before they are used for the test. Sterilise the required equipment and liquids at 121°C for 20 minutes in autoclaves.

**5.1.2** Fill the test tubes with 10 ml of the wetting solution under sterile conditions

and wet a cotton swab with this solution. Then wipe the spore material off

the nutrient agar plates using the cotton swab and transfer it to the filled

test tubes by rubbing it on the glass wall of the test tube. Use a test tube

shaker to mix up the preparation.

**5.1.3** Shake up the preparation again and then pour it through a funnel filter into

a centrifuge beaker. In the case of weakly sporulating mould cultures, additional spores should be collected from other nutrient agar plates. A separate funnel filter and a separate centrifuge beaker must be used for each mould.

**5.1.4** Centrifuge the suspensions for 10 minutes at 3,000 rpm and room

temperature. Then carefully remove the supernatant.

Wash the pellet with 10 ml of sterile, demineralised water and centrifuge

the preparation again for 10 minutes at 3,000 rpm and room temperature.

**5.1.5** Repeat the process described in 5.1.4 twice so that the spores have been

washed a total of three times.

**5.1.6** Add the pellet to 3-5 ml of sterile, demineralised water and determine the concentration of the spores using a suitable haemocytometer. (e.g. Thoma chamber, chamber depth 0.1 mm).

**5.1.7** The spore count of the individual spore suspensions should be

**8\*105 - 2\*106 spores/ml**

Store the prepared spore suspension in a refrigerator until it is ready

for use.

**5.1.8** In order to prepare a mixed suspension, combine and mix together identical volumes of each spore suspension.

The spore count for this spore suspension should also be

**8\*105 - 2\*106 spores/ml**

**5.1.9** Test the vitality of the moulds by spreading the spore suspension onto

PDA plates. Incubate the plates for a maximum of 7 days at 30 °C ±2 °C.

**5.2 Artificial ageing**

**5.2.1** Add the biocidal product to the paint/varnish at appropriate concentrations for the test series, vigorously mix the samples and store at room temperature for at least two days.

**5.2.2** Weigh out suitable portions (e.g. 50 g or 100 g) of the paint/varnish into sterile screw cap containers and close them tightly.

* + 1. Six non-preserved samples should be used as control samples. For this purpose, inoculate three samples with spore suspensions (positive control) and leave the remaining three samples uninoculated (negative control or retained sample).

**5.2.4** Store the samples for 4 weeks at 40 °C ± 2 °C or alternatively 18 weeks at 30 °C ± 2 °C.

**5.2.5** After the 4-week ageing process, carry out the mould load test as described in Paragraph 5.3.

* 1. **Mould load test**

**5.3.1** Inoculate each sample (except for the negative control) with the same volume of spore suspension equivalent to 1.0 % of the sample weight. Distribute the spore suspension homogeneously onto the surface of the sample by carefully tilting the sample. Then screw the cap onto the container in such a way that oxygen can still enter the container.

**5.3.2** In order to check the vitality of the mould, spread the spore suspension onto nutrient agar plates and incubate the plates for a maximum 7 days at 30 °C ± 2 °C.

**5.3.3** Incubate all samples for 7 days at 30 ± 2°C.

**5.3.4** Visually inspect the surface growth of the mould.

0: No growth

1: Weak growth (up to 10% of the surface covered)

2: Medium growth (up to 30 % of the surface covered)

3: Strong growth (up to 70 % of the surface covered)

4: Full growth (up to 100 % of the surface covered)

**5.3.5** Repeat steps 5.3.1 to 5.3.4 at weekly intervals until at least 3 inoculation cycles have been completed. The test is concluded after a total incubation time of 6 weeks at 30 °C ± 2 °C.

**5.3.6** In order to determine the effectiveness of different biocidal concentrations depending on the incubation times, additional cell counts can be determined, e.g. 1 and 3 days after inoculation.

**5.4 Validity of the test**

The test is valid if the growth capacity of the mould can be verified on PD agar in petri dishes (see 5.1.9).

* 1. **Pass/Fail criteria**

**5.5.1**  The curative effect of the biocide in the paint/varnish at the added

concentration is verified if no mould growth can be observed at the

end of the test.

**5.5.2** The effectiveness of a tested biocide cannot be verified if no mould

growth in the biocide-free paint/varnish after inoculation can be verified.

**5.6 Verification of a significant difference**

Use suitable statistical measures to verify any significant differences

between the cell count in the samples treated with in-can preservatives and in the

untreated samples.

**Annex 1**

**Nutrient medium**

**Nutrient agar**

Potato glucose agar is used to isolate, determine the number and identify yeasts and moulds.

Typical composition (g/L)

Potato infusion 4.0

Glucose 20.0

Agar 15.0

pH 5.6 +/- 0.2

Preparation

Suspend 39 g of nutrient agar in 1 L of distilled water, stir until completely dissolved and then autoclave for 15 minutes at 121°C. In order to suppress the undesired growth of bacteria, add the nutrient medium chloramphenicol to produce a final concentration of 30µg/ml.

Alternatively, sabouraud agar or malt extract agar can also be used as a nutrient medium.

1. Test to be completed by a testing institution accredited according to DIN EN ISO/IEC 17025 [↑](#footnote-ref-1)
2. Product-type 6 according to the Biocidal Products Regulation (EU No. 528/2012) [↑](#footnote-ref-2)
3. Only those substances or substance combinations stated in the list of approved in-can preservatives are permitted. [↑](#footnote-ref-3)