International Standard

ISO 16000-22

First edition
2025-02

Indoor air —

Part 22: Detection and quantification of fungal biomass by fungal β-N-acetylhexosaminidase enzyme activity

Air intérieur —

Partie 22: Détection et quantification de la biomasse fongique par caractérisation de l'activité de l'enzyme fongique β-N-acétyl-hexosaminidase

Reference number
ISO 16000-22:2025(en)

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Published in Switzerland

Contents

Page

Foreword

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This document was prepared by Technical Committee ISO/TC 146, *Air quality*, Subcommittee SC 6, *Indoor air*.

A list of all parts in the ISO 16000 series can be found on the ISO website.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

Fungi from different taxonomic groups form filamentous cells (mycelium) and asexual spores (conidia). Most spores are in the size range of 2 µm to 10 µm, some up to 30 µm and only few up to 100 µm. Spores of some fungi genera are small and become airborne very easily (e.g. *Aspergillus*, *Penicillium*) while others are bigger and/or embedded in a slime matrix (e.g. *Stachybotrys*, *Fusarium*) and less mobile.

Fungi spores are widely distributed in the outdoor environment and, therefore, also occur in varying concentrations indoors. The growth of fungi in indoor environments, however, is considered to be a hygiene problem because epidemiological studies have revealed that dampness and/or mould growth in homes is closely related to health problems affecting the occupants.

Harmonized methods for sampling, detection and enumeration of moulds, including standards for sampling strategies, are important for the comparative assessment of indoor mould problems. Before doing any measurements, a plan for the measurement strategy (on the basis of ISO 16000-19[14]) should be made.

This document describes the measurement of fungal material by enzymatic biochemical analysis.

It describes a rapid quantitative method to determine the total fungal material in air, on surfaces or in material samples by measuring a naturally occurring enzyme found in the chitinolytic system of all filamentous fungi (β-N-acetylhexosaminidase or NAHA (EC 3.2.1.52)).[1][2][3][4][5][6][7][8][9][10][11]

It describes the analytical procedure that can be performed on-site or in a laboratory and refers to applicable sampling procedures for air, surfaces and material samples.

This method does not enumerate or differentiate genera or species of fungi such as those found in ISO 16000-17[12], ISO 16000-18[13] and ISO 16000-20[15].

It is a quantitative method used to rapidly assess conditions found in indoor spaces or post-remediation.

Indoor air —

Part 22: Detection and quantification of fungal biomass by fungal β-N-acetylhexosaminidase enzyme activity

1 Scope

This document specifies requirements for the sampling and analysis of air, surface or bulk material samples analysed by fluorometric detection of an enzyme activity present in filamentous fungi[1] (US Patent No. 6,372,446) to quantitatively determine the total fungal biomass density. It describes the analytical procedure that can be performed on-site or in a laboratory. This method does not enumerate or differentiate genera or species of fungi.

2 Normative references

There are no normative references in this document.

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminology databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at https://www.electropedia.org/

3.1
enzyme
substance, produced by living cells that acts as a catalyst to bring about a specific biochemical reaction

3.2
filamentous fungus
fungus growing in the form of filaments of cells known as hyphae

Note 1 to entry: The term "filamentous fungi" differentiates fungi with hyphal growth from yeasts.

[SOURCE: ISO 16000-20:2014, 2.3[15]]

3.3
mould
<air quality> *filamentous fungi* (3.2) from several taxonomic groups; namely Ascomycota, Basidiomycota, Mucoromycota, and their asexual states

Note 1 to entry: Mould form different types of spores depending on the taxonomic group they belong to, namely conidiospores (conidia), sporangiospores, basidiospores or ascospores.

[SOURCE: ISO 16000-20:2014, 2.6[15], modified — "Basidiomycota, Mucoromycota, and their asexual states" replaced "Zygomycota, and their anamorphic states former known as Deuteromycota or fungi imperfecti". "basidiospores" added to Note 1 to entry.]

3.4
mycelium
branched hyphae network

[SOURCE: ISO/TS 10832:2009, 3.5[16]]

4 Principle of enzyme-targeted fluorescent detection

The principle of enzyme-targeted fluorescent detection (ETFD) is to identify and target a specific naturally occurring enzyme (β-n-acetylhexosaminidase) present in the cell walls of the fungi, and therefore present in all fungal particles including hyphae, hyphal fragments, mycelium and spores. This enzyme is used by the fungal cells to act on certain substances or substrates in the environment and break them down.

Enzyme activity follows Michaelis Menten kinetics, and if the amount of substrate is in surplus during the reaction, the rate of reaction is constant (V_{max}). If the enzyme reaction is running for a certain amount of time (e.g. 30 min), the amount of substrate formed by the reaction will depend on:

— the Michaelis Menten constant (K_m);

— the temperature;

— the enzyme concentration.

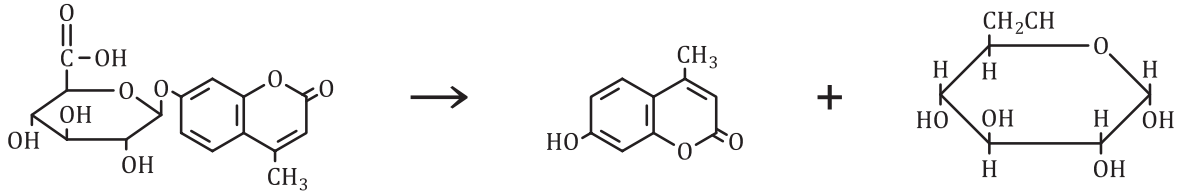
K_m is a constant, and if the temperature is kept constant during the reaction time, the amount of product formed will be linear with time and proportional to the concentration of enzyme. The concentration of enzyme depends on the concentration of fungi, and the concentration of product is therefore, proportional to the fungal concentration.

The technology described in this method uses this naturally occurring reaction by using an artificial substrate that mimics what the enzyme would act on in the environment. Inside this artificial substrate, an inert fluorescent molecule (fluorophore) is embedded. The signature of this particular fluorophore (4-MU) is that it absorbs light at 365 nm wavelength and emits equal energy at 441 nm wavelength.

When the artificial substrate is exposed to a sample with fungal material present, the enzyme in the fungal cells breaks down the substrate releasing the fluorophore. The more fungal particles present, the more enzyme activity to break down the substrate, which then releases fluorophore as a surrogate measure of the amount of activity. The fluorescent signal measured at the end of the reaction is proportional to the amount of fungi (the biomass density) present in the sample.

This reaction does not require cells to be lysed. The reaction happens at the cell wall interface, as it would in the environment.

Figure 1 describes the process using a target fungal enzyme's activity to break down the substrate and release the fluorescent marker, a measure of the activity, which is proportional to the amount of fungal material.



a) b) c) d)

Key

a artificial enzyme substrate with embedded fluorescent marker compound: 4-methylumbelliferyl β-D glucopyranoside

b reaction of sample with fungal enzyme cleaving artificial substrate: fungal B-D-N-acetylhexosaminidase

c fluorophore: 4-methylumbelliferyl (4-MU)

d fluorescent marker compound released by cleaving of substrate: glucose

Figure 1 — Illustration of enzyme activity reaction with artificial substrate and subsequent release of fluorescent marker

5 Apparatus and materials

5.1 Sterile cotton swabs, for sampling.

5.2 Sample area templates, for surface sampling (e.g. 9 cm^2 if using the example criteria in Clause A.2).

5.3 Alcohol wipes, to sterilize the scraping tool, forceps and surface of the porous material to be sampled.

5.4 Scraping tool and forceps, to use for collecting porous material samples.

5.5 Clean plastic bags, with firm closure containers for porous material samples.

5.6 High-volume indoor air pump, an oil-free rotary vane or diaphragm, capable of pulling air through the filter cassette at a rate of at least 10 l/min.

To obtain a representable sample, the sampling time and the flow rate are the variables. For most practical purposes, a relative short sampling time is favoured, so a high-volume pump is preferable.

5.7 Filter cassette, for air sampling, 27 mm mixed cellulose ester (MCE) filter, 0,8 um pore size.

5.8 Clean nitrile or similar gloves, for handling reagents, vials and cuvettes.

5.9 Fluorometer, with an excitation wavelength of 365 nm and emission wavelength of 445 nm.

5.10 Ultraviolet (UV) transparent cuvettes, for measuring samples in the fluorometer.

5.11 Sterile test tubes, at least 3 ml, glass or plastic, to hold the substrate and swab during the reaction period.

5.12 100 µl auto–pipette, with sterile tips for transferring unreacted and reacted substrate to developer reagent to measure the sample blank value and analysis value, respectively.

5.13 Sterile syringes, 2,5 ml with luer lock fitting, for transferring reagents to vials, cuvettes or test tubes.

5.14 Calibration cuvette (black), to set zero point during fluorometer calibration.

5.15 Thermometer, with a range of at least 18 °C to 30 °C (64,4 °F to 86,0 °F).

5.16 Timing device, to monitor the reaction period.

5.17 0,45 µm pore size filter, with luer lock fitting for turbidity filtration in final sample, if necessary.

6 Reagents

6.1 Fluorescent labelled enzyme substrate (ES), such as 4-methylumbelliferyl-N-acetyl-beta-D-glucosaminide with an inhibitor to exclude non-fungal NAHA.[8]

6.2 Developer solution (DS), alkaline buffer solution (pH10) used to stop the reaction between the ES and NAHA fungal enzyme while enhancing the fluorescence for measurement.

6.3 Swab wetting agent (SWA), sterile saline buffer to aid sample collection and stabilize the swab after collection.

6.4 4-MU standard solution, serial concentrations, for fluorometer calibration.

7 Sampling

7.1 Surface sampling

Dip the sterile swab in the wetting agent once and discard the remaining. Place the sample template on the surface to be sampled (if using the interpretation criteria in Clause A.2, a sampling area of 9 cm^2 shall be used).[9] The fungal biomass should be removed as quantitatively as possible. A swab with a rigid stick improves the amount of force that can be used, making swabs with a wooden shaft preferable. Clean the area as thoroughly as possible. Keep the swab in a low angle to use as much of the cotton as possible. Rotate the swab during sampling and use as much force as the stick allows. Also, the area should be evenly swabbed in two perpendicular directions. When the concentration of fungi on the surface is very high, it is not always possible to remove all fungal biomass. This is important to consider as surfaces are often uneven and mycelia are also growing inside the material. Samples collected from visually clean surfaces, such as after remediation, require a minimum of three passes over the area of the template before sampling is complete. Return the swab to the swab container and label with the location and sample number.

7.2 Porous materials

7.2.1 High-density materials (e.g. cementitious materials, such as concrete, plaster, mortar)

To detect fungal biomass inside this type of porous materials situated *in situ* in a building, use alcohol to sterilize the surface where the sample is to be taken with a sterile tool. Then use the tool to scrape the outer layer of the high-density materials (HDMs). Use a clean plastic bag with a zip fastener. With the tool, scrape the material into the plastic bag. Collect at least 300 mg of the material since this is the amount needed for the analysis.

7.2.2 Low-density materials (e.g. insulation materials such as glass wool or mineral wool)

Use sterile forceps to take samples. Keep samples in a clean plastic bag with a zip fastener. Collect at least 100 mg of the material as since this is the amount used for analysis.

For both HDMs and low-density materials (LDMs): If materials are moist or wet, they should only be kept in a closed plastic bag for a short period of times (a few hours), and they should be analysed as soon after sampling as possible.

7.3 Air sampling

For air sampling, a filter cassette containing a methyl cellulose ester membrane filter (pore size 0,8 µm) is used. The sampling time is 10 min with a minimum flow rate of 10 l/min. The minimum sample volume should be 100 l. Rotary vane pumps or some membrane pumps can be used.

A preferred method for sampling is by use of activated air sampling, where fungal propagules that are settled on the floor or furniture are re-aerosolized using a small hand-held blower with an airspeed of 3,3 m/s to 3,8 m/s. Activated air sampling is designed to mimic a high level of activity in a space and to improve the reproducibility of the air sample (see Annex B for the details of the protocol).[10]

8 Preparation of the fluorometer

Calibrate the fluorometer with the black calibration cuvette (0,0 fluorescent units, FLU) and the calibration standard in a new cuvette. Compare to the standard value (STD.VAL) of the fluorometer. If the value is within ±4 % of the STD.VAL of the instrument, note the value on the calculation sheet.

NOTE Sometimes manufacturers have specific fluorometer preparation and calibration requirements. If the fluorometer does not fall within calibration tolerance, then the results will not be valid.

Alternatively, calibrate the fluorometer using a dilution series in the relevant concentrations of the fluorophore to establish a linear response.

Relevant concentrations of the fluorophore should be used to obtain a minimum of a four-point calibration. Dilutions are made using the developer solution. Make the concentrations up in 2 ml solutions in each cuvette. Read the fluorescence from each cuvette and generate a curve of fluorescence intensity versus the standard solution.

9 Analysis

9.1 General

Allow the chemistry to equilibrate to room temperature where the analysis will be performed. Analysis should be performed at ambient temperatures between 18 °C to 30 °C (64,4 °F to 80 °F) to be in the most linear range of the reaction.

9.2 Surface samples

Transfer 100 µl of the enzyme substrate solution to a UV cuvette and add 2 ml buffer to the cuvette. Measure the fluorescence and note the value as the blank value (BV).

Set the time according to the temperature/reaction time, see Table A.1.

Transfer the samples (swabs) into tubes containing at least 2 ml of enzyme substrate solution and start the timer.

When the timer signals, remove the swab from the tube and transfer 100 µl of the reacted substrate to a UV cuvette and add 2 ml of buffer.

Measure the fluorescence and note the value as the analysis value (AV).

If using the 9 cm^2 template, the interpretation criteria in Clause A.2 can be used. If not using the template, compare the results to the sample control.

9.3 Analysis of porous materials (both high- and low-density materials)

Analysis is similar to 9.1, except that instead of a swab, 100 mg of LDM or 300 mg of HDM are added to the substrate solution after the BV has been determined.

Compare the data to the interpretation criteria for the type of material sampled.

NOTE Interpretation criteria will be different from surface samples, and there are differences between HDM and LDM.

9.4 Analysis of air samples

Transfer 1 ml of substrate solution to a UV cuvette and add 2 ml of buffer to the cuvette. Measure the fluorescence and note the value as the BV.

Set the time according to the temperature/reaction time, see Table A.1.

Add 1 ml of substrate solution directly to the filter (in the filter cassette) and start the timer.

When the timer signals, add 2 ml of buffer to the filter. Extract the solution from the filter using a syringe attached to the top port of the filter and push the liquid through the cassette through the bottom port of the filter into a UV cuvette.

Measure the fluorescence and note the value as the AV.

Subtract the BV from the AV and compare the value to the interpretation criteria for air samples.

Annex A
(informative)

Reaction time and temperature correction and example data interpretation categories

A.1 Reaction time and temperature correction

Table A.1 — Reaction time and temperature correction chart

Reaction time (min:sec)	**Celsius**	**Fahrenheit**
42:00	18,0	64,4
40:35	18,5	65,3
39:14	19,0	66,2
37:55	19,5	67,1
36:40	20,0	68,0
35:27	20,5	68,9
34:17	21,0	69,8
33:09	21,5	70,7
32:04	22,0	71,6
31:01	22,5	72,5
30:00	23,0	73,4
29:02	23,5	74,3
28:05	24,0	75,2
27:11	24,5	76,1
26:18	25,0	77,0
25:28	25,5	77,9
24:39	26,0	78,8
23:52	26,5	79,7
23:06	27,0	80,6
22:22	27,5	81,5
21:40	28,0	82,4
20:59	28,5	83,3
20:20	29,0	84,2
19:41	29,5	85,1
19:05	30,0	86,0

A.2 Example interpretation categories

A.2.1 Development of interpretation categories

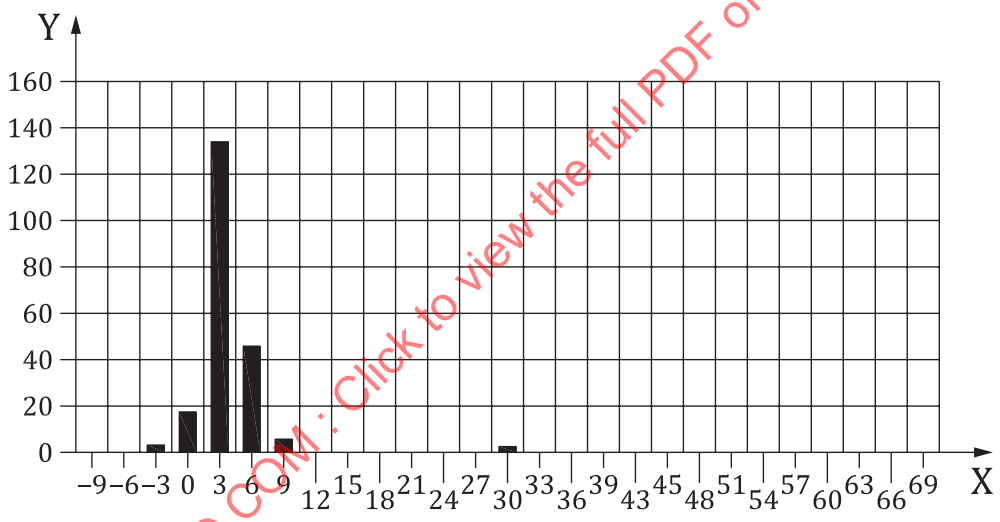
Surface interpretation categories (A, B and C) were developed as described in A.2.2 to A.2.4. A summary of the categories is given in Table A.2.

Table A.2 — Surface interpretation categories

Interpretation category	Interpretation criteria value (FLU)	Description
A	< 21	The level of fungi is not above normal background level.
B	21 to 135	The level of fungi is above normal background level. This is typically due to high concentrations of spores in dust deposits but can, in some cases, indicate the presence of an old fungal damage (fungal growth).
C	> 135	The level of fungi is high, above normal background level, due to fungal growth.

A.2.2 Category A

To develop the interpretation criteria, samples were collected from a 9 cm^2 area of visually clean surfaces (n = 101), with no visible dust or dirt, in a variety of well-maintained buildings with no fungal or moisture problems. The samples were analysed with enzyme activity (see Figure A.1). All samples showed MV-values (FLU-values) below 20. Therefore, FLU-values below 20 were defined as the normal background level of fungal biomass. It should be noted that this category is not indicative of sterile or no fungal material whatsoever. It is universally recognized that all buildings have a natural background level of fungal spores. Appropriately, samples having a FLU-value of 20 or less are not sterile and a low level of spores is likely to be present. This level of fungal biomass is characterized as Category A.



Key

X enzyme activity (fluorescence units)

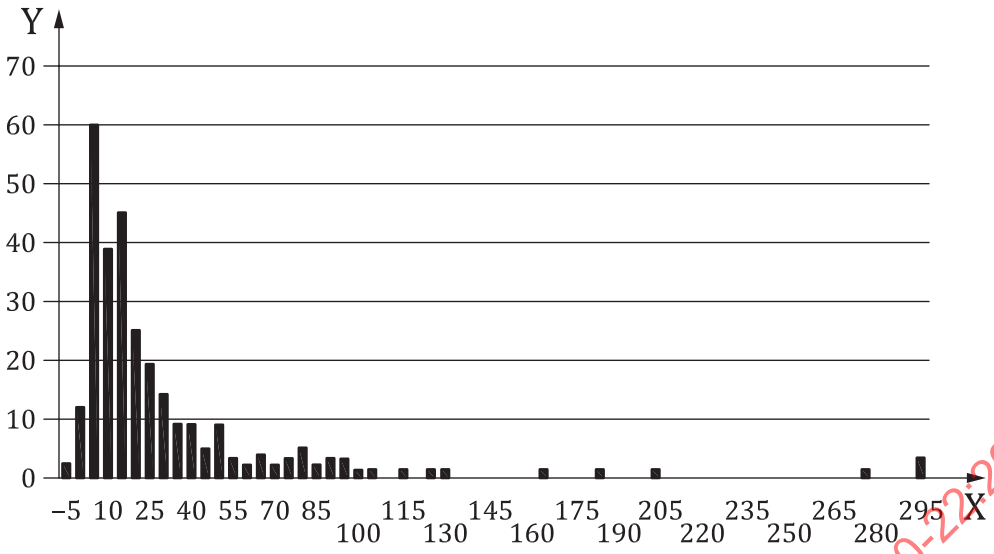
Y number samples

Figure A.1 — Frequency distribution from samples defining Category A — Samples from visually clean surfaces (n = 217)

A.2.3 Category B

Samples were collected from surfaces with visible accumulation of dust or dirt, varying from barely visible to very dirty, including locations where it was not possible to collect all the dirt with the swab (n = 127). The test results were described by a log normal distribution (see Figure A.2). Of the samples collected, 96 % showed a FLU-value of less than 136. Therefore, Category B was defined as FLU-values higher than 25 but lower than 136.

Levels of dry fungal biomass above this background level can be due to either accumulated spores, such as in dust or actual low-density fungal growth. However, the level of dry fungal biomass will almost always be substantially higher on surfaces with growth compared to surfaces with accumulated spores. This quantitative difference constitutes the basis that distinguishes Category B from Category C.



Key

X enzyme activity (fluorescence units)

Y number samples

Figure A.2 — Frequency distribution from samples defining Category B — Samples from dusty/dirty surfaces (n = 290)

A.2.4 Category C

The FLU values obtained from surfaces with fungal growth that are above 136 (samples with values up to 40 000 have been registered) are termed Category C.

Category C results mean that the dry biomass density of fungi is very high, a clear indication of fungal growth.

FLU-values below 10 should be reported as below detection limit (BDL) (significance level: 99 %).

Annex B
(informative)

Activated sampling — Protocol for air sampling

B.1 General conditions for sampling

The general conditions for sampling are as follows:

a) Sampling height: 130 cm ± 5 cm.

b) Flow rate: minimum 15 l/min.

c) Sampling time: 10 min.

d) Windows should be closed 6 h before sampling.

e) Doors should be closed during blowing and sampling.

f) Use 27 mm diameter, 0,8 µm pore size MCE filters in a cowl housing.

g) Use one sample per 60 m^2.

h) Collected samples should be stored at room temperature for up to seven days before analysis.

B.2 Materials for activated sampling protocol

Materials for the activated sampling protocol are as follows:

a) High volume rotary vane or membrane pump.

b) Rotameter on pump or manifold.

c) Tripod, preferably with a manifold and rotameter(s).

d) Timing device.

e) Small hand-held leaf blower with airspeed of 3,3 to 3,8 m/s.

f) The sample filters specified in Clause B.1 f).

B.3 Sampling protocol

The sampling protocol is as follows:

a) Set up the pump and tripod and attach the tubing to the manifold.

b) Test the flow rate by placing a “Test filters” in the filter holder. Remove the lid from the filter and start the pump. Adjust the flow rate to the highest possible (minimum 10 l/min).

c) Replace the “Test filters” with the sample filter and give it an identification number (ID#). Note the flow rate.

d) Set a timer on 10:30 (min:sec).

e) Put on the respiratory protection.