Normalisation of the certification, distribution and use of microbial reference material

Wetenschappelijk ondersteuningsprogramma voor de normalisatie en technische regelgeving

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CONTENTS

ABBREVIATIONS

AFNOR	Association Française de Normalisation (France)
ASTM	American Society for Testing and Materials (USA)
ATCC	American Type Culture Collection (Virginia, USA)
BCCM	Belgian Co-ordinated Collections of Micro-organisms (Belgium)
BS	British Standard
CFU	Colony Forming Units
CIP	Collection de l'Institut Pasteur (Paris, France)
CTB	Collection of the CTBA
CTBA	Centre Technique du Bois et de l'Ameublement (Bordeaux, France)
DIN	Deutsches Institut für Normung (Germany)
DSM	Deutsche Sammlung von Mikroorganismen = DSMZ (Braunschweig, Germany)
EN	European Norm
ENV	European prestandard
FDA-BAM	Food and Drug Administration, Bacterological Analytical Manual (USA)
FIL	Fédération Internationale de Laiterie = International Dairy Federation = IDF
FPRL	Forest Products Research Laboratory (Watford, United Kingdom)
IBN – BIN	Institut Belge de Normalisation - Belgisch Instituut voor Normalisatie = Belgian Institute for Standardisation (Brussels, Belgium)
IEC	International Electrotechnical Commission
IHEM	BCCM/IHEM Collection of the Mycology section of the Institute of Public Health (Brussels, Belgium)
ISO	International Organization for Standardization
LMG	BCCM/LMG Bacteria Collection of the Laboratorium voor Microbiologie, Universiteit Gent (Ghent, Belgium)
MUCL	BCCM/MUCL Collection of the Microbiology Unit of the Université catholique de Louvain = Mycothèque de l'Université catholique de Louvain (Louvain-la-Neuve, Belgium)
NBN	Norme Belge = Belgische Norm = Belgian Norm
NCCLS	National Committee for Clinical Laboratory Standards (Pennsylvania, USA)
NCTC	National Collection of Type Culture (United Kingdom)
NF	Norme Française = French Norm
NMKL	Nordic Committee on Food Analysis (Oslo, Norway)
Belgian Science Policy	Belgian Science Policy (Brussels, Belgium)
SP-VG	Santé Publique – Volksgezondheid = Public Health (Brussels, Belgium)
TCB	Test, Control or Bioassay
USP	United States Pharmacopoeia (USA)
XP	Norme Française Expérimentale = Experimental French Norm

SUMMARY

A. Context

Microbiological reference strains are imposed for the validation of methods described in national and international norms and standardised methods (NBN, ISO, EN, NF, Pharmacopoeia, …) in fields related to microbiology. Most of these strains are available in the Belgian collections of the BCCM consortium (Belgian Coordinated Collections of Microorganisms). Belgian laboratories and industrial companies concerned need to have access to this reference material in a user-friendly form and with proven properties.

B. Objectives

BCCM/IHEM (biomedical fungi and yeasts), BCCM/LMG (bacteria) and BCCM/MUCL (agro-industrial fungi and yeasts) wished to raise its profile and to meet the needs of the Belgian laboratories and industrial companies concerned.

Norms necessitating the use of micro-organisms were defined. The search of these standards was focused on the areas related to the specialisation of our 3 collections: health (disinfectants, antiseptics, drugs), foodstuffs (food and water analysis) and materials (wood, plastics, paints, textile, paper).

The performances of the strains imposed in the norms were tested following the test method described in the norms, and compared with those of alternative strains. Potential strains were tested when the norm didn't specify the strains.

The possibility was assessed to distribute the strains under a user-friendly form as ready-to-use working stock.

C. Conclusions

BCCM/IHEM selected 3 European and one French norms related to disinfectants and antiseptics necessitating 2 fungal and 4 yeast strains. The performances of these strains were studied and compared to those of 4 other strains (2 fungi and 2 yeasts) of species particularly important in hospitals. Species listed in the norms were found rather suitable for the defined objective. Only one of the alternative yeast species could be more judicious than those cited in the norms. Two of the strains studied and 4 bacterial strains tested by BCCM/LMG are listed in two methods from the European Pharmacopoeia; performance tests of those strains gave conform results.

BCCM/LMG tested 37 bacterial strains chosen for the validation of 48 standardised methods and norms in the food sector that didn't specify any strain(s). Ninety six percent of the performance tests realised (detection, enumeration, confirmation), *i.e*. 358 out of 373, gave a result conform to the corresponding method. Consequently, 33 of the strains studied will be available to the clients with a certificate mentioning the realised performance tests.

BCCM/MUCL selected 20 fungal strains involved in wood degradation to check their performances according to 4 European standards concerning wood preservation products. Wood test specimens were exposed to the strains for determining the mass loss allowing to evaluate the virulence of the strains and to validate the assays. Only one strain out of the 4 mentioned in standards, and 9 out of the 16 alternative strains have shown a sufficient virulence.

Furthermore, each of the 3 collections selected representative strains to assess the preservation on cryobeads at –20°C, in order to meet the need of clients to receive strains

under a user-friendly form as a ready-to-use working stock. The viability of 18 strains (2 from BCCM/IHEM, 7 from BCCM/LMG and 9 from BCCM/MUCL) was followed during a period of 14 to 18 months. In most cases, this preservation method was satisfactory. Twelve clients from BCCM/LMG and BCCM/MUCL agreed to test strains on cryobeads with regard to viability and performance and to give their opinion in a detailed questionnaire. This form of supply was highly appreciated. Following this project, 26 out of the BCCM/LMG reference strains can be distributed to the clients under this form.

A catalogue available for the users of these reference micro-organisms has been established specifying all the norms studied and the corresponding strains tested (imposed or proposed).

D. Contribution of the project in a context of support to the processes of standardisation and technical regulations

After carrying out the performance tests on strains and studying of the norms we expressed some remarks about the normative texts in the different fields considered and suggestions to improve them. These remarks and suggestions were sent to the Belgian Institute for Standardisation and Beltest.

E. Keywords

Antiseptic, bacteria, bioassay strain, control strain, disinfectant, food, fungi, micro-organisms, norm, pharmacopoeia, reference strain, standard, test strain, wood, yeast.

SAMENVATTING

A. Context

Microbiële referentie stammen worden opgelegd voor het valideren van methodes beschreven in nationale en internationale normen en gestandaardiseerde methoden (NBN, ISO, EN, NF, Pharmacopoeia, ...) in verschillende sectoren verwant aan de microbiologie. De meeste van deze stammen zijn ter beschikking in de Belgische collecties van het BCCM consortium (Belgian Coordinated Collections of Microorganisms). De betrokken Belgische laboratoria en industriële bedrijven wensen dit referentie materiaal te ontvangen onder een gebruiksvriendelijke vorm met gecontroleerde eigenschappen.

B. Doelstellingen

BCCM/IHEM (biomedische schimmels en gisten), BCCM/LMG (bacteriën) and BCCM/MUCL (agro-industriële schimmels en gisten) wensten hun imago te versterken en wensten zich beter af te stemmen op de noden van de betrokken Belgische laboratoria en industriële bedrijven.

Normen die het gebruik van micro-organismen vereisen, werden gedefiniëerd. Het onderzoek naar deze standaarden was gefocusseerd op de sectoren gerelateerd aan de specialisaties van onze 3 collecties: gezondheid (desinfectantia, antiseptica, geneesmiddelen), voedingsmiddelen (voedsel en water analyses) en materialen (hout, kunststof, verf, textiel, papier).

De performantie van de opgelegde stammen in de normen werd getest volgens de test methode beschreven in de normen, en deze werden ook vergeleken met alternatieve stammen. Indien de normen geen specifieke stammen opleggen, werden potentiële stammen uitgetest.

De mogelijkheid om de stammen onder een gebruiksvriendelijke vorm te leveren als kant-en-klare werkstock werd onderzocht.

C. Besluiten

BCCM/IHEM heeft 3 Europese en 1 Franse norm verwant met desinfectantia en antiseptica geselecteerd waarbij er 2 schimmel en 4 gist stammen nodig zijn. De performantie van deze stammen werd bestudeerd en vergeleken met deze van 4 andere stammen (2 schimmels en 2 gisten) van species die in het bijzonder belangrijk zijn in ziekenhuizen. De vermelde species in de normen zijn geschikt bevonden voor de gedefinieerde doelstelling. Slechts een van de alternatieve gist species zou oordeelkundiger blijken te zijn dan deze vermeldt in de normen. Twee van de bestudeerde stammen en anderzijds 4 bacteriële stammen, getest door BCCM/LMG, worden ook vermeld in twee methoden van de Europese Pharmacopoeia; performantie testen van deze stammen hebben een conform resultaat opgeleverd.

BCCM/LMG heeft 37 bacteriële stammen geselecteerd voor de validatie van 48 gestandaardiseerde methoden en normen uit de voedingssector die geen stammen opleggen. Negenenzestig procent van de gerealiseerde performantie testen (detectie, telling, bevestiging), zijnde 358 van de 373, hebben een conform resultaat gegeven t.o.v. de overeenkomstige methode. Als een gevolg hiervan, zullen 33 van de bestudeerde stammen ter beschikking worden gesteld van de klanten; deze zullen worden verdeeld met een certificaat waarop de uitgevoerde performantie testen worden vermeld.

BCCM/MUCL heeft 20 schimmel stammen met betrekking tot hout degradatie geselecteerd om hun performantie te controleren volgens 4 Europese standaarden betreffende hout

bewaringsproducten. Hout stalen werden blootgesteld aan de stammen om het verlies in massa te kunnen bepalen, dit laat toe de virulentie van de stammen te evalueren en de testen te valideren. Slechts één stam van de vier vermeld in de standaarden, en 9 van de 16 alternatieve stammen hebben de vereiste virulentie vertoond.

Om tegemoet te komen aan de noden van de klanten die de stammen wensen te ontvangen onder een gebruiksvriendelijke vorm als kant-en-klare werkstock heeft elk van de 3 collecties een aantal representatieve stammen geselecteerd om de bewaring op cryoparels bij –20°C uit te testen. De viabiliteit van 18 stammen (2 van BCCM/IHEM, 7 van BCCM/LMG en 9 van BCCM/MUCL) werden gedurende een periode van 14 tot 18 maanden gevolgd. Deze bewaringsmethode is in de meeste gevallen geschikt bevonden. Twaalf klanten van BCCM/LMG en BCCM/MUCL hebben toegezegd om de stammen op cryoparels te testen op viabiliteit en performantie en om hun advies te geven via een gedetailleerde vragenlijst. Deze leveringsmethode werd zeer gewaardeerd. Als een gevolg van dit project, zal BCCM/LMG 26 van hun referentie stammen kunnen verdelen onder deze vorm aan hun klanten.

Een cataloog die alle bestudeerde normen en geteste corresponderende stammen (opgelegd of voorgesteld) vermeld, werd opgesteld en wordt ter beschikking gesteld van de gebruikers van deze referentie micro-organismen.

D. Bijdrage van het project in een context van ondersteuning aan het proces inzake normalisatie en technische regelgeving

Het uitvoeren van de performantie testen op de stammen en de studie van de normen heeft ons toegelaten om een aantal opmerkingen te formuleren over de normatieve teksten van de verschillende beschouwde domeinen en suggesties te formuleren om deze te verbeteren. Deze opmerkingen en suggesties werden overgemaakt aan het Belgisch Instituut voor normalisatie en Beltest.

E. Trefwoorden

Antiseptica, bacteriën, bioassay stam, controle stam, desinfectantia, gisten, hout, micro-organismen, norm, pharmacopoeia, referentie stam, schimmels, standaard, test stam, voedingsmiddelen.

RESUME

A. Contexte

Dans divers secteurs liés à la microbiologie, des souches de référence sont imposées pour valider les méthodes décrites dans les normes nationales et internationales et dans les méthodes standardisées (NBN, ISO, NE, NF, Pharmacopée,...). La plupart de ces souches sont disponibles dans les collections belges du consortium BCCM (Belgian Coordinated Collections of Microorganisms). Les laboratoires et entreprises belges qui utilisent ce matériel de référence le demandent sous une forme facile à l'emploi. Ils demandent également que les propriétés de ces souches soient contrôlées.

B. Objectifs

BCCM/IHEM (moisissures et levures biomédicales), BCCM/LMG (bactéries) et BCCM/MUCL (moisissures et levures d'intérêt agro-industriel) ont voulu améliorer leur image et rencontrer les besoins des laboratoires et entreprises belges qui utilisent ces souches de référence.

Les normes impliquant l'usage de microorganismes ont été recherchées. Cette recherche s'est focalisée sur les secteurs en relation avec les spécialisations de nos 3 collections: la santé (désinfectants, antiseptiques, médicaments), l'agro-alimentaire (analyse des denrées alimentaires et des eaux) et les matériaux (bois, plastiques, peintures, textile, papier).

Les performances des souches imposées dans les normes ont été testées selon les méthodes décrites dans ces normes et comparées à celles de souches alternatives. Des souches potentielles ont été testées lorsque les normes ne renseignaient pas de souches précises.

La possibilité a été évaluée de distribuer ces souches sous une forme facile à l'emploi comme stock de travail prêt à l'emploi.

C. Conclusions

BCCM/IHEM a sélectionné 3 normes européennes et une norme française relatives aux désinfectants et antiseptiques qui impliquaient un total de 2 souches fongiques et 4 souches de levures. Les performances de ces souches ont été étudiées et comparées avec celles de 4 autres souches (2 champignons et 2 levures) d'espèces particulièrement importantes en milieu hospitalier. Les espèces mentionnées dans les normes conviennent assez bien au but défini; seule une des espèces alternatives de levure pourrait s'avérer plus judicieuse que celles citées dans les normes. Deux des souches déjà étudiées sont également imposées dans 2 méthodes de la Pharmacopée européenne, de même que 4 des souches bactériennes testées par ailleurs par BCCM/LMG; les performances de ces souches ont donné des résultats conformes à ceux attendus.

BCCM/LMG a testé 37 souches bactériennes choisies pour valider 48 méthodes standardisées et normes du domaine alimentaire qui ne spécifiaient pas les souches à utiliser. Nonante six pour cent des tests de performance réalisés (détection, énumération et confirmation), soit 358 des 373 tests, ont donné un résultat conforme à la méthode correspondante. En conséquence, 33 des souches étudiées seront distribuées aux clients avec un certificat précisant les tests de performance effectués.

BCCM/MUCL a sélectionné 20 souches fongiques impliquées dans la dégradation du bois pour contrôler leurs performances selon 4 normes européennes concernant les produits de protection du bois. Des échantillons de bois ont été exposés aux souches afin d'en déterminer la perte de masse, ce qui permet d'évaluer la virulence des souches et de valider

les essais. Une seule des 4 souches mentionnées dans les normes et 9 des 16 souches alternatives a atteint la limite de virulence imposée.

Par ailleurs, chacune des 3 collections a sélectionné des souches représentatives pour en tester la conservation sur cryobilles à - 20°C, pour répondre au souhait des clients de recevoir les souches sous une forme d'emploi aisé comme stock de travail prêt à l'emploi. La viabilité de 18 souches (2 de BCCM/IHEM, 7 de BCCM/LMG et 9 de BCCM/MUCL) a été suivie pendant une période allant de 14 à 18 mois. Dans la grande majorité des cas, ce mode de préservation s'avère satisfaisant. Douze clients des collections BCCM/LMG et BCCM/MUCL ont accepté de tester la viabilité et les performances des souches sur cryobilles et de nous communiquer leur avis via un questionnaire détaillé. Cette forme d'envoi des souches a été fort appréciée. A la suite du présent projet, 26 des souches de référence de la collection BCCM/LMG pourront être fournies aux clients sous cette forme.

Toutes les normes étudiées et les souches testées correspondantes (imposées ou proposées) ont été reprises dans un catalogue disponible pour les utilisateurs de microorganismes de référence.

D. Apport du projet dans un contexte d'appui au processus de normalisation et de réglementations techniques

La réalisation des tests de performance sur les souches et l'étude des normes nous a amené à formuler diverses remarques sur les textes normatifs des domaines considérés et suggestions pour les améliorer. Elles ont été communiquées à l'Institut Belge de Normalisation et à Beltest.

E. Mots-clés

Antiseptique, bactérie, bio-essai, bois, denrées alimentaires, désinfectant, levure, microorganismes, moisissure, norme, pharmacopée, souche de contrôle, souche de référence, souche de test.

1 INTRODUCTION

According to national and international norms and standardised methods, accredited or certified laboratories and companies active in the fields related to microbiology have to use specific micro-organisms (bacteria, fungi and yeasts) as Test, Control or Bioassay strains (TCB strains). Criteria to check identity and performances of these TCB strains before use are lacking as well as handling recommendations for the supplier or for the end user. For the Belgian Coordinated Collections of Microorganisms (BCCM), the stake of this project was to promote the BCCM collections as source of TCB strains and to offer to their clients strains of proven quality. For that reason BCCM/LMG (bacteria), BCCM/IHEM (medical yeasts and fungi) and BCCM/MUCL (agro-industrial fungi and yeasts) decided to collaborate on a common project in the framework of the scientific support from the Belgian Science Policy to a federal policy concerning the whole of activities relating to standardisation and technical regulations.

The first step of this project was to collect the norms necessitating the use of reference strains in the competence sphere of our 3 collections, using various sources of information (among others Internet, literature, visit to the Belgian Institute for Standardisation,…).

In parallel an inquiry using a questionnaire was set up in order to define the specific needs of the clients with regard to the reference strains they need to validate their work. The data obtained would also help determining the list of norms to be worked on by each partner.

At the layout of the project, one of the defined aims was to publish internal procedures to be used for handling the reference strains and to insure their quality, as well as recommendations for the clients concerning the use of the strains or remarks about application of the norms.

During the project, the publication of internal procedures was considered inadequate as they were confidential and did not contain interesting information to the client. Furthermore, no important recommendation for the client about the use of the strains or the application of the norms emerged during the realisation of this project.

On the other hand, the results of the inquiry conducted us to add a new aim to this project: to look for a more user-friendly form of supply than the ampoules with freeze-dried cells. Various possibilities concerning preservation were considered and the preservation of the TCB strains on cryobeads maintained at -20°C was studied in order to offer to the clients ready-to-use controlled working stocks. Some strains from each collection were chosen and preserved with this method to assess stability of viable count and of the performances of the strains during 1 or 2 years.

Testing the TCB strains from the BCCM collections according to the normative methods selected, should also allow to have a critical view on these norms. This could possible result in suggestions or remarks for the Belgian normalisation body (IBN – BIN) and for the Belgian accreditation body (Beltest).

It was also planned to edit a catalogue with the strains studied and their features tested, that should be distributed not only to the current BCCM clients but also to potential new users of TCB strains to increase the visibility of the BCCM collections.

To end this introduction, it is important to point out the particularity of this collective project. Already in the first phase (collection of information and results of the inquiry), it turned out that there is much more demand for bacterial reference strains than for fungi and yeasts. The collaboration between 3 collections oriented to very different sectors, and the unbalanced importance of the use of the TCB strains in these sectors, explains the heterogeneous

composition of the present report. We have nevertheless preferred to present a common report because the objectives at the beginning were the same.

2 CHOICE OF STANDARDS AND STRAINS

2.1 Methodology

The research of standards was focused on several industrial sectors with activities related to microbiology such as health (disinfectants, antiseptics and pharmacopoeia), foodstuff (food and water analysis) and materials (wood, plastics, paints, textile and paper).

2.1.1 Sources

Several sources were used to collect the relevant standards and the corresponding list of reference strains:

- Catalogues and websites of international normalisation bodies: Belgian Institute for Standardisation (BIN-IBN), International Organization for Standardization (ISO), Association Française de Normalisation (AFNOR);
- The CD-Rom Perinorm available at BIN-IBN;
- The literature: National Committee for Clinical Laboratory Standards (NCCLS), European and United States Pharmacopoeia, handbooks, courses;
- BCCM collections databases;
- Participation in the microbial sector-related meeting of Beltest (Belgian Organisation for Accreditation and conformity assessment) chaired by Mrs Vanlaethem on 6 April 2000;
- Visits to several laboratories to gather specific information in May and June 2000:
    - Prof. De Zutter, Universiteit Gent, Faculteit Diergeneeskunde, Diergeneeskundig toezicht op eetwaren;
    - Mrs De Loy, Universiteit Gent, Landbouwfaculteit, Levensmiddelentechnologie en voeding;
    - Mrs De Roose, Stadslabo Gent;
    - Prof. Nelis, Universiteit Gent, Faculteit Farmaceutische Wetenschappen, Farmaceutische analyse;
    - Mr Iliano, Labo Iliano;
    - Dr Dierick, IHE, Dienst Voedingswaren;
    - Prof. Daube and Dr Ghafir, Université de Liège, Faculté de Médecine Vétérinaire, Département des Sciences des Denrées Alimentaires;
    - Mrs Thomassin and Le Bayon, Centre Technique du Bois et de l'Ameublement (CTBA) at Bordeaux (France) in June 2000.
- The course "Le point sur les méthodes normalisées et méthodes validées en microbiologie des aliments" followed at the Institut Pasteur de Lille (France) in November 2000.

2.1.2 Inquiry

An inquiry was set up and distributed to laboratories and companies (especially in Belgium), that use microbiological TCB strains in order to determine their needs as well as what is requested by Beltest for their accreditation.

The objectives of this inquiry were to gather information on:

- The most frequently used norms and TCB strains;
- The test criteria for checking and maintaining the TCB strains, the occurrence and the recorded performance stability expected by the norms;
- The nature of the certificates wanted by the clients;
- The remarks and suggestions concerning TCB strains useful for the BCCM collections;
- The quality of the provided information concerning the manipulation of TCB strains (use, storage);
- The quality of recommendations for norms application;
- Conditions of application of TCB strains (packaging, transportation, use);

- The satisfaction in registered results in the norms implementation (reproducibility of results, level of confidence);
- The preservation of TCB strains (techniques, duration, stability).

The addresses of (potential) clients to whom the inquiry was sent, was collected from:
- Our own BCCM clients databases: buyers/users of TCB strains;
- Beltest website: list of accredited laboratories and companies;
- Federal, Walloon and Flemish Ministries: list of approved laboratories and companies;
- CD-Rom Kompass: selected addresses in Belgium in relevant sectors (cosmetics and pharmaceutics).

The inquiry form, presented in the following pages, was sent by fax or by post.

Re: Use of microbial reference material **FAXNR:**

Dear ,

▶ As you may know, **BCCM** (Belgian Coordinated Collections of Microorganisms) constitute a consortium of complementary research-based service culture collections co-financed by the Belgian Federal Office for Scientific, Technical and Cultural Affairs (OSTC). One of the main tasks of the BCCM consortium is to collect, to preserve and to distribute cultures of bacteria, fungi and yeasts.
Certain microbial strains are imposed to validate test methods described in **national and international norms/standards** (BN, ISO, EN, FN, Pharmacopoeia...) which are applied in various sectors performing activities related to microbiology. Most of these strains are available at the BCCM collections.
Concerning **these microbial test strains**, BCCM/LMG (bacteria), BCCM/IHEM (medical yeasts, fungi) and BCCM/MUCL (agro-industrial yeasts, fungi) wish to meet the needs of the laboratories and industrial companies involved.

▶ In this frame, BCCM has elaborated a **project** in concertation with the Belgian accreditation body (BELTEST) and the Belgian Institute for Standardization (BIN-IBN), and which is financed by OSTC:
The aims are:

▷ to build a quality control system on the microbial reference material most frequently used. Test methods will be established to check these strains by BCCM on their performance (properties) in relation to the official norms/standards. This will allow BCCM to distribute the strains with a BCCM attestation.

▷ to propose reference strains to validate the test methods described in the official norms which are not imposing specific cultures. These strains will be subjected to the quality control system mentioned above.

▷ to meet the needs of the laboratories that do not have the infrastructure to preserve the reference strains at an optimal low temperature or that do not have experience in performing entrance control on these cultures. Therefore the project will study the possibility to prepare large working stocks of microbial test strains checked on their performance (properties) to be distributed to the laboratories in a userfriendly form, on a regular basis and with a BCCM attestation.

▶ The BCCM collections wish to focus their activities during this project on **your specific needs**.
Therefore, **if you are performing microbiological analyses**, we would appreciate it if you could spare us a few minutes to complete this inquiry and to fax it back to the fax number given below, preferably this week. Suggestions on any aspects of microbial reference material are welcome.

Please reply to fax nr: +32/9/264 53 46

We already thank you for your cooperation and await your reply with interest.

Mrs. K. Vanhonacker

Project Coordination
BCCM/LMG Bacteria Collection
K.L. Ledeganckstraat 35, B-9000 Gent
Belgium

Laboratory :

Name :

▶ General

1. Do you know the BCCM collections ? ☐ yes ☐ no
2. Did you already make use of the services of the BCCM collections? ☐ yes ☐ no
3. Your laboratory is ☐ accredited
   ☐ certified
   ☐ working on an accreditation
   ☐ working on a certification
   ☐ none of these
4. The quality system in your laboratory is based on (e.g. ISO 9002, EN 45001, GLP,...):.....
   .....
   .....
   .....
5. Your laboratory is active in the sector :
   ☐ animal feed ☐ environment
   ☐ biodegradation ☐ human food
   ☐ clinical sector ☐ pharmacy
   ☐ cosmetics ☐ other:.....
6. Do you use microbial reference strains? ☐ yes ☐ no

▶ Microbiological analyses

7. Concerning the microbiological analyses performed and the norms/standards applied at your laboratory, please complete columns 1 and 2 of the table on page 3.
8. Concerning the culture media used at your laboratory, please complete column 3 of the table on page 3. Please add what media you buy under a ready-to-use* form with a manufacturer's certificate.
9. Concerning the microbial reference strains used at your laboratory, please complete column 4 of the table on page 3.

▶ Culture Media

10. Do you check the batches of culture media you prepared yourself? ☐ yes ☐ no
11. Do you check the ready-to-use*, certified culture media? ☐ yes ☐ no
12. The checks you perform on the culture media consist of:
    ☐ checking colour ☐ checking sterility
    ☐ checking pH ☐ checking microbiological efficiency

▶ Microbial reference strains

13. The microbial reference strains you use are provided by:
    ☐ the BCCM collections
    ☐ other public culture collections
    Please specify: .....
    ☐ producer/private company: ☐ Oxoid (Cultiloops)
    ☐ RIVM (gelules)
    ☐ other:.....
14. Do you prepare a working stock from the microbial reference strains bought? ☐ yes ☐ no
15. How do you preserve this working stock? ☐ -20°C ☐ active by subculturing
    ☐ -80°C ☐ lyophilized
    ☐ other:.....
    .....

* such as agar plates, tubes or bottles with solidified agar or liquid medium

16. If the working stock is below minimum,
    do you prepare a new working stock from the remaining working stock? ☐ yes ☐ no
    or do you buy a new microbial reference strain for preparing a new stock? ☐ yes ☐ no
    In the latter case, at which interval? ☐ 6 monthly ☐ yearly ☐ other:.....
17. Which checks do you perform on the microbial reference strains you buy?
    ☐ upon receipt: ☐ viability ☐ identification
    ☐ purity ☐ performance
    ☐ none ☐ other:.....
    .....
    .....
    ☐ after preparing the working stock:
    ☐ viability ☐ identification
    ☐ purity ☐ performance
    ☐ none ☐ other:.....
    .....
    .....
18. The checks you perform on the microbial reference strains are based on:
    ☐ official norm(s)/standard(s) ☐ literature
    ☐ own experience ☐ other:.....
    .....
    .....
19. Which checks on the microbial reference strains do you want to be performed and do you want to be guaranteed on an attestation by the BCCM collections?
    ☐ viability ☐ identification
    ☐ purity ☐ performance
    ☐ none ☐ other:.....
    .....
    .....
20. What remarks do you have on handling, checking and preserving the microbial reference strains?
    .....
    .....
    .....
21. What remarks did you receive from the Belgian accreditation body (BELTEST) or any certifying institute on handling, checking and preserving microbial reference strains ? .....
    .....
    .....
    .....
22. What information would you like to receive from the BCCM collections on handling, checking and preserving the microbial reference strains?.....
    .....
    .....
    .....

▶ Project

23. If the BCCM will organise a central working stock of well controlled (with attestation) microbial reference strains combined with a fast delivery system on a regular basis for direct use, would you be interested to make use of it? ☐ yes ☐ no
    Please indicate on the table for what microbial reference strains (column 5) and at what interval (column 6).
24. What remarks or suggestions in the frame of this project do you want to give to the BCCM collections? .....
    .....
    .....
    .....

(1) **Description of the analysis**	(2) **Corresponding norm/standard**	(3) **Culture medium used**		(4) **Microbial reference material**		(5)	(6) **w=weekly m=monthly 6m=6-monthly y=yearly other: *please specify***
			Ready-to use	**Species name**	**Strain reference**		
Examples: Enumeration of *Bacillus cereus (Colony-count technique at 30°C)*	*ISO 7932/1993*	*MYP agar* *Glucose agar* *VP medium* *Nitrate medium*	X X X X	*Bacillus cereus (positive control)* *Bacillus coagulans (negative control)*	*LMG 8221* *LMG 6326*	X X	w w
Detection of fungicide activity	*NF EN 1275*	*GEM*	-	*Candida albicans* *Aspergillus niger*	*ATCC 10231* *ATCC 16404*	X X	m m

2.2 Results

2.2.1 Results of the collection of data

BCCM/IHEM has gathered four standards and six reference methods using 13 strains in the field of health. BCCM/LMG has listed 78 standards using 47 strains in the food industry sector. Concerning resistance of materials, 27 standards and 73 strains have been collected by BCCM/MUCL.

2.2.2 Results of the inquiry

The number of dispatches and replies about the inquiry are presented in Table I.

Table I: number of dispatches and replies about the inquiry.

Clients	IHEM	LMG	MUCL	Potential	Total
Number of dispatches	31	173	87	331	571
Number of replies	6 (19%)	53 (31%)	17 (20%)	27(8%)	85 (15%)

General information
- 73% of the potential clients didn't know the BCCM collections (especially in the clinical sector);
- Accreditation is the most used quality system;
- TCB strains are used by a lot of laboratories.

Culture media
- 75% of the laboratories check their homemade culture media. Only 34% verify the bought ready-to-use culture media batches. 80% of the laboratories inspect the microbiological efficiency.

TCB strains
- Private companies supply TCB strains more frequently than public culture collections.
- Among the various international public culture collections, BCCM is the most cited (63% of the respondents are clients from BCCM).
- More than 80% of the laboratories prepare a working stock from the bought TCB strains. This working stock is mainly preserved at –20°C or kept active by sub-culturing. Approximately two thirds of the laboratories buy a new microbial reference strain for preparation of a new working stock.
- The checks performed on the microbial reference strains by the laboratories are particularly based on standards. The second source is the literature.
- The laboratories want that identification, purity, viability and performance checks (in order of importance) be performed on the reference strains by BCCM.
- More than 80% of the respondents are interested in using a central working stock of well-controlled microbial reference strains.

Microbiological analyses
- The used standards are mostly methods of detection and enumeration of micro-organisms in food and water microbiology and standards relative to antiseptics and chemical disinfectants.
- Among the used TCB strains, bacteria hold a dominant place in comparison with fungi and yeasts.

Emerging needs or remarks
- Freeze-dried ampoules are not easy to use, users prefer ready-to-use strains;
- Some users prefer to get TCB strains with a quantitative guarantee;
- The users wish to receive as much information as possible on the strains, especially on the conditions for preservation of the strains;

- The interest of the users in this project is function of the price of the controlled TCB strains.

2.3 Conclusions

From the inquiry, it becomes clear that routine laboratories need TCB strains delivered in a user-friendly form with a guaranteed storage life (expiry date). Therefore the partners of the project have decided to study the possibility to deliver the TCB strains under frozen conditions in cryovials containing 25 ceramic cryobeads. The strains are thus easier to handle and the 25 cryobeads constitute the working stock that can be preserved at -20°C. The BCCM collections check these stocks for their viability, their purity, their identification and their performances (point 3).

The three criteria used to select the standards and the strains are: (1) the number of buyers/users and potential clients, (2) the norms and TCB strains most commonly applied according to the inquiry, (3) the technical feasibility.

BCCM/IHEM has selected four standards and two methods of the European pharmacopoeia. The performances of 4 fungal and 6 yeast strains were checked. BCCM/LMG has selected 37 bacterial TCB strains available in freeze-dried form from their public collection, to check their identity and their performances according to 48 standards used in the food sector and two methods of the European pharmacopoeia. BCCM/MUCL has selected 20 non-sporulating basidiomyceta involved in the wood degradation to check their performances according to four European standards, and nine sporulating fungi to check their viability and their purity on cryobeads.

The complete information is presented in Table II; the selected norms that will be studied and which constitute the scientific and technical activities and the other norms collected that will be not studied because they don't fulfil the criteria quoted above.

Table II: Relevant standards and number of microbial strains specified for each standard.

	Health		
	Standard number	Title	Number of strains
Selected norms	EN 1275	Chemical disinfectants and antiseptics – Basic fungicidal activity (phase 1)	2
	EN 1650	Chemical disinfectants and antiseptics - Quantitative suspension test for the evaluation of fongicidal activity of chemical disinfectants and antiseptics used in food, industrial, domestic, and institutional areas – Test method and requirements (phase 2, step 1)	4
	EN 1657	Chemical disinfectants and antiseptics - Quantitative suspension test for the evaluation of fongicidal activity of chemical disinfectants and antiseptics used in veterinary field – Test method and requirements (phase 2, step 1)	2
	European Pharmacopoeia 2.6.1	Sterility test	2
	European Pharmacopoeia 2.6.12	Microbiological examination of non-sterile products (Total viable aerobic count)	2
	NF T 72-281	Disinfectants. Methods of airborne disinfection of surfaces – Determination of bactericidal, fongicidal and sporicidal activity	2
Other norms collected	European Pharmacopoeia 2.7.2	Microbiological assay of antibiotics	2
	NCCLS M27-A	Reference method for broth dilution antifungal susceptibility testing of yeasts	6
	USP 24/ NF 19 <71>	Sterility tests	2
	USP 24/NF 19 <81>	Biological tests and assays - Antibiotics	1

	Food industry		
	Standard number	Title	Number of strains
Selected norms	AFNOR 3M-01/2-09/89	Petrifilm Coliform Count Plate	
	AFNOR 3M-01/5-03/97	Petrifilm Rapid Coliform Count Plate	
	AFNOR 3M-01/6-09/97	Petrifilm *Enterobacteriaceae* Count Plate	
	AFNOR 3M-01/7-03/99	Petrifilm High-Sensitivity Coliform Count Plate	
	AFNOR 3M-01/8-06/01	Petrifilm Select *E. coli* Count Plate	
	AFNOR BIO-12/5-01/99	Coli-ID	
	AFNOR SDP 07/01-07/93	Rapid *E. coli* 2	2
	EN 12824	Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Salmonella*	1
	FDA-BAM p. 6.01-6.06	*Shigella*	
	FDA-BAM p. 9.01-9.27	*Vibrio*	
	FIL 138	Dried milk – Enumeration of *Staphylococcus aureus* – Colony count technique at 37°C	
	FIL 143A	Milk and milk products – Detection of *Listeria monocytogenes*	
	FIL 145A	Milk and milk-based products – Enumeration of coagulase-positive *Staphylococci* – Colony count technique	
	FIL 73B	Milk and milk products – Enumeration of Coliforms – Part 1: Colony count technique at 30°C without resuscitation	
	FIL 93B	Milk and milk products – Detection of *Salmonella*	
	ISO 10272	Microbiology of food and animal feeding stuffs – Horizontal method for detection of thermotolerant *Campylobacter*	2
	ISO 10273	Microbiology – General guidance for the detection of presumptive pathogenic *Yersinia enterocolitica*	1
	ISO 11290	Microbiology of food and animal feeding stuffs – Horizontal method for the detection and enumeration of *Listeria monocytogenes*	7
	ISO 11866-3	Milk and milk products – Enumeration of presumptive *Escherichia coli* – Part 3: Colony-count technique at 44°C using membranes	1
	ISO 13720	Meat and meat products – Enumeration of *Pseudomonas* spp.	2
	ISO 4832	Microbiology – General guidance for the enumeration of Coliforms – Colony count technique	2
	ISO 6340	Water quality – Detection of *Salmonella* species	6
	ISO 6579	Microbiology – General guidance on methods for the detection of *Salmonella*	2
	ISO 6888	Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of coagulase-positive *Staphylococci*	5
	ISO 7402	Microbiology – General guidance for the enumeration of *Enterobacteriaceae* without resuscitation – MPN technique and colony-count technique	2
	ISO 7899-2	Water quality – Detection and enumeration of intestinal *Enterococci* – Part 2: Membrane fltration method	4
	ISO 7932	Microbiology – General guidance for the enumeration of *Bacillus cereus* – Colony-count technique at 30°C	3
	ISO 7937	Microbiology of food and animal feeding stuffs – Horizontal method for enumeration of *Clostridium perfringens* – colony count technique	3
	ISO 9308-1	Water quality – Detection and enumeration of Coliform organisms, thermotolerant Coliform organisms and presumptive *Escherichia coli* – Part 1: Membrane filtration method	8
	Mossel p. 416-418	Test for *Enterococcus* species	
	Mossel p. 421-422	Test for *Shigella* species	
	NF V04-503	Meat and meat products – Enumeration of lactic bacteria	7
	NF V04-504	Microbiology of food and animal feeding stuffs – Enumeration of *Pseudomonas* spp. in meats and meat products	5
	NF V08-050	Microbiology of food and animal feeding stuffs – Enumeration of Coliforms by colony-count technique at 30°C – Routine method	3
	NF V08-052	Microbiology of food and animal feeding stuffs – Detection of *Salmonella* – Routine method	5
	NF V08-053	Food Microbiology – Enumeration of b-glucuronidase positive *Escherichia coli* by colony count technique at 44°C – Routine method	3
	NF V08-054	Microbiology of food and animal feeding stuffs – Enumeration of the Enterobacteria by colony count technique at 30°C – Routine method	5

	NF V08-055	Microbiology of food and animal feeding stuffs – Detection of *Listeria monocytogenes* – Routine method	5
	NF V08-056	Food microbiology – Enumeration of *Clostridium perfringens* by colony count technique at 37°C – Routine method	2
	NF V08-057	Food microbiology – Routine method for enumeration of coagulase positive *Staphylococcu*s by colony-count technique at 37°C	4
	NF V08-060	Microbiology of food and animal feeding stuffs – Enumeration of thermotolerant Coliforms by colony-count technique at 44°C – Routine method	3
	pr ISO/TS 11133-2	Microbiology of food and animal feeding stuffs – Guidelines on preparation and production of culture media – Part 2: Practical guidelines on performance testing of culture media (under approval – not yet published)	
	SP-VG M001	Microbiology of food – Method for detection of presumptive pathogenic *Escherichia coli* O157 (2000)	1
	SP-VG M002	Microbiology of meats and meat products – Method for detection of *Salmonella*	
	SP-VG M003	Microbiology of food of animal origin – Method for detection of thermotolerant *Campylobacter* (1998)	1
	SP-VG M004	Microbiology of meats and meat products – Method for the detection of *Yersinia enterocolitica* O:3	
	SP-VG M006	Microbiology of food – Method for the detection of *Vibrio cholerae* or *Vibrio parahaemolyticus*	
	XP V08-058	Food and animal feeding stuffs microbiology -Enumeration of *Bacillus cereus* by colony-counting technique at 30°C – Routine method	5
Other norms collected	AFNOR 3M 01/3-07/92	Kit Tecra *Salmonella*	2
	AFNOR BIO 12/1-04/94	Vidas *Salmonella*	2
	AFNOR BIO 13/3-03/96	Vidas *Listeria monocytogenes*	2
	AFNOR SDP 07/04-09/98	Rapid L. mono	2
	Belgian Pharmacopoeia V.2.1.1	Sterility	2
	BS 541	Determination of Rideal Walker coefficient	1
	BS 6471	Method for determination of the antimicrobial value of QAC disinfectant formulations	1
	DIN 10106	Microbiological analysis of meat and meat products; determination of *Enterococcus faecalis* and *Enterococcus faecium*; spatula method (reference method)	2
	EN 1174	Sterilization of medical devices. Estimation of the population of micro-organisms on product.	6
	EN 1276	Chemical disinfectants and antiseptics - Quantitative suspension test for the evaluation of bactericidal activity of chemical disinfectants and antiseptics used in food, industrial, domestic and institutional areas - Test method and requirements (phase 2, step 1)	7
	EN 26461	Water quality. Detection and enumeration of the spores of sulfite-reducing anaerobes ({*Clostridia*}).	4
	EN 6265	*Legionella*	2
	EN 6573	Counting of *Pseudomonas*	1
	European Pharmacopoeia 2.6.13	Microbiological examination of non-sterile products (test for specified micro-organisms)	7
	ISO 11731	Water quality. Detection and enumeration of *Legionella*.	1
	ISO 13722	Meat and meat products. Enumeration of Brochothrix THERMOSPHACTA. Colony-count technique.	2
	ISO 17025	General requirements for the competence of testing and calibration laboratories	4
	ISO 4833	Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of microorganisms - Colony-count technique at 30° C	3
	ISO 6222	Water quality. Enumeration of culturable micro-organisms. Colony count by inoculation in a nutrient agar culture medium.	2
	ISO 7899	Water quality - Detection and enumeration of intestinal *enterococci*	1
	ISO 7954	Microbiology. General guidance for enumeration of yeasts and moulds. Colony count technique at 25 degrees C.	1
	Kirby-Bauer/NCCLS	Antibiogram	6
	NBN V21 029	Counting of *Streptococcus thermophilus*	2
	NF T90-421	Testing water. Bacteriological examinations of swimming pool water.	1
	NMKL 71	Detection of *Salmonella*	2
	prEN 12780	Water quality - Detection and enumeration of *Pseudomonas aeruginosa* by membrane filtration	1
	SP-VG M005	Counting of total anaerobic micro-organisms	1

	V08-051	Microbiology of food and animal feeding stuffs. Enumeration of microorganisms by colony-count technique at 30 degrees Celsius. Routine method.	4
	XP V08-059	Microbiology of food animal feeding stuffs - Enumeration of yeasts and moulds by colony-count technique at 25 °C - Routine method	2
	XP V08-061	Counting of sulphite reducing anaerobic micro-organisms	2

	Materials		
	Standard number	Title	Number of strains
Selected norms	EN 113	Wood preservatives - Test method for determining the protective effectiveness against wood destroying basidiomycetes - Determination of the toxic values	14
	EN 350-1	Durability of wood and wood-based products - Natural durability of solid wood - Part 1: Guide to the principles of testing and classification of the natural durability of wood	4
	ENV 839	Wood preservatives - Determination of the preventive efficacity against wood destroying basidiomycete fungi	4
	ENV 12038	Durability of wood and wood-based products - Wood-based panels - Method of test for determining the resistance against wood-destroying basidiomycetes	10
Other norms collected	ASTM D3273	Standard Test Method for Resistance to Growth of Mold on the Surface of Interior Coatings in an Environmental Chamber	3
	ASTM G21	Standard Practice for Determining Resistance of Synthetic Polymeric Materials to Fungi	5
	BS 1982-1	Fungal resistance of panel products made of or containing materials of organic origin Part 1. Method for determination of resistance to wood-rotting Basidiomycetes	6
	BS 1982-3	Fungal resistance of panel products made of or containing materials of organic origin Part 3. Methods for determination od resistance to mould or mildew	7
	BS 3900	Part G6. Assessment of the resistance to fungal growth	9
	BS 6085	Methods for determination of the resistance of textiles to microbiological deterioration	8
	EN 1104	Paper and board intented to come into contact with foodstuff - Determination of transfer of antimicrobic constituents	1
	EN 152	Test methods for wood preservatives; Laboratory method for determining the preventive effectiveness of a preservative treatment against blue stain in service	2
	ENV 12404	Durability of wood and wood-based products - Assessment of the effectiveness of a masonry fungicide to prevent growth into wood of Dry Rot *Serpula lacrymans* (Schumacher ex Fries) S. F. Gray - Laboratory method	1
	ENV 807	Wood preservatives - Determination of the toxic effectiveness against soft rotting micro-fungi and other soil inhabiting micro-organisms	6
	IEC 68-2-10	Basic environmental testing procedures Part 2: Tests - Test J and guidance: mould growth	8
	ISO 846	Plastics - Evaluation of the action of microorganisms	9
	NF B51-295	Particle boards - Test method for determining rot resistance (Basidiomycetes fungi)	4
	NF T72-083	Products for protecting wood surfaces - Method of testing resistance to microorganisms	10
	NF X41-513	Protection des matières plastiques 1e partie Methode d'essai de resistance des constituants aux microorganismes	12
	NF X41-515	Protection des matières plastiques 3e partie Methode d'essai de resistance des matériels et appareillages aux microorganismes	12
	NF X41-517	Protection du papier Methode d'essai des propriétés fongistatiques des papiers et cartons	10
	NF X41-520	Protection. Method of test of the resistance of paints to micro-organisms and their protective capacity	10
	NF X41-547	Wood preservatives - Determination of fongicide efficacity of temporary wood protectives for green sawn timber - Laboratory method	8
	NF X41-548	Wood preservatives - Determination of fongicide efficacity of temporary wood protectives for fresh cut wood billets - Laboratory method	8
	NF X41-555	Wood preservatives - Determination of the toxic values against *Chaetomium globosum* Kunze - Soft rotting agent	1
	NF X41-600	Tests for the resistance to micro-organisms of natural or artificial cellulose textiles Method by mixed inoculation (spores/mycelium)	10
	NF X41-603	Protection of textiles - Method of test for the resistance to microorganisms of natural fibre ropes and cordage intented for marine use	12

3 PRESERVATION OF THE TCB STRAINS ON CRYOBEADS

3.1 Methodology

3.1.1 Introduction

To meet the needs of the laboratories which do not have the infrastructure to preserve the reference strains at an optimal ultra-low temperature or which prefer not to perform entrance controls on these cultures, the possibility was studied to prepare at BCCM large working stocks of microbial reference strains checked on their identity and performances, to be distributed to the laboratories in a user-friendly form, on a regular basis and with a guaranteed storage life (expiry date).

The viability and the stability of the relevant features of some TCB strains on cryovials preserved at –20°C was followed in time to guarantee the storage life. When using reference strains it is recommended to go back to the distributor on a regular basis (Beltest advice: every 1 or 2 year). For that reason a storage life of 1 or 2 years was pursuit.

Following tests were performed during the assumed storage life of 1 or 2 years:
- Viability tests at regular intervals (statistical evaluation)
- Performance tests at the beginning and the end of the preservation period (according the norms) (see 4 Performance testing)

The result of the counts would make it possible to evaluate the possibility to deliver the TCB strains with a known range of colony forming units (CFU).

3.1.2 Working method

The selected microbial TCB strains available in freeze-dried form from the public BCCM collections, checked for viability, purity and identification, were rehydrated and cultivated on a general medium. For each strain an experimental stock was prepared in cryovials with 25 cryobeads and preserved at –20°C during 1-2 years, simulating the suboptimal conditions used by the customer.

To test the viability, counts were performed at regular times on a subset of the experimental stocks. BCCM/IHEM selected 2 strains for the subset, BCCM/LMG 7 strains and BCCM/MUCL 9 strains. For each of these strains, 20 cryovials with cryobeads were prepared and frozen at –20°C. From these cryovials, 10 cryovials were used to perform the counts and from those 10 cryovials 3 or 2 beads were taken to count the CFU.
The microbial cells of each bead were subjected to specific dilutions depending on the organism. From certain dilutions, a quantity inoculum was spread over the surface of the agar plates according to the organism. After incubation of the agar plates, the colonies on each plate were counted and the CFU per bead were calculated.
These counts were repeated at regular intervals during a period of 1-2 years.

BCCM/LMG: A subset of the experimental stock (5 vials used for the counts) was brought 25 times for 5 minutes at room temperature after a preservation period of 1 year to simulate the use by the client as good as possible.
From the 37 selected bacterial TCB strains, 7 were tested on viability when performing the counts. For the other strains, the viability was tested by performing the performance tests at the beginning and the end of the preservation period (point 4 Performance testing).

BCCM/IHEM checked the effect on the viability when defrosting cryovials from the 2 strains during 48 hours after a preservation period of 5 months to examine the impact on the viability of the strains when sending them to the customer without dry ice.

Prof. Palm of the University of Gembloux (Faculté Universitaire des Sciences Agronomiques, Unité de Statistique et Informatique) made a statistical processing of the first set of counts (after 0 month preservation). He performed a variation analysis ANOVA.

3.2 Results of the viability tests: counts

- The results of the 2 selected strains from BCCM/IHEM:

The conservation method is better appropriate to the yeast *C. albicans* IHEM 3731 (Figure 1) then the filamental fungus *A. niger* IHEM 3766 (Figure 2). For this last strain, the cryobeads proved to be less charged with micro-organisms from the beginning and the viability reduced more rapidly. The recovery of *A. niger* demonstrated at several repetitions numbers smaller then 100 CFU/cryobead while *C. albicans* had always numbers bigger then 1000 CFU/cryobead.
This preservation method is provided for a preservation at –80°C and not –20°C by the producer of the cryovials. The temperature of –20°C was chosen to meet the needs of our customers.

The defrosting of the cryovials during 48 hours had no effect on the strain *A. niger* while the yeast *C. albicans* lost its viability for a part or completely.

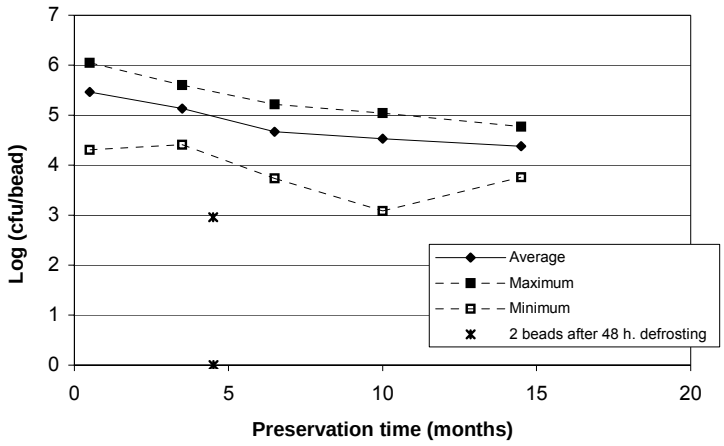


Figure 1: evolution of the viability for *Candida albicans* IHEM 3731.

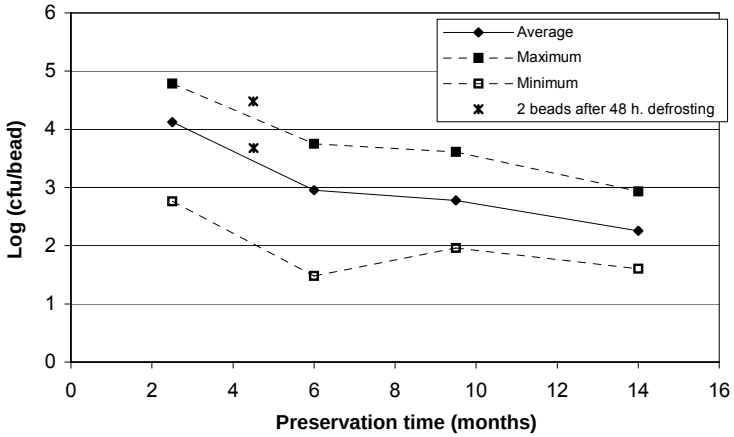


Figure 2: evolution of the viability for *Aspergillus niger* IHEM 3766.

- The results of the 7 selected strains from BCCM/LMG:

For the strain *Bacillus cereus* LMG 8221 (Figure 3), there were almost no CFU recovered after a preservation of 18 months at –20°C. A reduction of a little less than factor 100 was determined after 18 months for *Pseudomonas aeruginosa* LMG 1242 (Figure 4). A reduction of a little more than factor 10 was determined after 12 months for *Escherichia coli* LMG 8223 (Figure 5), but after 18 months the reduction was less than factor 10. The reduction was less than factor 10 after a preservation of 18 months for the strains *Listeria monocytogenes* LMG 13305 (Figure 6), *Staphylococcus aureus* LMG 8224 (Figure 7), *Salmonella choleraesuis* subsp. *choleraesuis* LMG 14933 (Figure 8) and *Enterococcus faecalis* LMG 7937 (Figure 9).

Bacillus cereus LMG 8221 will not be available for the customer on cryovials with cryobeads because the recuperation after 1-2 years preservation at –20°C is very poorly.

Removing out 5 of the 10 vials 25 times during 5 minutes at room temperature has no effect on the number of CFU per bead (time of defrosting is indicated on the figures).

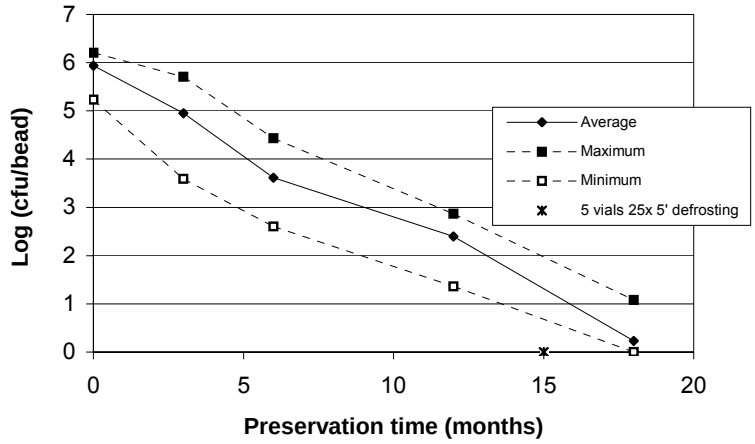


Figure 3: evolution of the viability for *Bacillus cereus* LMG 8221.

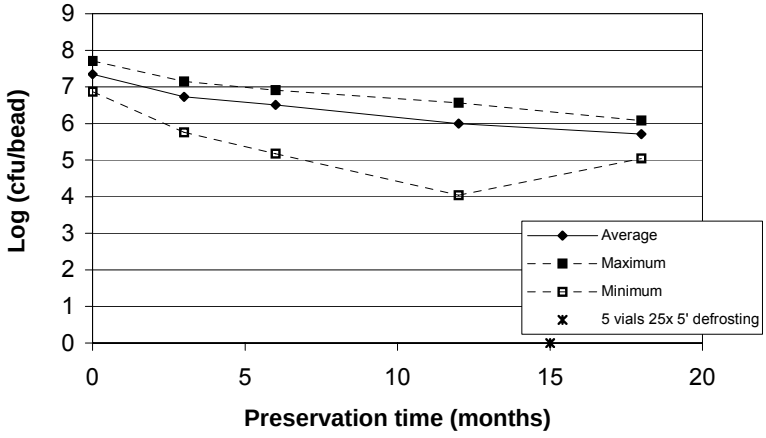


Figure 4: evolution of the viability for *Pseudomonas aeruginosa* LMG 1242.

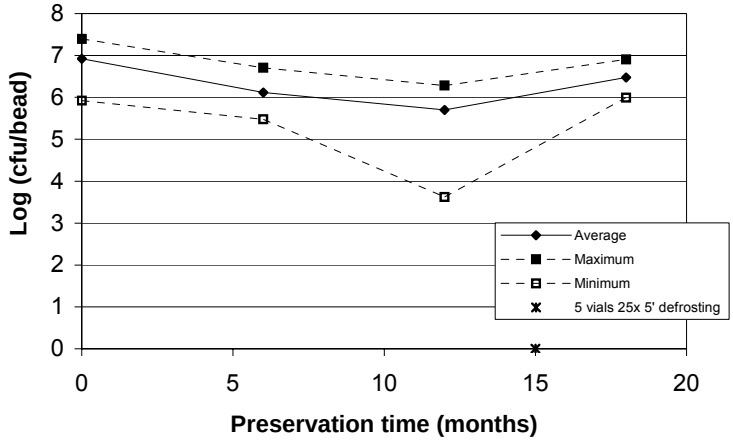


Figure 5: evolution of the viability for *Escherichia coli* LMG 8223.

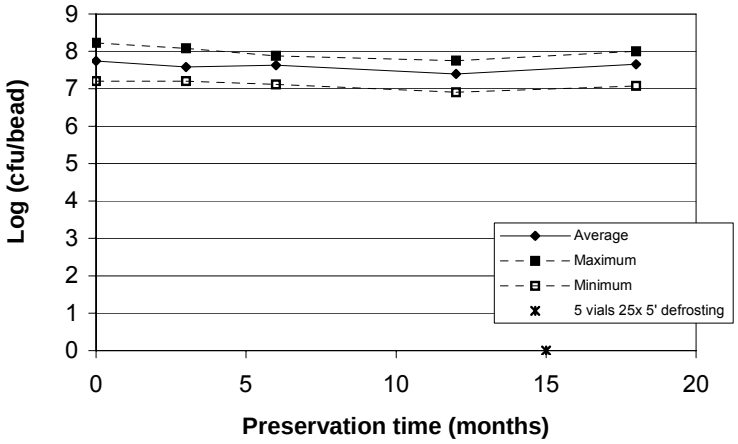


Figure 6: evolution of the viability for *Listeria monocytogenes* LMG 13305.

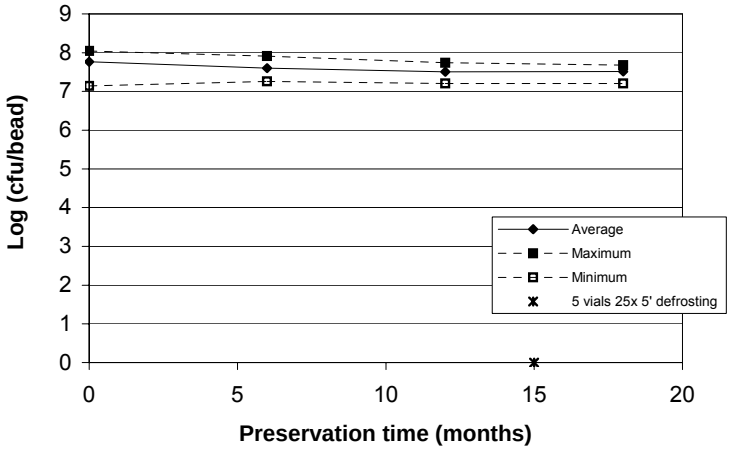


Figure 7: evolution of the viability for *Staphylococcus aureus* subsp. *aureus* LMG 8224.

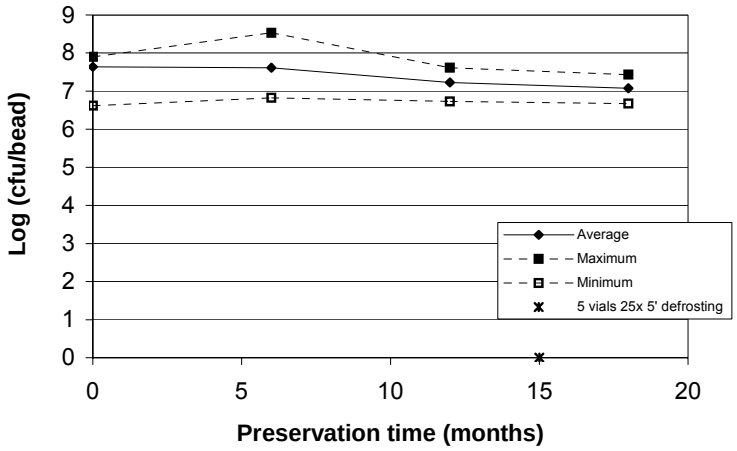


Figure 8: evolution of the viability for *Salmonella choleraesuis* subsp. c*holeraesuis* serotype *typhimurium* LMG 14933.

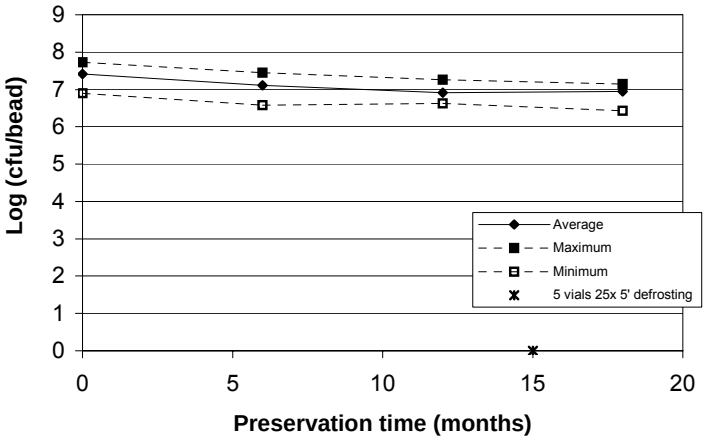


Figure 9: evolution of the viability for *Enterococcus faecalis* LMG 7937.

- The results of the 9 selected strains from BCCM/MUCL:

The reduction was less than factor 10 for the strains: *Aspergillus niger* MUCL 19002 (Figure 10), *Aureobasidium pullulans* MUCL 19020 (Figure 11), *Chaetomium globosum* MUCL 1984 (Figure 12), *Cladosporium herbarum* MUCL 19275 *(*Figure 13), *Paecilomyces variotii* MUCL 31697 (Figure 14), *Penicillium pinophilum* MUCL 38548 (Figure 15) and *Scopulariopsis brevicaulis* MUCL 31699 (Figure 16).A reduction of the CFU per bead of about factor 100 was determined after a preservation of 18 months at –20°C for *Stachybotrys chartarum* MUCL 19022 (Figure 17). For *Trichoderma virens* MUCL 31700 (Figure 18), there was a reduction of factor 1000 after 18 months preservation.

The conservation system of the cryobeads at –20°C were appropriate for the majority of the tested strains. Only the strains *S. chartarum* MUCL 19022 and *T. virens* MUCL 31700 show a regular and important loss of viability and even a total loss of viability for *T. virens* MUCL 31700.

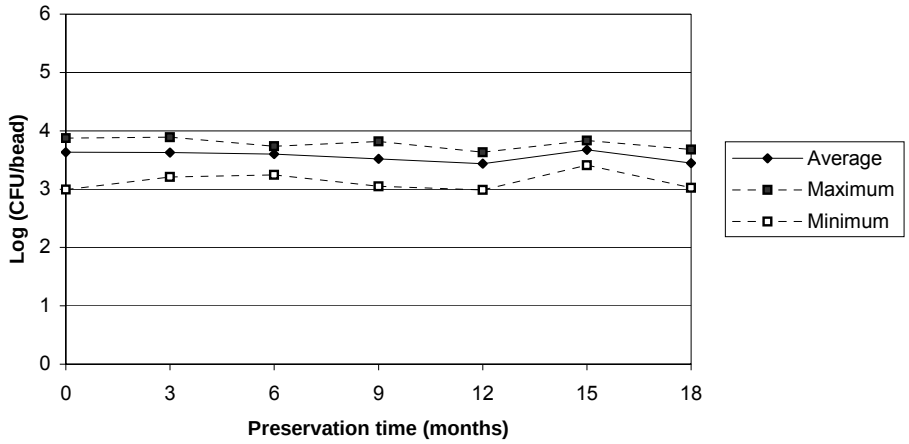


Figure 10: evolution of the viability for *Aspergillus niger* MUCL 19002.

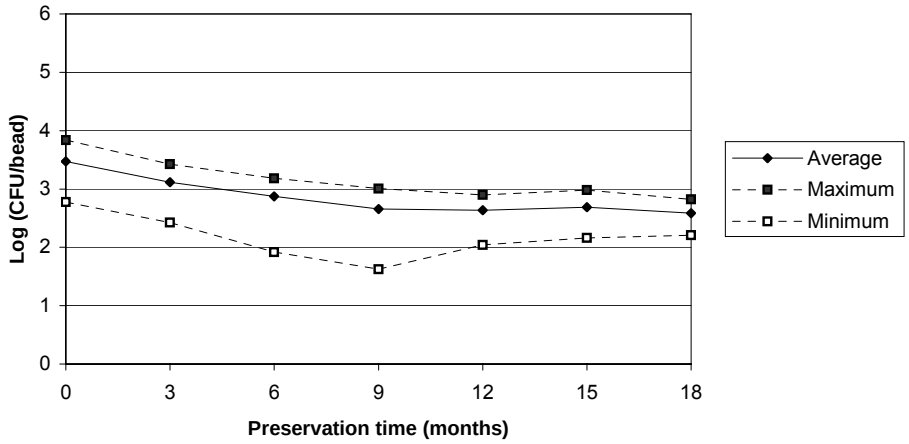


Figure 11: evolution of the viability for *Aureobasidium pullulans* MUCL 19020.

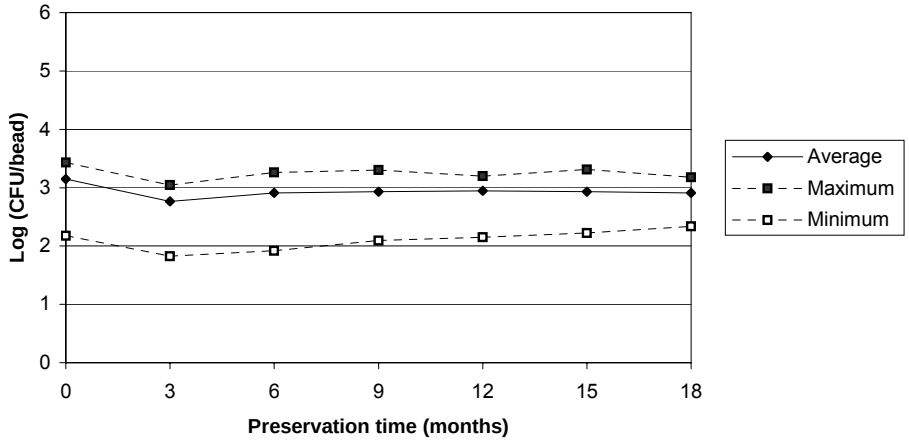


Figure 12: evolution of the viability for *Chaetomium globosum* MUCL 1984.

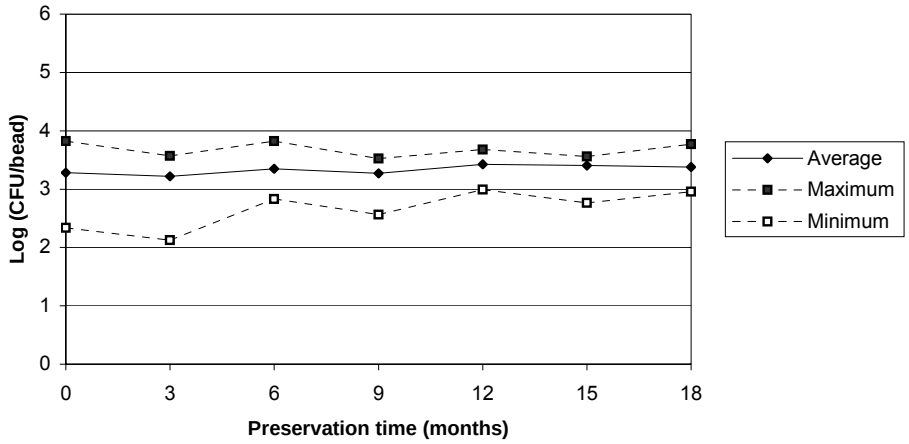


Figure 13: evolution of the viability for *Cladosporium herbarum* MUCL 19275.

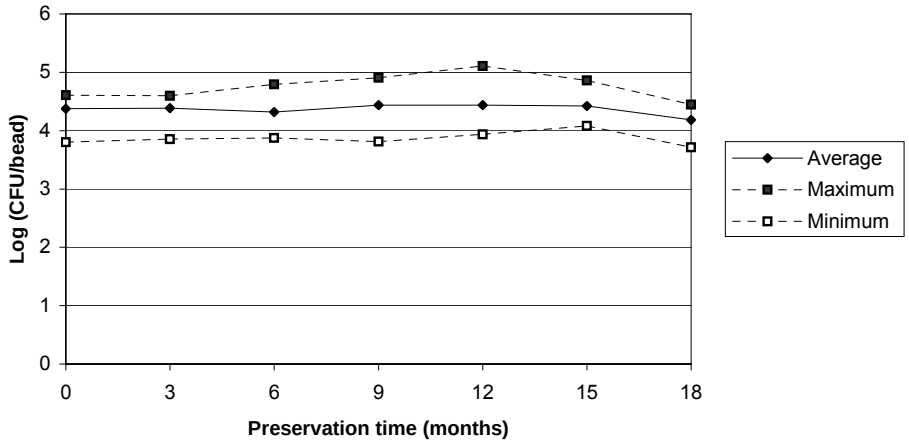


Figure 14: evolution of the viability for *Paecilomyces variotii* MUCL 31697.

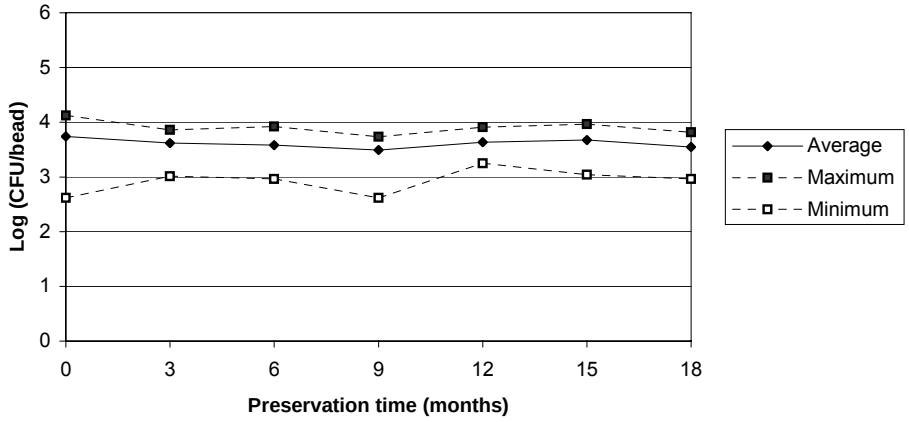


Figure 15: evolution of the viability for *Penicillium pinophilum* MUCL 38548.

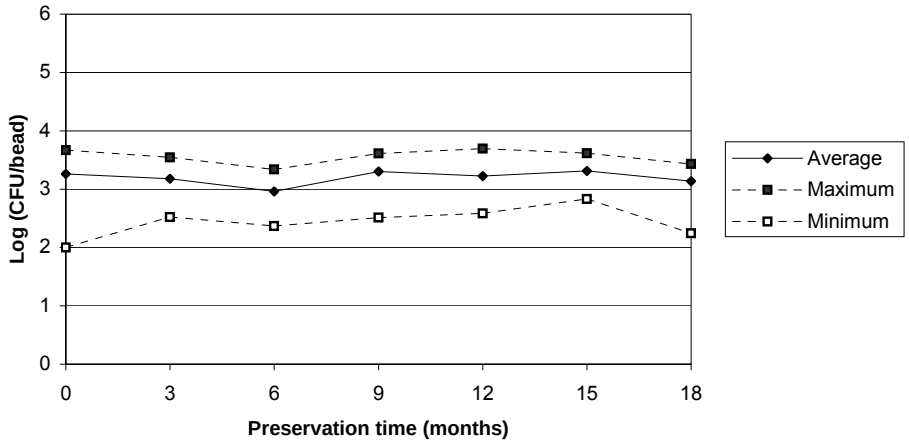


Figure 16: evolution of the viability for *Scopulariopsis brevicaulis* MUCL 31699.

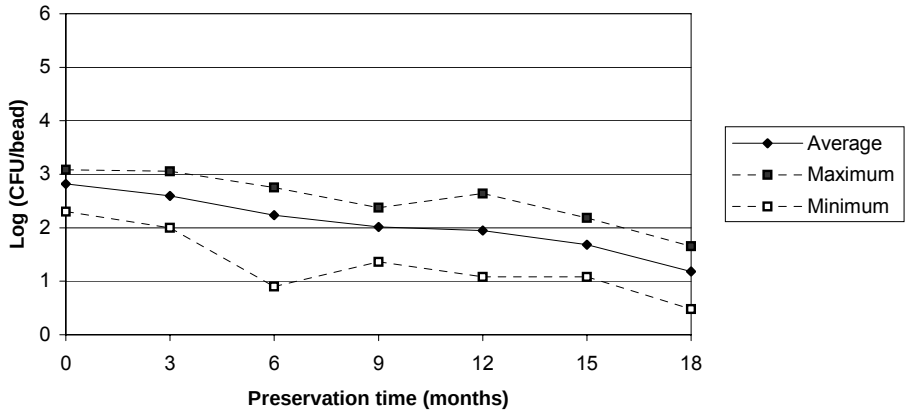


Figure 17: evolution of the viability for *Stachybotrys chartarum* MUCL 19022.

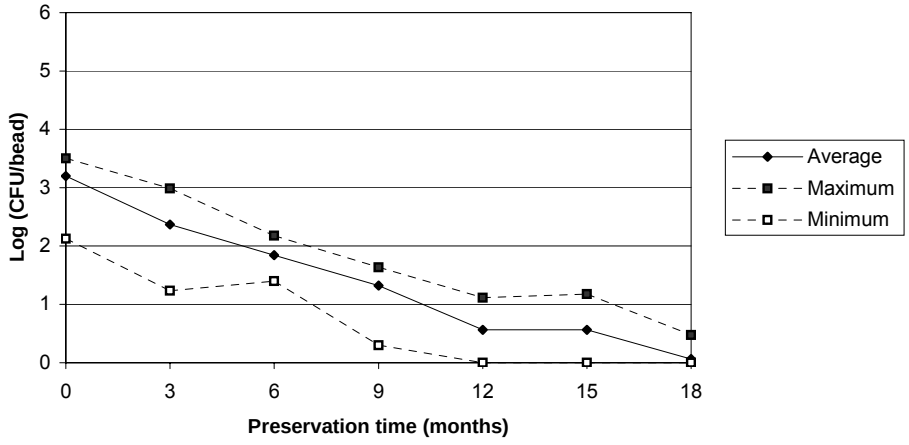


Figure 18: evolution of the viability for *Trichoderma virens* MUCL 31700.

- The results of the statistical analyses from Prof. Palm of the Gembloux Agricultural University:

The variation of the number CFU between the cryobeads from a TCB strain is for the greatest part due to the bead and not to the vial (the production of the beads is not standardized according to Pro-Lab Diagnostics). This means that the number of CFU can strongly vary if you take an arbitrary bead of an arbitrary vial.
So it is not possible to deliver the TCB strains on cryovials with a quantitative guarantee.

4 PERFORMANCE TESTING

4.1 TCB strains related to norms on antiseptics and disinfectants

4.1.1 Norms and strains considered

As explained in point 2.3, 4 norms in the field of antiseptics and disinfectants were selected to work on. Two to four strains are quoted in each, for a total of 6 different strains:

Strains quoted in norms NBN EN 1275 and NBN EN 1657:

Aspergillus niger	IHEM 3766	ATCC 16404
Candida albicans	IHEM 3731	ATCC 10231

Strains quoted in norms NBN EN 1650:

Aspergillus niger	IHEM 3766	ATCC 16404
Candida albicans	IHEM 3731	ATCC 10231
S. cerevisiae	IHEM 3961	ATCC 9763
S. cerevisiae var. *distacus*	HEM 17987	DSM 70487

Strains quoted in norms NF T72-281:

C. albicans	IHEM 3740	CIP 1180-79
P. aurantiogriseum var. *aurantiogriseum* (= *P. verrucosum* var. *cyclopium*)	IHEM 18001	CIP 1186-79

Moreover we decided to test also some others strains belonging to medically important species:

Aspergillus fumigatus	IHEM 10045
Aspergillus flavus	IHEM 5903
Candida glabrata	IHEM 17730
Candida tropicalis	IHEM 15905

4.1.2 Methodology

Testing the reference strains following a norm in the field of antifungal substances implies to test them against disinfectants. Such products were thus selected: hypochlorite as reference substance and 2 commercial compound products having satisfied to the norm following the manufacturer:

a) Bacterianos D: association of glutaraldehyde, glyoxal, formaldehyde and a quaternary ammonium compound.

b) Phagosept spray: biguanide and a quaternary ammonium compound in a propyl alcohol base.

The EN1275 norm "Chemical disinfectants and antiseptics – Basic fungicidal activity – Test method and requirements" is more basic than the 3 other selected and was studied first. The method is summarised in the following figure.

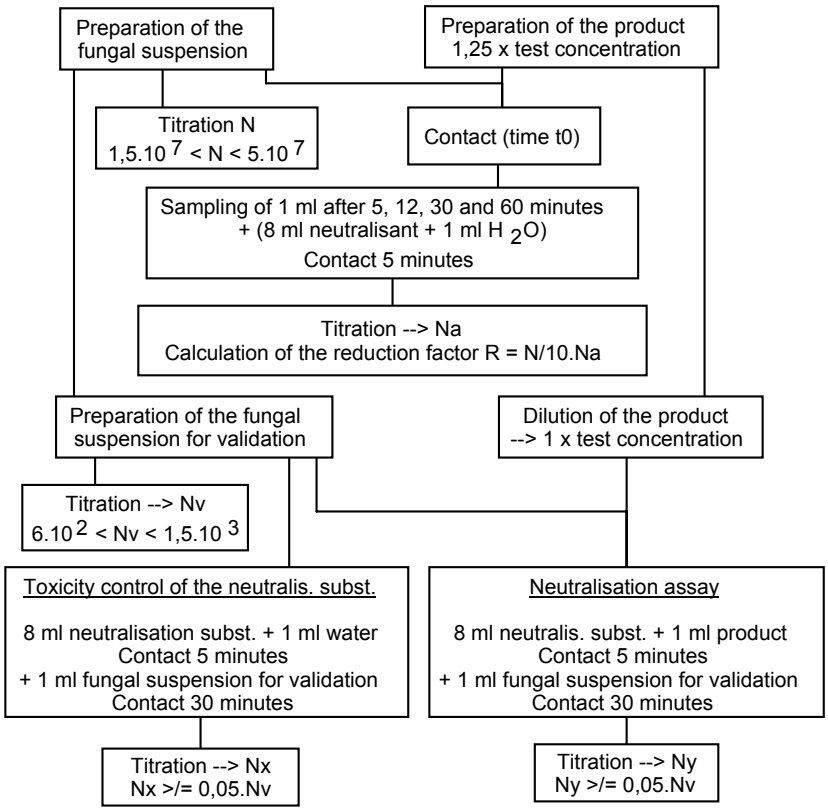


Figure 19: scheme of the operative procedure.

Following the normative text, the antifungal activity of a product is satisfactory when the reduction factor R is equal or higher then 10^4 for both reference strains. As we actually intended to test the strains and not a product, we have considered that a strain was sensitive to a given product, used at the recommended concentration by the manufacturer, when R reached or exceeded this value.

The principle of the norms EN1650 and EN1657 are similar to the previous one, apart from 2 parameters: hard water must be used and an interfering substance has to be add (bovine albumine, milk, … depending on the area of utilisation of the product), in order to test the antifungal products in bad conditions; it suggests also to add specific strains for specific areas as breweries. The norm NF T 72-281, adapted to antifungal products to be sprayed on surfaces, requires special equipment (a watertight chamber for example) and was first discarded for practical reasons.

As these experiments depends highly on the quality of commercial products (the first results obtained for EN1275 showed sometimes a difference with the assertions of the manufacturer), it was decided to replace the strict application of these methods to the strains by a characterisation of each strain determining its sensitivity limit to hypochlorite, chosen as reference antiseptic (Fleurette *et al.* 1995). This limit of sensitivity was established using the method of the norm EN1275 with a time of contact of 5 minutes; this was performed with the strains previously tested in the context of EN1275, with the optional strain of the norm EN1650 and with the strains from the norm NF T 72-281.

4.1.3 Results

EN1275

The results relative to the sensitivity of the strains to the 3 antifungal substances following the norm EN1275 are given in the following tables (light grey area correspond to sensitivity, dark grey area to resistance). It appeared clearly that all the strains were killed by hypochlorite, even after only 5 minutes, while filamentous fungi were not so easily inhibited by the 2 commercial products; especially *Aspergillus niger* survived after 60 minutes of contact with these antifungals.

Table III: sensitivity of the strains to hypochlorite.

Strains listed in EN1275	Reduction factor R			
	5 min.	15 min.	30 min.	60 min.
Aspergillus niger IHEM3766	$> 8.10^4$	$> 8.10^4$	$> 8.10^4$	$> 8.10^4$
Candida albicans IHEM3731	$> 2{,}5.10^5$	$> 2{,}5.10^5$	$> 2{,}5.10^5$	$> 2{,}5.10^5$
Other strains				
Aspergillus fumigatus IHEM10045	$> 6{,}4.10^4$	$> 6{,}4.10^4$	$> 6{,}4.10^4$	$> 6{,}4.10^4$
Aspergillus flavus IHEM5903	$> 1{,}5.10^5$	$> 1{,}5.10^5$	$> 1{,}5.10^5$	$> 1{,}5.10^5$
Candida glabrata IHEM17730	$> 1{,}3.10^5$	$> 1{,}3.10^5$	$> 1{,}3.10^5$	$> 1{,}3.10^5$
Candida tropicalis IHEM15905	$> 1{,}4.10^5$	$> 1{,}4.10^5$	$> 1{,}4.10^5$	$> 1{,}4.10^5$

Table IV: sensitivity of the strains to Bacterianos D.

Strains listed in EN1275	Reduction factor R			
	5 min.	15 min.	30 min.	60 min.
Aspergillus niger IHEM3766	$< 2.10^2$	$< 2.10^2$	$1{,}6.10^2$	$3{,}2.10^2$
Candida albicans IHEM3731	$6{,}7.10^4$	$> 10^6$	$> 10^6$	$> 10^6$
Other strains				
Aspergillus fumigatus IHEM10045	$4{,}8.10^3$	$5{,}5.10^4$	8.10^5	2.10^5
Aspergillus flavus IHEM5903	$< 3.10^3$	$< 3.10^3$	3.10^3	10^4
Candida glabrata IHEM17730	$> 1{,}2.10^5$	$> 1{,}2.10^5$	$> 1{,}2.10^5$	$> 1{,}2.10^5$
Candida tropicalis IHEM15905	$> 1{,}6.10^5$	$> 1{,}6.10^5$	$> 1{,}6.10^5$	$> 1{,}6.10^5$

Table V: sensitivity of the strains to Phagosept Spray.

Strains listed in EN1275	Reduction factor R			
	5 min.	15 min.	30 min.	60 min.
Aspergillus niger IHEM3766	$< 4.10^3$	$< 4.10^3$	$< 4.10^3$	$4{,}3.10^3$
Candida albicans IHEM3731	$> 3.10^5$	$> 3.10^5$	$> 3.10^5$	$> 3.10^5$
Other strains				
Aspergillus fumigatus IHEM10045	$1{,}4.10^4$	$3{,}2.10^4$	$1{,}4.10^5$	$2{,}4.10^6$
Aspergillus flavus IHEM5903	$< 10^4$	$1{,}5.10^4$	3.10^5	$1{,}8.10^6$
Candida glabrata IHEM17730	$> 1{,}1.10^5$	$> 1{,}1.10^5$	$> 1{,}1.10^5$	$> 1{,}1.10^5$
Candida tropicalis IHEM15905	$> 1{,}5.10^5$	$> 1{,}5.10^5$	$> 1{,}5.10^5$	$> 1{,}5.10^5$

Limit of sensitivity of the strains to hypochlorite

Some strains were confronted first with 3 concentrations of hypochlorite. Depending on this preliminary result, other concentrations were used in order to determine precisely the limit of sensitivity of each strain. In the table here under, results are presented, strains being classified by resistance to hypochlorite.

Table VI: sensitivity of the strains to various concentrations of hypochlorite.

Strains	Hypochlorite concentration (° chl)								
	2	1	0,5	0,25	0,1	0,05	0,025	0,01	0,005
A. niger IHEM 3766	7.10^6	4.10^5	$< 10^3$		$< 10^3$			$< 10^3$	$< 10^3$
A. flavus IHEM 5903	$> 10^6$	2.10^5	8.10^3	$< 10^3$	$< 10^3$				
A. fumigatus IHEM 10045		2.10^6	10^6	$3\ 10^5$	$< 10^3$				
P. aurantigriseum IHEM 18001		$> 10^6$	$> 10^6$	$> 10^6$	$< 10^3$				
C. tropicalis IHEM 15905			$> 10^6$		$> 10^6$	$< 10^3$	$< 10^3$	$< 10^3$	
C. albicans IHEM 3731	$> 10^6$	$> 10^6$	$> 10^6$		$> 10^6$	$> 10^6$	$> 10^6$	$< 10^3$	$< 10^3$
C. albicans IHEM 3740			$> 10^6$		$> 10^6$		10^6	$< 10^3$	
S. cerevisiae IHEM 3961	$> 10^6$	$> 10^6$			$> 10^6$	$> 10^6$	$> 10^6$	$< 10^3$	$< 10^3$
S. cerevisiae IHEM 17987	$> 10^6$	$> 10^6$			$> 10^6$	$> 10^6$	$> 10^6$	$< 10^3$	$< 10^3$
C. glabrata IHEM 17730	$> 10^6$	$> 10^6$			$> 10^6$	$> 10^6$	$3\ 10^3$	$< 10^3$	$< 10^3$

Filamentous fungi appeared clearly more resistant to the antifungal action of hypochlorite than yeasts. *Aspergillus niger* and *A. flavus* were the most resistant strains, not only to hypochlorite, but also to the 2 compound products tested previously. Concerning yeasts, the strains of *Candida albicans* (2 strains), *Saccharomyces cerevisiae* (2 strains), and *C. glabrata*, are more sensitive than *C. tropicalis*.

These observations justify the choice of *A. niger* in the norms, but suggest that *C. tropicalis*, as emergent species of medical importance, should be a good optional strain for antiseptics in the health care area.

4.2 TCB strains related to norms on microbial food analyses

4.2.1 Methodology

BCCM/LMG has focused on the Food sector based on the results of the inquiry.
The performance tests, carried out at the beginning and the end of the preservation period, are situated on 3 different levels: detection, enumeration and confirmation. The tests are clustered in 16 test groups named after the relevant bacterial species or group of species. In each test group several TCB strains (either as positive or negative control) were subjected to performance testing based on one or several norms. The Detection and Enumeration tests were performed on selective culture media. The Confirmation tests are (mostly) biochemical tests. The phenotypical characteristics as displayed on the (selective) culture media and in the biochemical tests were examined and compared to those described in the norm/standard.
If the selectivity of certain culture media was based on the same characteristics, the TCB strain was checked on only one of this culture media. The culture media and reagents were bought as much as possible ready-to-use with certificate. The second option was to buy them in the dehydrated form. Otherwise the culture media and reagents had to be prepared with the separate ingredients.
For testing the performance (protocol reproduced in Figure 20), 3 vials were used from the prepared experimental stock.

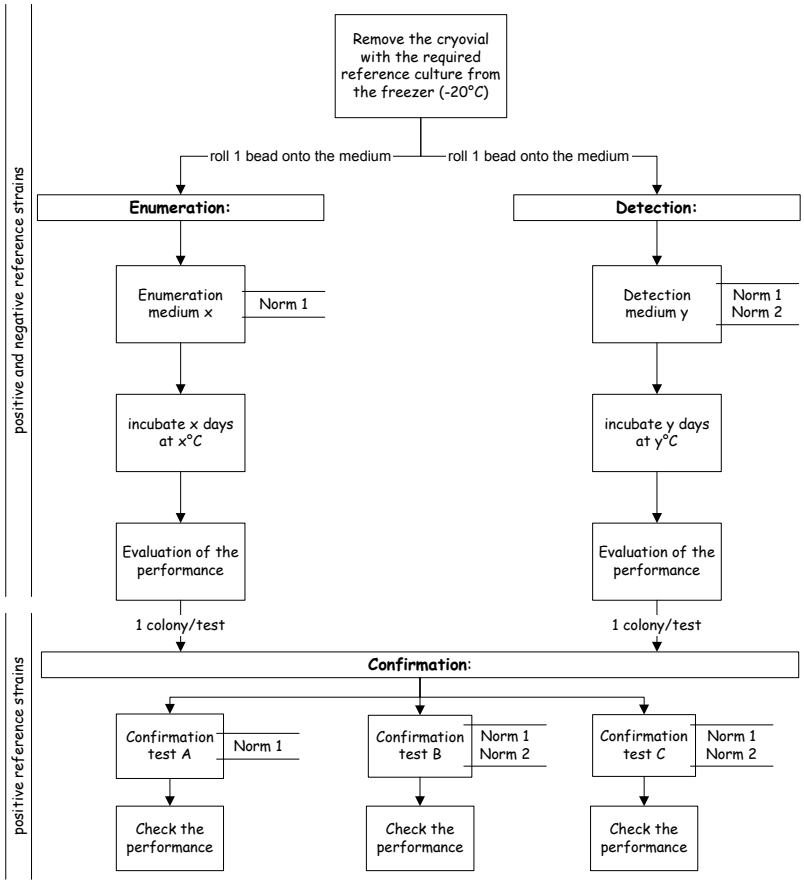


Figure 20: protocol performance tests for BCCM/LMG

Each performance test of a TCB strain was carried out in duplicate: one cryobead of 2 different cryovials was rolled on the considered culture media for testing the detection and/or enumeration. One colony was picked from the detection and/or enumeration plate to carry out each confirmation test. After each test the performance was checked.

A subset of the experimental stock (1 vial used for testing the performance) was brought 25 times for 5 minutes at room temperature after a preservation period of 1 year to simulate the use by the client as good as possible.

The third vial was used for the test phase with some clients. 24 strains were sent to 10 different clients (5 strains per client). The strains were included in the routine analyses of each client. Several aspects were asked for evaluation (see point 5).

The results of the performance tests were evaluated in collaboration with Beltest auditor Mrs. K. Dierick.

4.2.2 Results

A total of 373 performance tests were carried out with the 37 selected bacterial reference strains (see Table VII) in relation to the 48 selected norms/standards (see Table II) used in the Food sector.
The results are summarized in Table VIII.
The results are represented per test group. For each test group the positive and negative TCB control strains tested according to some norms/standards are presented. The results are presented following the Detection, Enumeration and Confirmation tests and are divided in the tests performed at the beginning (B) and the end (E) of the preservation period. The number of tests performed is presented and the number of tests giving a result conform to the norm are in brackets. The doubtful results are in brackets indicated in bold.
At the beginning of preservation period, 350 tests were carried out. Of those, 336 tests (96%) gave a result conform to the norms/standards and 5 results gave a doubtful result.
At the end of preservation period, 373 tests were carried out. Of those, 358 tests (96%) gave a result conform to the norms/standards and 5 results gave a doubtful result.

Remarks:
-At the end of the preservation period *Campylobacter coli* didn't recover on the detection culture media, this strain isn't suitable for preservation on cryobeads.
-*Enterobacter aerogenes* LMG 2094 is not a good reference strain for testing coliforms and *Enterobacteriaceae*. The strain forms no very specific coliform or *Enterobacteriaceae* colonies on the detection and enumeration culture media and one confirmation test is doubtful. This strain will not be recommended as TCB strain in those cases.
-*Pseudomonas aeruginosa* LMG 6395 used as negative control in the test groups coliforms and *Escherichia coli,* forms on certain culture media colonies similar to the colonies formed by some positive control strains in this test groups. *Pseudomonas aeruginosa* LMG 6395 will not be recommended for those culture media.
-LMG 12692 was first chosen as *Enterococcus faecium* TCB strain but since this strain is not listed in the on-line catalogue, it was decided to use its duplicate LMG 11423 as TCB strain.
-At the beginning of the normalisation project, the collection had only a toxic representative of serovar O157:H7, *E. coli* LMG 15068. During the project, the collection obtained a non-toxigenic variant of serovar O157:H7, *E. coli* LMG 21756, this strain will be used as TCB strain.
-The confirmation test β-glucuronidase from the test group *Escherichia coli* O157:H7 was performed on different ways (test following SP-VG M001, Coli ID, PTG medium, Petrifilm selective *E. coli* count plate). *Escherichia coli* O157:H7 LMG 21756 (and LMG 15068) gave a positive reaction when using the test following SP-VG M001 but when using the other test methods, *Escherichia coli* O157:H7 LMG 21756 (and LMG 15068) gave a negative reaction as expected.

Table VII: selected bacterial reference strains

Name	LMG number
Bacillus cereus	LMG 8221
Bacillus coagulans	LMG 6326
Bacillus subtilis subsp. *spizizenii*	LMG 8197
Campylobacter coli	LMG 21266
Campylobacter jejuni subsp. *jejuni*	LMG 18455
Citrobacter freundii	LMG 21265
Clostridium perfringens	LMG 11264
Enterobacter aerogenes	LMG 2094
Enterococcus faecalis	LMG 7937
Enterococcus faecium	LMG 11423 / LMG 12692
Escherichia coli	LMG 8063
Escherichia coli	LMG 8223
Escherichia coli serotype O157:H7	LMG 15068 / LMG 21756
Lactobacillus delbrueckii subsp. *bulgaricus*	LMG 6901
Lactobacillus gasseri	LMG 13047
Lactobacillus sakei subsp. *sakei*	LMG 9468
Lactococcus lactis subsp. *lactis*	LMG 6890
Listeria innocua	LMG 11387
Listeria ivanovii	LMG 11388
Listeria monocytogenes	LMG 13305
Listeria monocytogenes	LMG 21263
Listeria monocytogenes	LMG 21264
Pediococcus damnosus	LMG 11484
Proteus mirabilis	LMG 3257
Pseudomonas aeruginosa	LMG 1242
Pseudomonas aeruginosa	LMG 6395
Rhodococcus equi	LMG 18452
Salmonella choleraesuis subsp. *choleraesuis* serotype *abony*	LMG 18222
Salmonella choleraesuis subsp. *choleraesuis* serotype *enteritidis*	LMG 10395
Salmonella choleraesuis subsp. *choleraesuis* serotype *typhimurium*	LMG 14933
Shigella sonnei	LMG 10473
Staphylococcus aureus subsp. *aureus*	LMG 8195
Staphylococcus aureus subsp. *aureus*	LMG 8224
Staphylococcus epidermidis	LMG 10273
Vibrio parahaemolyticus	LMG 2850
Yersinia enterocolitica subsp. *enterocolitica* serotype 0:8	LMG 7899
Yersinia enterocolitica subsp. *enterocolitica* serotype 0:3	LMG 15558

Table VIII: results of the performance tests from the bacterial TCB strains.

Test group	Used Norms/standards	TCB strain		+ control	- control	Detection		Enumeration		Confirmation	
						B	E	B	E	B	E
Bacillus cereus	ISO 7932 XP V 08-058	*Bacillus cereus*	LMG 8221	+		0	0	1(1)	1(1)	5(5)	5(5)
		Bacillus coagulans	LMG 6326		-	0	0	1(1)	1(1)	0	0
		Bacillus subtilis subsp. *spizizenii*	LMG 8197		-	0	0	1(1)	1(1)	0	0
		Escherichia coli	LMG 8063		-	0	0	1(1)	1(1)	0	0
		Escherichia coli	LMG 8223		-	0	0	1(1)	1(1)	0	0
Campylobacter	ISO 10272 SP-VG M003	*Campylobacter coli*	LMG 21266	+		4(4)	4(0)	0	0	8(8)	0
		Campylobacter jejuni subsp. *jejuni*	LMG 18455	+		4(4)	4(4)	0	0	8(8)	8(8)
		Escherichia coli	LMG 8063		-	4(4)	4(4)	0	0	0	0
		Staphylococcus aureus subsp. aureus	LMG 8224		-	4(4)	4(4)	0	0	0	0
		Proteus mirabilis	LMG 3257		-	4(4)	4(4)	0	0	0	0
Clostridium perfringens	ISO 7937 NF V 08-056	*Clostridium perfringens*	LMG 11264	+		0	0	1(1)	1(1)	3(3)	3(3)
		Escherichia coli	LMG 8223		-	0	0	1(1)	1(1)	0	0
Coliforms	AFNOR 3M-01/2-09/89 AFNOR 3M-01/5-03/97 AFNOR 3M-01/7-03/99 FIL 73B ISO 4832 and 9308-1 NF V 08-050 and 08-060	*Escherichia coli*	LMG 8063	+		2(2)	2(2)	1(1)	4(4)	3(3)	3(3)
		Escherichia coli	LMG 8223	+		2(2)	2(2)	1(1)	4(4)	3(3)	3(3)
		Citrobacter freundii	LMG 21265	+		2(2)	2(2)	1(1)	4(4)	3(3)	3(3)
		Enterobacter aerogenes	LMG 2094	+		2(2)	2(2)	1(1)	4(4)	3(3)	3(**2+1**)
		Enterococcus faecalis	LMG 7937		-	2(2)	2(2)	1(1)	4(4)	0	0
		Pseudomonas aeruginosa	LMG 6395		-	2(1**+1**)	2(1**+1**)	1(1)	4(4)	0	0
Enterobacteriaceae	AFNOR 3M-01/6-09/97 ISO 7402 NF V 08-054	*Escherichia coli*	LMG 8063	+		0	0	1(1)	2(2)	2(2)	2(2)
		Escherichia coli	LMG 8223	+		0	0	1(1)	2(2)	2(2)	2(2)
		Salmonella choleraesuis subsp. *choleraesuis* serotype *typhimurium*	LMG 14933	+		0	0	1(1)	2(2)	2(2)	2(2)
		Enterobacter aerogenes	LMG 2094	+		0	0	1(1)	2(2)	2(2)	2(2)
		Enterococcus faecalis	LMG 7937		-	0	0	1(1)	2(2)	0	0
Enterococci	ISO 7899-2 Mossel p. 416-418	*Enterococcus faecalis*	LMG 7937	+		1(1)	1(1)	0	0	1(1)	2(2)
		Enterococcus faecium	LMG 12692	+		1(1)	1(1)	0	0	1(1)	2(2)
		Enterococcus faecium	LMG 11423	+		1(1)	0	0	0	4(4)	0
		Escherichia coli	LMG 8063		-	1(1)	0	0	0	0	0
		Escherichia coli	LMG 8223		-	0	1(1)	0	0	0	0
		Lactococcus lactis subsp. *lactis*	LMG 6890		-	1(1)	1(1)	0	0	0	0
Escherichia coli	AFNOR 3M-01/8-06/1 AFNOR BIO-12/5-01/99 ISO 9308-1 ISO 11866-3 NF V 08-053	*Escherichia coli*	LMG 8063	+		3(3)	3(3)	3(3)	3(3)	3(3)	3(3)
		Escherichia coli	LMG 8223	+		3(3)	3(3)	3(3)	3(3)	3(3)	3(3)
		Escherichia coli serotype O157:H7	LMG 15068	+		3(3)	3(3)	3(3)	3(3)	3(3)	3(3)
		Escherichia coli serotype O157:H7	LMG 21756	+		3(3)	0	3(3)	0	3(3)	0
		Pseudomonas aeruginosa	LMG 6395		-	3(1**+2**)	3(1**+2**)	3(3)	3(3)	0	0
Escherichia coli O157:H7	SP-VG M001	*Escherichia coli* serotype O157:H7	LMG 15068	+		1(1)	1(1)	0	0	8(5**+1**)	10(8**+1**)
		Escherichia coli serotype O157:H7	LMG 21756	+		1(1)	0	0	0	10(8**+1**)	0
		Escherichia coli	LMG 8063		-	1(1)	0	0	0	0	0
		Escherichia coli	LMG 8223		-	1(1)	1(1)	0	0	0	0
Lactic acid bacteria	NF V 04-503	*Lactobacillus delbrueckii* subsp. *bulgaricus*	LMG 6901	+		0	0	1(0)	1(0)	2(2)	2(2)
		Lactobacillus gasseri	LMG 13047	+		0	0	1(0)	1(0)	2(2)	2(2)
		Lactobacillus sakei subsp. *sakei*	LMG 9468	+		0	0	1(1)	1(1)	2(2)	2(2)
		Lactococcus lactis subsp. *lactis*	LMG 6890	+		0	0	1(1)	1(1)	2(2)	2(2)
		Pediococcus damnosus	LMG 11484	+		0	0	1(1)	1(1)	2(2)	2(2)
		Escherichia coli	LMG 8063		-	0	0	1(1)	1(1)	0	0
		Escherichia coli	LMG 8223		-	0	0	1(1)	1(1)	0	0
		Bacillus cereus	LMG 8221		-	0	0	1(1)	1(1)	0	0

Listeria	FIL 143A ISO 11290 NF V 08-055	*Listeria monocytogenes*	LMG 13305	+		2(2)	2(2)	1(1)	1(1)	8(8)	8(8)
		Listeria monocytogenes	LMG 21263	+		2(2)	2(2)	1(1)	1(1)	8(8)	8(8)
		Listeria monocytogenes	LMG 21264	+		2(2)	2(2)	1(1)	1(1)	8(8)	8(8)
		Listeria innocua	LMG 11387	+		2(2)	2(2)	1(1)	1(1)	8(8)	8(8)
		Listeria ivanovii	LMG 11388	+		2(2)	2(2)	1(1)	1(1)	8(8)	8(8)
		Escherichia coli	LMG 8063		-	2(2)	2(2)	1(1)	1(1)	0	0
		Enterococcus faecalis	LMG 7937		-	2(2)	2(2)	1(1)	1(1)	0	0
		Staphylococcus aureus subsp. *aureus*	LMG 8224			0	0	0	0	1(1)	1(1)
		Rhodococcus equi	LMG 18452			0	0	0	0	1(1)	1(1)
Pseudomonas	ISO 13720 NF V 04-504	*Pseudomonas aeruginosa*	LMG 1242	+		0	0	1(1)	1(1)	2(2)	2(2)
		Pseudomonas aeruginosa	LMG 6395	+		0	0	1(1)	1(1)	2(2)	2(2)
		Escherichia coli	LMG 8223		-	0	0	0	1(1)	0	0
		Staphylococcus aureus subsp. *aureus*	LMG 8224		-	0	0	0	1(1)	0	0
Salmonella	EN 12824 FIL 93B ISO 6340 ISO 6579 NF V 08-052 SP-VG M002	*Salmonella choleraesuis* subsp. *choleraesuis* serotype *abony*	LMG 18222	+		4(4)	4(4)	0	0	5(5)	6(6)
		Salmonella choleraesuis subsp. *choleraesuis* serotype *enteritidis*	LMG 10395	+		4(4)	4(4)	0	0	5(5)	6(6)
		Salmonella choleraesuis subsp. *choleraesuis* serotype *typhimurium*	LMG 14933	+		4(4)	4(4)	0	0	5(5)	6(6)
		Escherichia coli	LMG 8063		-	0	4(4)	0	0	0	0
		Escherichia coli	LMG 8223		-	4(4)	4(4)	0	0	0	0
		Enterococcus faecalis	LMG 7937		-	4(4)	4(4)	0	0	0	0
Shigella	FDA-BAM p. 6.01-6.06 Mossel p. 421-422	*Shigella sonnei*	LMG 10473	+		3(0)	3(0)	0	0	2(1)	7(7)
		Enterococcus faecalis	LMG 7937		-	3(3)	3(3)	0	0	0	0
		Proteus mirabilis	LMG 3257		-	3(3)	3(3)	0	0	0	0
Staphylococcus aureus	FIL 138 FIL 145A ISO 6888 NF V 08-057	*Staphylococcus aureus* subsp. *aureus*	LMG 8195	+		0	0	1(1)	1(1)	2(2)	2(2)
		Staphylococcus aureus subsp. *aureus*	LMG 8224	+		0	0	1(1)	1(1)	2(2)	2(2)
		Staphylococcus epidermidis	LMG 10273		-	0	0	1(1)	1(1)	0	0
		Escherichia coli	LMG 8063		-	0	0	1(1)	1(1)	0	0
		Escherichia coli	LMG 8223		-	0	0	1(1)	1(1)	0	0
Vibrio	FDA-BAM p. 9.01-9.27 SP-VG M006	*Vibrio parahaemolyticus*	LMG 2850	+		1(1)	1(1)	0	0	12(12)	15(15)
		Escherichia coli	LMG 8063		-	1(1)	0	0	0	0	0
		Escherichia coli	LMG 8223		-	0	1(1)	0	0	0	0
		Proteus mirabilis	LMG 3257		-	1(1)	1(1)	0	0	0	0
		Pseudomonas aeruginosa	LMG 6395		-	1(1)	1(1)	0	0	0	0
Yersinia enterocolitica	ISO 10273 SP-VG M004	*Yersinia enterocolitica* subsp. *enterocolitica*	LMG 7899	+		2(2)	2(2)	0	0	13(13)	13(13)
		Yersinia enterocolitica subsp. *enterocolitica*	LMG 15558	+		2(2)	2(2)	0	0	0	13(13)
		Escherichia coli	LMG 8223		-	2(2)	2(2)	0	0	0	0
		Escherichia coli	LMG 8063		-	2(2)	2(2)	0	0	0	0
		Staphylococcus aureus subsp. *aureus*	LMG 8224		-	2(2)	2(2)	0	0	0	0

Escherichia coli O157:H7 LMG 21756 (and LMG 15068) gave a positive reaction for the β-galactosidase test and not a negative reaction as described as normally expected in the standard.
-At the end of the preservation period, in some cases *Escherichia coli* LMG 8223 was used instead of *Escherichia coli* LMG 8063 because LMG 8063 was no longer available on the cryobeads.
-Lactic acid bacteria: the strains *Lactobacillus delbrueckii* subsp. *bulgaricus* LMG 6901 and *Lactobacillus gasseri* LMG 13047 don't grow on MRS at 25°C. These strains will not be recommended for this test as TCB strains.
-The colonies formed by *Shigella sonnei* LMG 10473 on the Detection culture media from the test group *Shigella* are not conform to those described in the standards. This strain will not be recommended as TCB strain.
-*Vibrio parahaemolyticus* LMG 2850 gave a negative reaction for the ornithine decarboxylase test and not a positive reaction as the most *V. parahaemolyticus.*

Removing one of the three vials 25 times during 5 minutes at room temperature has no effect on the viability and the performance of the strains.

For the results of the test phase, see point 5.2.

4.2.3 Conclusion

Storage during 1-2 years at –20°C on cryobeads does not affect performance and maintains good viability for 28 of the TCB strains.
The following strains will not be available on cryobeads but will be available only in freeze-dried form:

- *Bacillus cereus* LMG 8221
- *Bacillus subtilis* subsp. s*pizizznii* LMG 8197
- *Campylobacter coli* LMG 21266
- *Campylobacter jejuni* subsp. *jejuni* LMG 18455
- *Clostridium perfringens* LMG 11264
- *Lactobacillus sakei* subsp. *sakei* LMG 9468
- *Vibrio parahaemolyticus* LMG 2850

The following strains will not be recommended as TCB strains for the used norms/standards because the performance of these strains is not satisfied:

- *Enterobacter aerogenes* LMG 2094
- *Lactobacillus delbrueckii* subsp. *bulgaricus* LMG 6901
- *Lactobacillus gasseri* LMG 13047
- *Shigella sonnei* LMG 10473

33 bacterial reference strains will be available in freeze-dried form with a certificate of proven identity and performance.
From these, 26 bacterial reference strains will also be available as ready-to-use controlled working stocks in frozen cryovials with 25 cryobeads, which can be preserved at –20°C during 1-2 years at the user's location.

An example of a certificate and the instructions for using the cryobeads are given on the next pages.

CERTIFICATE

Bacillus cereus **LMG 8221**

culture batch: 01/01

Following items have been checked by BCCM™/LMG:

VIABILITY

confirmed *on LMG medium Nr. 14 at 28°C after 24 hours*

PURITY

confirmed *on LMG medium Nr. 14 at 28 °C during 7 days*

IDENTIFICATION

confirmed *by the following technique:*
Gaschromatographic determination of the fatty acid composition of the cells, and comparison of the profile with the commercial TSBA database (Microbial Identification System, Inc., Delaware U.S.A., Rev. 3.90). The extraction and the analysis of the fatty acids were performed conform to the recommendations of the system producer.

PERFORMANCE

as positive control:

- *for MYP agar according to norms ISO 7932 / XP V 08-058 / pr ISO/TS 11133-2* **confirmed**
- *for glucose fermentation according to norm ISO 7932* **confirmed**
- *for Voges-Proskauer reaction according to norm ISO 7932* **confirmed**
- *for reduction of nitrate to nitrite according to norm ISO 7932* **confirmed**
- *for hemolysis according to norm XP V 08-058* **confirmed**
- *for motility according to norm XP V 08-058* **confirmed**

as negative control:

- *for MRS agar at 25°C according to norm NF V 04-503 / pr ISO/TS 11133-2* **confirmed**

Date: 28/01/03

Dr. D. Janssens
Curator

Form of supply:
the culture is delivered frozen on carboglass in Microbank™ vial with cryobeads

Expiry date*: 01/2003
** if cultures are kept frozen at a temperature ≤ - 20°C and if the attached instructions for handling have been carefully followed.*

Information from the depositor and/or extracted from public collection catalogues:

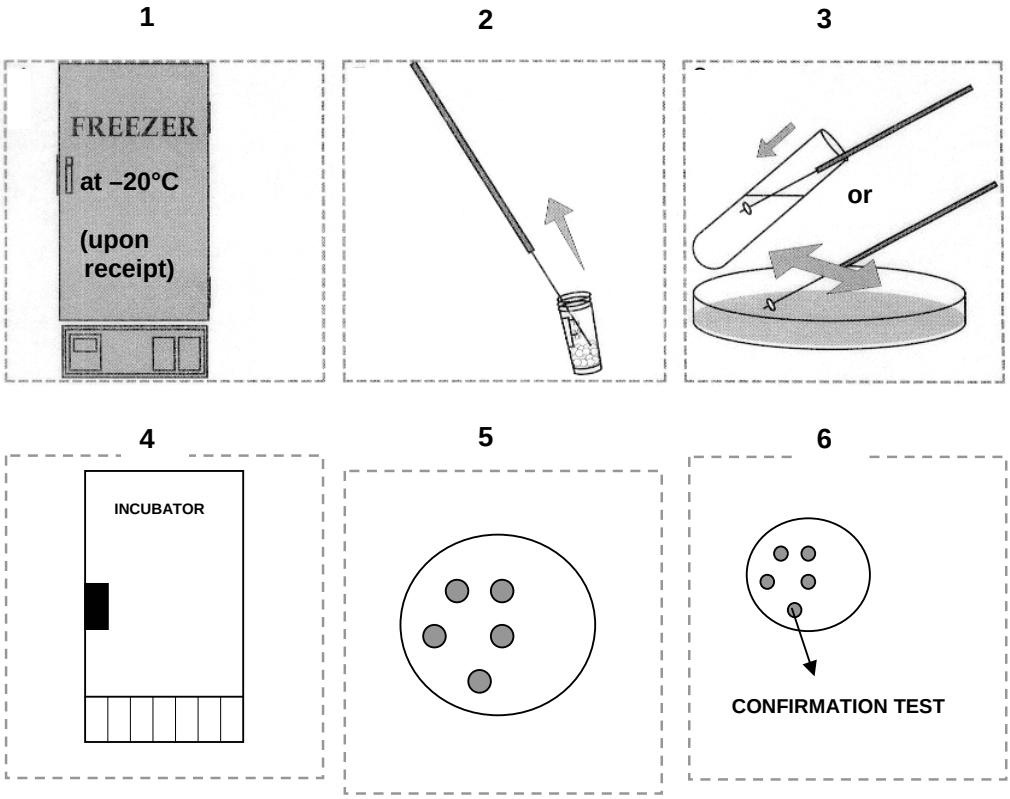
Equivalent strain numbers:

LMG 8221 = ATCC 9634 = ATCC 11778 = DSM 345 = NCIB 8012 = NCIB 9231 = NCTC 10320 = FDA strain PCI213 = Waksman strain O.

Strain history:

BCCM/LMG ← 1988, ATCC 11778 ← FDA (*Bacillus mycoides*).

INSTRUCTIONS FOR USING THE BCCM/LMG REFERENCE CULTURES ON MICROBANK CRYOVIALS WITH CRYOBEADS



1. Upon receipt, store the cryovials with frozen cultures in a freezer at (at least) –20°C. In case the vials are no longer frozen upon receipt, please notify BCCM/LMG (☏: 09/264 51 08).

2. Remove the cryovial with the required reference culture from the freezer and place it in a pre-cooled cryobloc or ice bath to prevent the vial from defrosting. Harvest aseptically one cryobead from the vial with a sterile needle. After use, close the vial immediately and store it again at -20°C without delay.

3. To recover a reference culture, roll a bead onto a culture medium or place it into recovery broth. Each cryotube contains 25 identical potential subcultures.
For testing the performance of a medium (for counting or detection), the bead can immediately be rolled on the medium that needs to be checked. No entrance control is necessary, since the cryobeads are checked as specified on the certificate enclosed.

4. Incubate the culture in proper conditions.
For testing the performance of a medium (for counting or detection), apply the conditions as described in the corresponding norm/standard.

5. After incubation, observe the growth of colonies. To check the performance of a culture medium, evaluate the colonies for specific characteristic(s) conform with norm/standard.

6. For testing the performance of a confirmation test, harvest one or more colonies from the culture medium mentioned above and perform the confirmation test as described in the corresponding norm/standard. Evaluate the results.

Remarks:
1. Never use the reference culture if the expiry date on the vial has elapsed.
2. Aseptic technique should be practiced when manipulating the cryovials to ensure continued integrity of the stored microorganism.
3. Biohazard precautions should be taken whenever required based on the biohazard class of the microorganism.
4. Once removed, the bead should not be returned to the cryovial for any reason

4.3 TCB strains related to norms on wood degradation

4.3.1 Scope

In the field of standards application using fungal material, a qualitative and a quantitative revaluation appears necessary for reasons linked essentially to the evolution of this biological material and the diversity of utilisation and storage conditions by users. This potential evolution entails important confusion risks as well as the absence of guarantee on quality, stability and accuracy of the expected results. European standard cannot be operational and normative in their application if the biological factor, the test fungus, is not monitored.

On the basis of information gathered in the initial statement (point 2), works of verification at BCCM/MUCL were undertaken on non-sporulating basidiomycetes which are fungi involved in wood degradation. Standards which are used to test the durability and the protection of wood and wood-based product depend on the use of well-selected test strains which bring about typical decay and which respond to preservatives in the same way as naturally occurring strains would in service (brown- and white-rot).

In order to improve the quality and accuracy of test results, it is essential that recommended normative strains (reference strains) are re-assessed, characterised and compared with other candidate cultures of the same species (alternative strains).

4.3.2 Methodology

Growth rate, decay virulence and enzymes activities were the quality characters investigated for re-assessment of optimal performance and stability of the strains.

4.3.2.1 Micro-organisms and culture conditions

All studied strains are preserved in the BCCM/MUCL culture collection and listed in Table IX.

Table IX: Fungal strains studied.

Species	Strain MUCL number	Locality	Admission year in the BCCM/MUCL collection	Type of decay
Coniophora puteana	**11662** [a]	Germany	1978	Brown-rot
C. puteana	**30714** [b]	Belgium	1990	Brown-rot
C. puteana	31046 [b]	Belgium	1991	Brown-rot
Coriolus versicolor	**11665** [a]	France	1968	White-rot
C. versicolor	28407 [b]	Belgium	1983	White-rot
C. versicolor	**38412** [b]		1993	White-rot
Gloeophyllum trabeum	**11353** [a]	Germany	1968	Brown-rot
G. trabeum	**31522** [b]	Belgium	1991	Brown-rot
G. trabeum	35053 [b]	Belgium	1992	Brown-rot
Lentinus lepideus	38721 [b]		1994	Brown-rot
L. lepideus	**40109** [b]		1996	Brown-rot
Pleurotus ostreatus	31534 [b]	Germany	1991	White-rot
P. ostreatus	**31535** [b]	Germany	1991	White-rot
P. ostreatus	38041 [b]	Belgium	1993	White-rot
Poria placenta	**20569** [a]		1979	Brown-rot
P. placenta	30789 [b]		1990	Brown-rot
P. placenta	**30853** [b]		1990	Brown-rot
Serpula lacrymans	7666 [b]	Belgium	1965	Brown-rot
S. lacrymans	28185 [b]	Belgium	1983	Brown-rot
S. lacrymans	**34572** [b]	Belgium	1992	Brown-rot

[a] Reference strains

[b] Alternatives strains

Strains tested both at BCCM/MUCL and at the CTBA

For growth rate, the cryopreserved strains were revivified and sub-cultured at 25°C during 21 days on three media in Petri dishes: 2% malt-agar medium (MA2) and water-agar medium with 1% small wood chips (6 x 6 mm) of Scots pine (brown-rot) or beech (white-rot) (WAW). These sub-cultures were stored under mineral oil and under sterilized distilled water at 13°C, and at -140°C.

For the EN 113 laboratory method, the strains tested were in BCCM/MUCL collection for many years. These strains were preserved under mineral oil and under sterilized distilled water at 13°C, and at -140°C.

4.3.2.2 Estimation of the growth rate

The growth rate was estimated to check the fitness of strains stored under different preservation methods and to detect potential relationship between growth and decay virulence.

After three and six months of preservation, strains were grown at 25°C during 12 days on MA2. Agar plugs (8 mm diameter) were cut from the growing edge of the colonies and used to inoculate Petri dishes (84 mm diameter) with 4% malt-agar medium, as registered on the EN 113 test. The plates were incubated in a dark room at 22°C (conform to EN 113 test). Each strain was assayed in triplicate. The distance covered by the mycelium was recorded every day during 15 days.

4.3.2.3 Decay virulence

EN 113 laboratory method

Six virulence control test specimens of a susceptible wood species are exposed to attack by basidiomycetes in pure culture. The mass loss of each test specimen is expressed as percentage of the initial dry mass. The EN 113 test can be accepted if the mean mass loss of the virulence control test specimens is equal or higher than the respective values for the minimum mass loss (20% (m/m)). The virulence of reference strains was compared to that of alternative strains isolated more recently. The following wood decaying fungi were used: i) *C. puteana*, *G. trabeum*, *L. lepideus*, *P. placenta* and *S. lacrymans* on Scots pine (brown-rot), ii) *C. versicolor* and *P. ostreatus* on beech (white-rot) (Table IX).

Screening test method

- The principle of the screening test method is similar to the EN 113 method, except that small test pieces (30 x 10 x 5 mm against 50 x 25 x 15 mm) and a short exposure time (6 weeks against 16 weeks) are used.
- In a first experiment, only cryopreserved strains were tested at CTBA (Table IX). For white-rot fungi, no test specimen support was used between the mycelium and the test specimen.
- In a second experiment, the tests were carried out at BCCM/MUCL using the same fungal material than that used in the estimation of the growth rate after three months of preservation (point 4.3.2.2).

4.3.2.4 Extra-cellular enzymes assays

Brown-rot fungi can degrade hollocellulose (cellulose and hemicellulose) in wood without first removing the lignin. The wood darkens, shrinks, and breaks into brick-shaped pieces that crumble easily into a brown powder (Green and Highley, 1997). White-rot fungi can degrade all cell wall components, including lignin. They often cause a bleaching of normal wood coloration (Blanchette, 2000). Cellulase (complex of enzymes), endoxylanase, laccase, Mn

dependant peroxydase (MnP), lignin peroxydase (LiP) are the major enzymes involved in wood degradation (Pointing, 2000).

The enzymatic production was followed to compare virulence decay and activity rate of wood cell wall decaying enzymes of the strains.

Culture conditions

In a first experiment, the course of enzymatic production of strains tested at CTBA (Table IX) was followed. Shaken liquid cultures were prepared in 100 ml Erlenmeyer flasks containing 50 ml of 2% malt medium (ML2) with or without 1% small wood chips (6 x 6 mm) of Scots pine (ML2P) or beech (ML2F). Three flasks per strain were inoculated with five agar plugs (3 mm diameter) cut from the actively growing parts of a colony sub-cultured on a Petri dish and incubated for 7 days at 25°C. The flasks were incubated at 22°C and 125 rpm during four weeks. Enzymatic activities were measured in each flask. In a second experiment, laccase activity of the white-rot fungi was assayed on the 8^{th} day of cultivation. The inocula came from the same fungal material than that used in the estimation of the growth rate after six months of preservation (point 4.3.2.2). Shaken liquid cultures were carried out in 100 ml Erlenmeyer flasks containing 50 ml of ML2.

Enzymes assays

- Laccase activity was measured following time oxidation of 0,5 mM ABTS (2,2'-azino-bis(3-ethyl benzthiazoline-6-sulphonic acid). The reaction was done in 100 mM Na acetate buffer, pH 5.0. An increase of absorbance was observed following the formation of the corresponding cation radical (ε_{420}=3,6x10^4 $M^{-1}.cm^{-1}$) (Bourbonnais and Paice, 1988).
- LiP activity was measured as the H_2O_2-dependent oxidation of veratryl alcohol to veratraldehyde. The reaction mixture contained 2 mM veratryl alcohol in 50 mM tartrate, pH 2.5, and 0,4 mM H_2O_2 (Tien and Kirk, 1988).
- MnP activity was measured by oxidation of Mn(II) to Mn(III). The reaction mixture contained 0,1 mM H_2O_2 and 1 mM $MnSO_4$ in 50 mM Na malonate, pH 4.5 (Wariishi *et al*., 1992).
- The xylanolytic activity was determined by measuring the reducing sugars formed from the birchwood xylan (Sigma X-0502). The reducing sugars were revealed with the dinitrosalicylic acid (DNS) (Bailey *et al*., 1992).
- The cellulase activity (filter paper assay) was determined using the DNS-method (Mandels *et al*., 1976; IUPAC, 1987). The activity was measured by the increase in reducing groups.

Results are expressed in U ml^{-1}, with one unit of enzyme activity defined as the amount of enzyme generating 1 nmole of product per minute under the conditions of assay.

4.3.3 Results

4.3.3.1 Fungal growth of the strains

The mean growth rates of strains are similar after three and six months (Table X). This character is similar under any medium and preservation methods. However the early recovery of the white-rot is more rapid on WAW medium.

Several strains of the same species presented different growth rates. Strains isolated more recently grow more rapidly. For example, *C. puteana* MUCL 31046 (1991) gives a rapid development and covers plate 7 days after inoculation. The reference strain, MUCL 11662 (1978), is regular but grows slowly. The strain MUCL 30714 (1990) gives a growth rate between these two strains.

The strain *S. lacrymans* MUCL 34572 grows very slowly. The revivification after preservation causes some problems, what explains some missing results. After three months of preservation, the sub-culture of *C. puteana* MUCL 30714 from cryopreservation have not grown on MA2.

Table X: Mean growth rate (mm/day) of strains preserved under different conditions.

Species	Strain MUCL number	Preservation method											
		Mineral oil				Sterilized distilled water				Cryopreservation			
		MA2		WAW		MA2		WAW		MA2		WAW	
		3 M	6 M	3 M	6 M	3 M	6 M	3 M	6 M	3 M	6 M	3 M	6 M
C. puteana	11662	4,9	4,6	4,8	4,4	4,9	4,4	5,0	4,7	4,8	4,4	4,8	4,5
C. puteana	30714	7,6	5,4	6,9	5,4	5,4	6,9	7,6	5,0	-	5,4	9,5	5,4
C. puteana	31046	10,9	9,5	10,9	9,5	10,9	9,5	10,9	9,5	10,9	9,5	10,9	9,5
C. versicolor	11665	7,6	7,6	6,9	7,6	7,6	7,6	7,6	7,6	7,6	7,6	7,6	7,6
C. versicolor	28407	7,6	7,6	7,6	5,4	7,6	6,9	6,9	6,9	8,4	6,9	7,6	6,9
C. versicolor	38412	10,9	10,9	10,9	10,9	10,9	9,5	10,9	10,9	10,9	10,9	10,9	10,9
G. trabeum	11353	4,7	4,8	4,2	4,2	4,7	4,3	4,4	3,7	5,0	4,8	4,8	4,1
G. trabeum	31522	5,4	5,4	5,4	5,1	6,9	5,4	5,4	5,0	5,4	4,3	5,4	5,1
G. trabeum	35053	6,9	5,4	6,9	5,4	6,9	4,8	5,4	5,4	6,9	5,4	6,9	5,4
L. lepideus	38721	8,4	8,4	5,4	6,9	8,4	7,6	5,4	5,4	8,4	8,4	8,4	8,4
L. lepideus	40109	5,4	5,4	5,1	5,4	5,4	9,5	5,4	5,4	5,4	5,1	5,4	5,1
P. ostreatus	31534	7,6	5,1	7,6	4,7	6,9	6,9	6,9	6,9	7,6	6,9	5,1	5,4
P. ostreatus	31535	5,4	4,6	6,9	5,1	8,4	7,6	7,6	7,6	7,6	5,4	7,6	7,6
P. ostreatus	38041	8,4	8,4	8,4	7,6	8,4	8,4	8,4	8,4	8,4	8,4	8,4	8,4
P. placenta	20569	8,4	9,5	9,5	8,4	8,4	8,4	8,4	7,6	8,4	9,5	9,5	8,4
P. placenta	30789	6,9	6,9	7,6	7,6	6,9	5,4	8,4	5,4	6,9	7,6	7,6	7,6
P. placenta	30853	9,5	5,4	9,5	9,5	9,5	9,5	9,5	9,5	9,5	9,5	9,5	9,5
S. lacrymans	7666	6,9	9,5	7,6	9,5	6,9	8,4	7,6	9,5	7,6	8,4	6,9	7,6
S. lacrymans	28185	10,9	10,9	10,9	10,9	10,9	10,9	10,9	10,9	10,9	10,9	10,9	10,9
S. lacrymans	34572	0,5	-	0,7	-	1,6	-	1,3	-	-	-	-	-

- No result

4.3.3.2 EN 113 laboratory method data

Wood degradation activity of the studied strains are given in Figure 21, except for the strains *G. trabeum* MUCL 11353 which is not preserved under sterilized distilled water and *P. placenta* MUCL 30789 from cryopreservation which has not grown.

Only 11 out of the 20 strains present a level of activity higher than the established limit of 20%, for at least one method of preservation. There is any method of preservation that seems better than another one.

The virulence of the reference strain *C. puteana* MUCL 11662 is far under the 20% limit while the two alternative strains tested showed mean mass losses of 30-40%. *C. versicolor* MUCL 11665 is the only reference strain that presents similar results in comparison of these recorded by the alternative strains of the same species. The two alternative strains of *G. trabeum* studied have the same level of virulence; the mean mass losses are under the limit. The strain *L. lepideus* MUCL 40109 is more virulent than the second alternative strain tested. The three strains of *P. ostreatus* demonstrated mean mass losses close to the limit of 20%. The strain *P. placenta* MUCL 30789 is much more virulent than the reference strain. Among the strain of *S. lacrymans*, the strain MUCL 34572 presents the highest virulence.

The loss of virulence of some strains can be explained by the metabolic dormancy produced when they are continuously maintained in non-optimum conditions for their development.

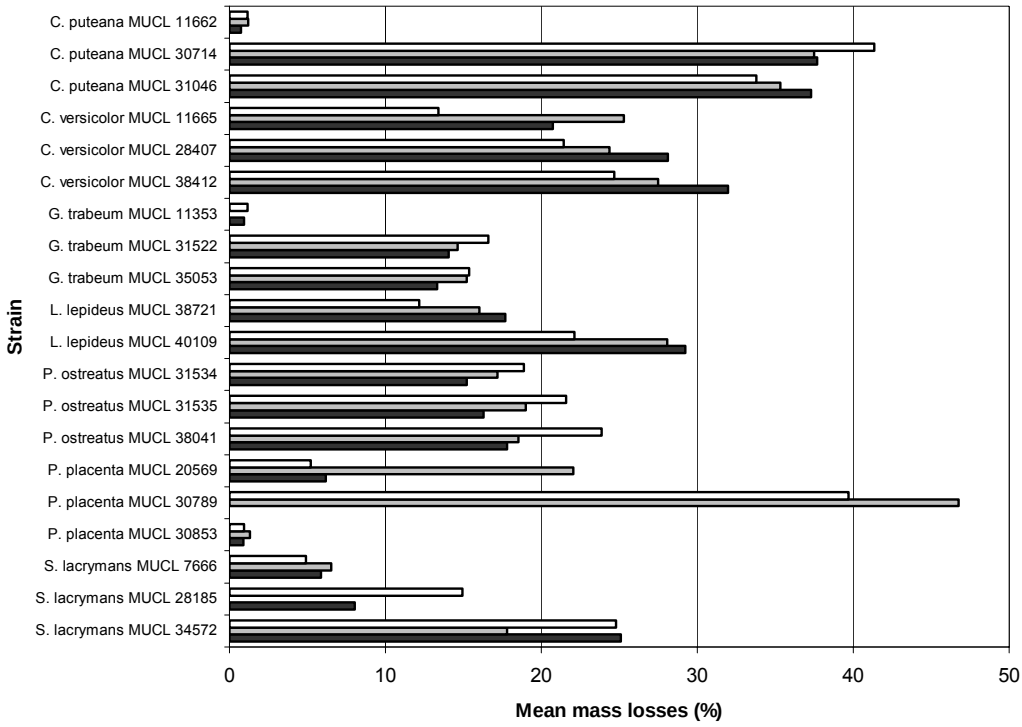


Figure 21: mean mass losses after exposure to decay fungi during 16 weeks. □, strains preserved under mineral oil; ■, under sterilized distilled water; ■, cryopreserved strains.

4.3.3.3 Screening test method at CTBA

Two reference strains of *P. placenta* and *G. trabeum* grew very slowly (Table IX). Therefore, the exposure of test specimens to these strains was delayed. The results about mass losses after six weeks are presented in the Figure 22.

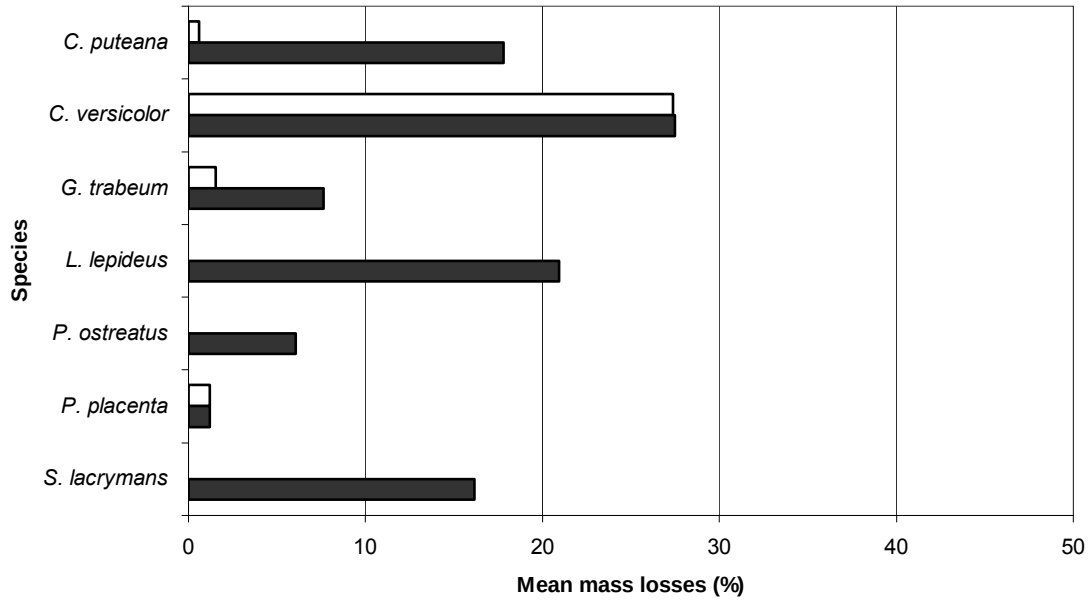


Figure 22: mean mass losses after six weeks of exposure. □, reference strains; ■, alternative strains.

For *C. puteana* and *G. trabeum*, the mean mass losses of the reference strains were clearly lower than the alternative strains. For *P. placenta* and *C. versicolor*, the means mass losses are similar for reference and alternative strains. However, the mean mass loss of *P. placenta* is weak (1%), whilst this of *C. versicolor* is important (27%). Among the reference strains, only the white-rot fungus *C. versicolor* is virulent.

Results obtained at the CTBA are similar to those obtained at BCCM/MUCL following the EN 113 method.

4.3.3.4 Screening test method at BCCM/MUCL

The results about mass losses after six weeks of exposure are presented in Figure 23. The sub-culture of *C. puteana* MUCL 30714 from cryopreservation has not grown on MA2. The strain *S. lacrymans* MUCL 34572 was excluded from the experiment due to its slow development under all conditions.

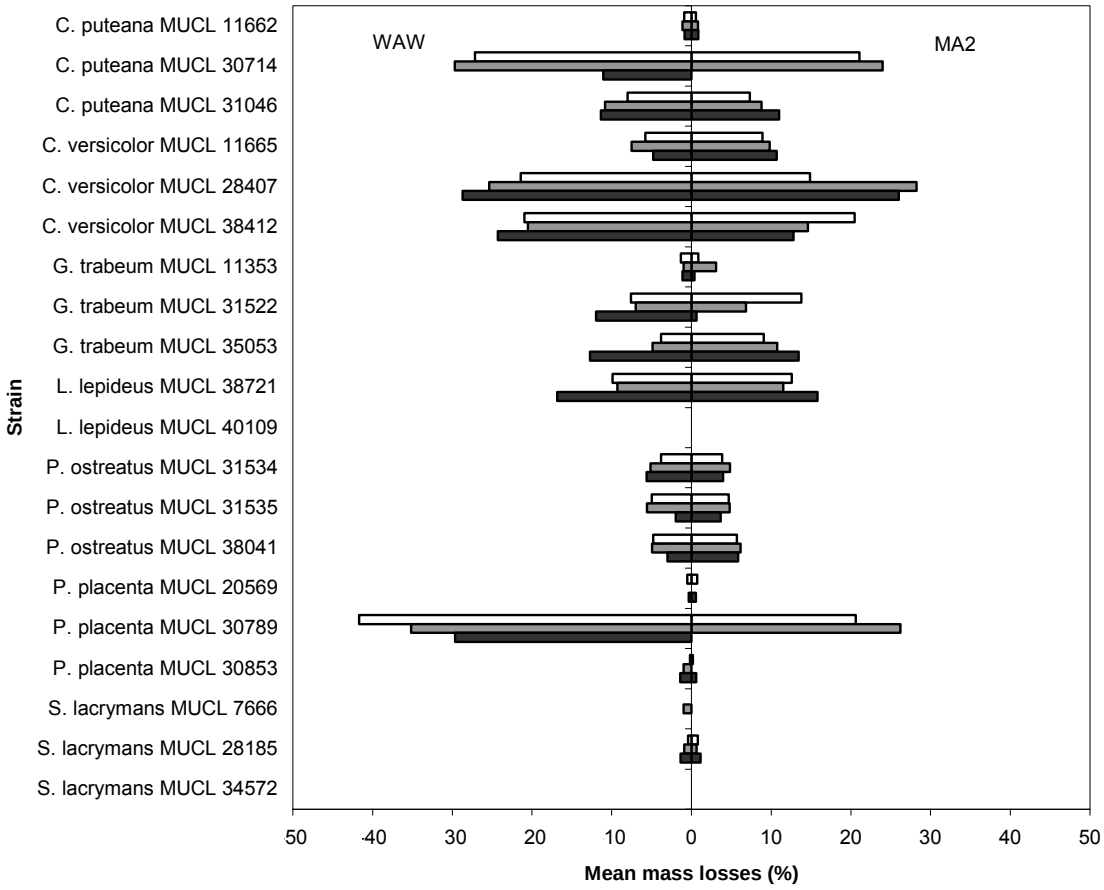


Figure 23: mean mass losses after exposure to decay fungi during six weeks. Strains sub-cultured on MA2 and WAW and preserved under mineral oil (□); under sterilized distilled water (■); cryopreserved strains (■).

- In comparison with the results of EN 113, the tendencies are similar. The screening test method allows a rapid evaluation of the decay virulence.
- Strangely enough in comparison with the previous results of virulence, the strain *L. lepideus* MUCL 40109 has not degraded the specimens of wood.
- Some strains give results of mass losses more important following on the presence of small wood chips on the culture medium.

4.3.3.5 Growth rate and virulence decay

The biological material used to estimate the growth rate after three months of preservation and the virulence by the screening method originated from the same sub-culture. We could thus compare the results of the two experiments (Table XI).

C. versicolor MUCL 11665 is the only reference strain which give mass loss higher than 3%. The results of this strain are however smallest compared with those recorded by two alternative strains of this species.

There is no correlation between the growth rate and the mass loss as already observed by Dirol (1986) and Navarrete and de Troya (1986).

Table XI: Mean growth rate (mm/day) and mass loss (%) obtained with the screening test method for strains preserved under different conditions.

Species	Strain MUCL number	Preservation method											
		Mineral oil				Sterilized distilled water				Cryopreservation			
		MA2		WAW		MA2		WAW		MA2		WAW	
		GR	ML	GR	ML	GR	ML	GR	ML	GR	ML	GR	ML
C. puteana	11662	4,9	*	4,8	*	4,9	*	5,0	*	4,8	*	4,8	*
C. puteana	30714	7,6	21,07	6,9	27,17	5,4	23,99	7,6	29,68	-	-	9,5	11,07
C. puteana	31046	10,9	7,33	10,9	8,01	10,9	8,80	10,9	10,82	10,9	11,00	10,9	11,38
C. versicolor	11665	7,6	8,91	6,9	5,78	7,6	9,82	7,6	7,52	7,6	10,67	7,6	4,80
C. versicolor	28407	7,6	14,89	7,6	21,41	7,6	28,26	6,9	25,39	8,4	26,03	7,6	28,69
C. versicolor	38412	10,9	20,48	10,9	20,95	10,9	14,62	10,9	20,51	10,9	12,81	10,9	24,30
G. trabeum	11353	4,7	*	4,2	*	4,7	3,09	4,4	*	5,0	*	4,8	*
G. trabeum	31522	5,4	13,79	5,4	7,59	6,9	6,82	5,4	6,98	5,4	*	5,4	11,98
G. trabeum	35053	6,9	9,07	6,9	3,80	6,9	10,78	5,4	4,86	6,9	13,45	6,9	12,71
L. lepideus	38721	8,4	12,57	5,4	9,88	8,4	11,53	5,4	9,30	8,4	15,83	8,4	16,83
L. lepideus	40109	5,4	*	5,1	*	5,4	*	5,4	*	5,4	*	5,4	*
P. ostreatus	31534	7,6	3,85	7,6	3,81	6,9	4,84	6,9	5,12	7,6	3,95	5,1	5,62
P. ostreatus	31535	5,4	4,69	6,9	4,97	8,4	4,80	7,6	5,58	7,6	3,65	7,6	1,96
P. ostreatus	38041	8,4	5,71	8,4	4,80	8,4	6,16	8,4	4,96	8,4	5,86	8,4	3,02
P. placenta	20569	8,4	*	9,5	*	8,4	*	8,4	*	8,4	*	9,5	*
P. placenta	30789	6,9	20,63	7,6	41,68	6,9	26,21	8,4	35,17	6,9	*	7,6	29,66
P. placenta	30853	9,5	*	9,5	*	9,5	*	9,5	*	9,5	*	9,5	*
S. lacrymans	7666	6,9	*	7,6	*	6,9	*	7,6	*	7,6	*	6,9	*
S. lacrymans	28185	10,9	*	10,9	*	10,9	*	10,9	*	10,9	*	10,9	*
S. lacrymans	34572	0,5	_	0,7	-	1,6	-	1,3	-	-	-	-	-

- No result

* Mass loss below 3%

4.3.3.6 Enzyme assays

No results were available from the cellulolytic enzymes experiments. The DNS-method for assaying cellulase and xylanolytic activities was not specific. Indeed, all reducing sugars were revealed with this method. As consequence, measures were influenced by the presence of malt in the growth media.

No lignolytic activity was found in any brown-rot culture tested. No LiP and MnP activities were detected in any culture of the white-rot fungi tested. The negative LiP and MnP test suggest that these strains either produce no significant levels of these enzymes, or their production requires different growth conditions. The course of production of laccase in white-rot fungi tested is shown in Figures 24 - 26.

A low level of laccase was produced by *C. versicolor* MUCL 11665 (1 U ml^{-1}). In the ML2P and ML2F media, an induction of this enzyme was observed due to the small wood chips in the growth medium. This induction happened earlier on medium added with chips of beech than with these of Scots pine. The peak of production was also higher in ML2F (85 U ml^{-1}) than ML2P (56 U ml^{-1}) (Figure 24).

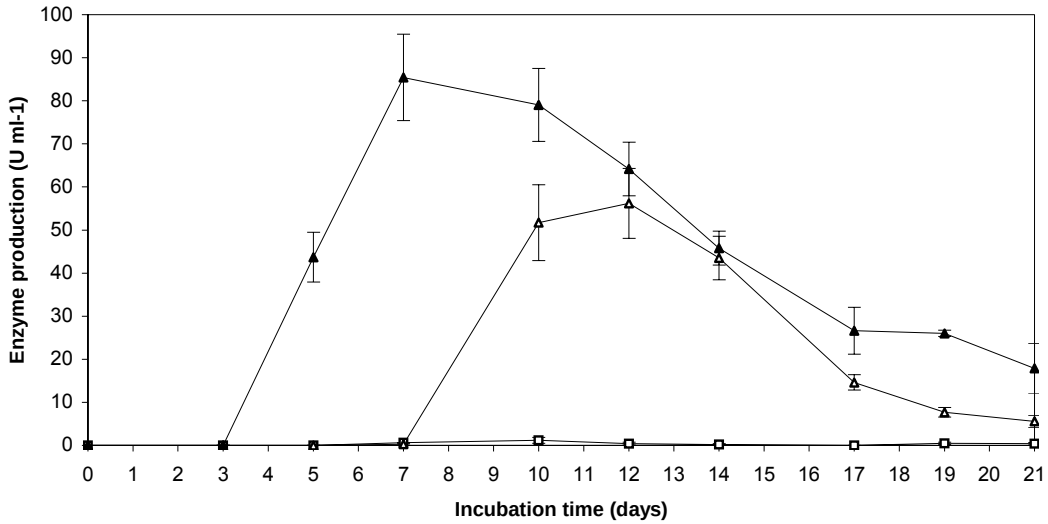


Figure 24: production of laccase by *C. versicolor* MUCL 11665 grown on ML2 (□); ML2P (Δ) and ML2F(▲) in shaken liquid cultures. Symbols represent the mean values of three replicate cultures. Error bars are the standard deviations. When not shown, the standard deviation error bars fall within the symbols.

Whatever the growth medium, the production of laccase by *C. versicolor* MUCL 38412 increased regularly up to a peak reached after 10-12 day of culture. In ML2F, the maximum registered by *C. versicolor* MUCL 38412 was 57 U ml^{-1}. In the ML2 and ML2P media, they had reached 26 and 16 U ml^{-1} respectively (Figure 25).

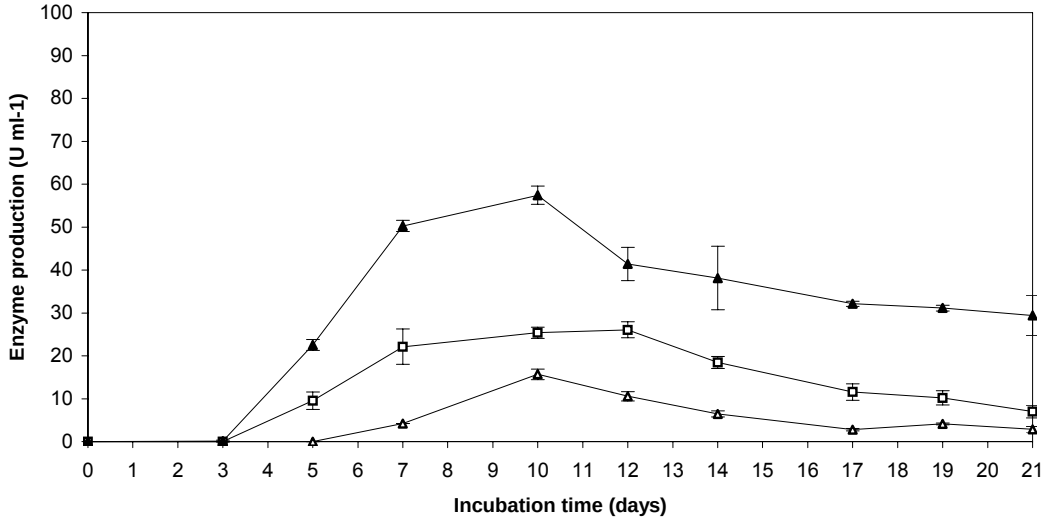


Figure 25: production of laccase by *C. versicolor* MUCL 38412 grown on ML2 (□); ML2P (Δ) and ML2F(▲) in shaken liquid cultures. Symbols represent the mean values of three replicate cultures. Error bars are the standard deviations. When not shown, the standard deviation error bars fall within the symbols.

The levels of laccase produced by *P. ostreatus* MUCL 31535 were lower than these observed for the two strains of *C. versicolor*. The peak of laccase was observed on the 7th day of culture and had reached 6 U ml^{-1}. A weak production was observed between the 17th and the 21st day of culture in the ML2P (1 U ml^{-1}). A second peak was registered in the ML2F. The first had reached 16 U ml^{-1}, the second 5 U ml^{-1} (Figure 26).

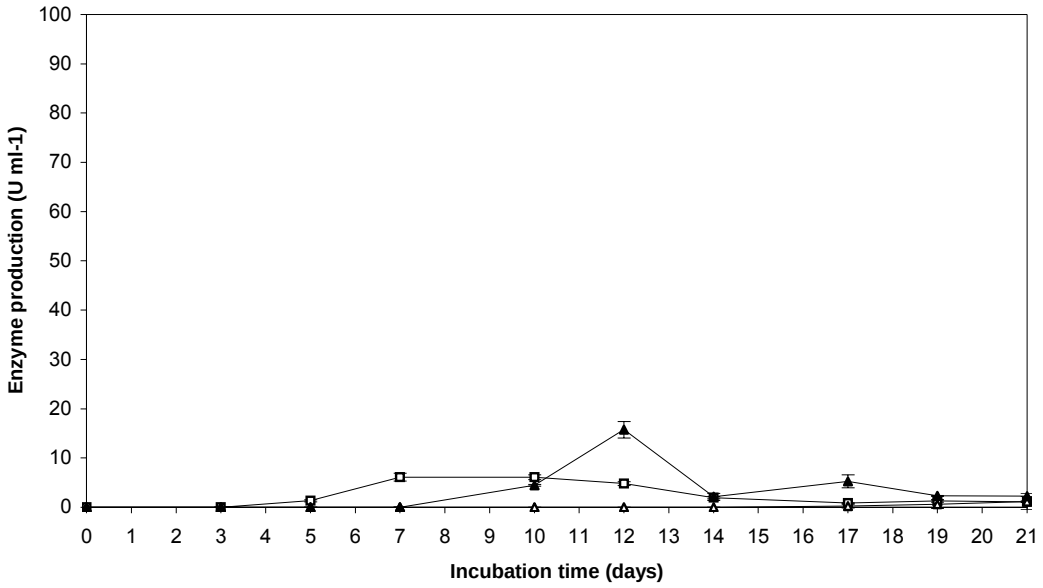


Figure 26: production of laccase by *P. ostreatus* MUCL 31535 grown on ML2 (□); ML2P (Δ) and ML2F(▲) in shaken liquid cultures. Symbols represent the mean values of three replicate cultures. Error bars are the standard deviations. When not shown, the standard deviation error bars fall within the symbols.

The laccase production was more important in ML2F than ML2P. The various structure of lignin between conifers and hardwoods can explain this observation. Aromatic rings of coniferyl aldehyde are the principal constituents in conifers, whereas aromatic nuclei of sinapylaldehyde are the major groups in hardwoods. In hardwoods, the lignin contains aromatic rings richer in groups electrons donor which will be oxidized by the laccase.

4.3.3.7 Laccase activity and decay virulence

Six strains of white-rot fungi were tested for the ability to produce laccase. The results are shown in the Figure 27.

After 8 days of culture in ML2, the production of laccase by *C. versicolor* MUCL 38412 is higher than this of MUCL 28407 under any preservation method and medium of sub-culture. The strain MUCL 11665 exhibited low activities of laccase. A possible explanation of the weak mass losses of the strain MUCL 11665 compared to these two alternatives strains in the screening test (Figure 23) is the low level of laccase production by this strain.

Concerning the three alternatives strains of *P. ostreatus*, the production of laccase after 8 days of culture is in the same range, around 5 U ml-1. The mass losses registered in the screening test method (Figure 23) are similar.

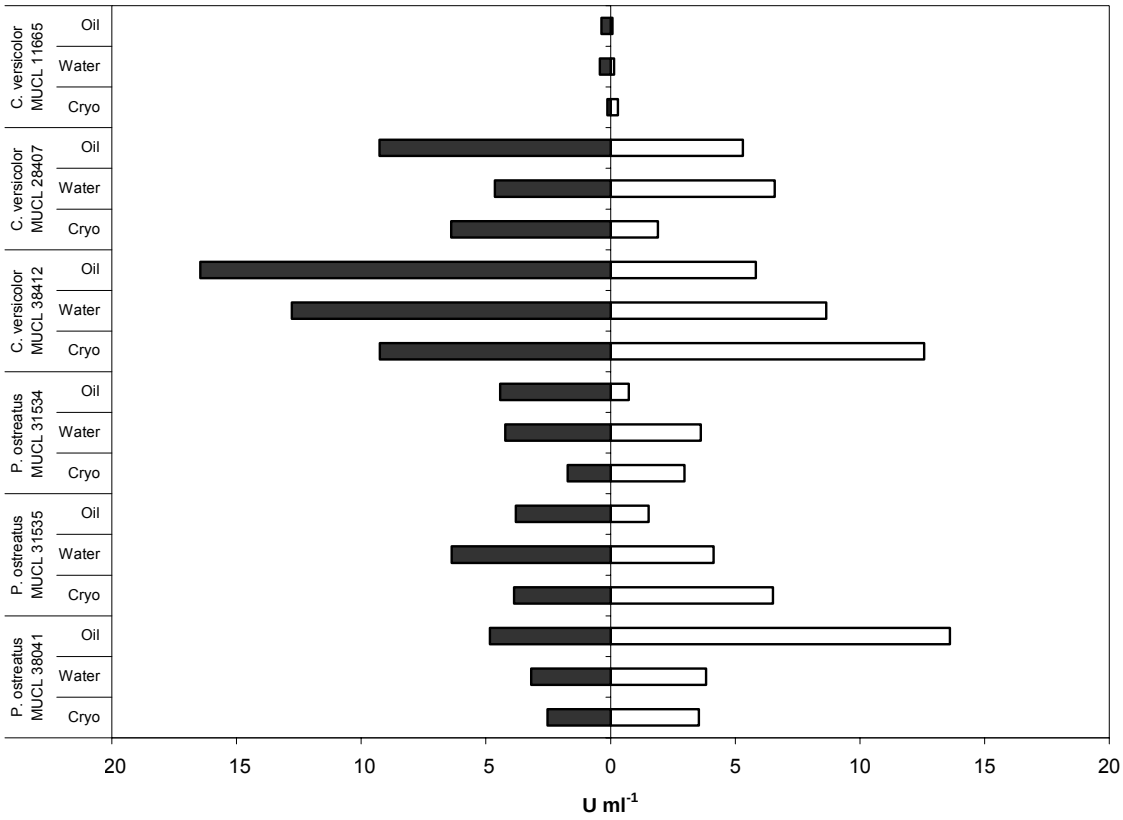


Figure 27: Mean values of laccase activities in different white-rot fungi. Strains sub-cultured on MA2 (□) and WAW (■).

4.3.4. Conclusion

Wood resistance to fungal attacks is an important parameter to be tested in the field of wood preservatives. Nevertheless validity of *in vitro* tests is dependent of the ability of the reference strain to maintain its level of virulence. The standard EN 113 indicates the minimum values of mass losses to be obtained on a given wood species.

Among the four reference strains tested, *C. versicolor* MUCL 11665 is the only one that has a virulence greater than the minimum recommended in the EN 113 standard and that showed reproducible results of virulence. Therefore alternative strains were tested under the EN 113 conditions. The virulence of these alternative strains was greater than the minimum value established in the standard, except for the species *G. trabeum*. The use of alternative strains proves to be judicious.

None of the three methods of preservation studied has proved to be more suitable for the maintenance of the strain virulence. For example, the use of small wood chips in the culture medium does not result in a relevant of virulence.

Compared to the EN 113 method, the screening test method was more rapid (6 instead of 16 weeks) and provides similar range of virulence.

4.4 TCB strains related to Pharmacopoeia

4.4.1 Methodology

The microbial strains quoted in the 2 tests of the European Pharmacopoeia considered were subcultured on the recommended media; the growth was observed (qualitative test only). Fungal strains were checked on TSB medium by BCCM/IHEM, bacterial strains on TSB and Thioglycolate liquid medium by BCCM/LMG.

4.4.2 Results

The results for the "Sterility test" (chapter 2.6.1.) of the European Pharmacopoeia 2001), and for "Total viable aerobic count" (chapter 2.6.12.) are the following:

Sterility test	
Aspergillus niger IHEM 3766	Positive
Candida albicans IHEM 3731	Positive
Bacillus subtilis LMG 8197	Positive
Pseudomonas aeruginosa LMG 6395	Positive
Staphylococcus aureus subsp. *aureus* LMG 8224	Positive
Total viable aerobic count	
Aspergillus niger IHEM 3766	Positive
Candida albicans IHEM 3731	Positive
Bacillus subtilis LMG 8197	Positive
Escherichia coli LMG 8063	Positive
Staphylococcus aureus subsp. *aureus* LMG 8224	Positive

5 TEST PHASE INVOLVING A FEW ROUTINE LABORATORIES

5.1 Methodology

A test phase in cooperation with 12 laboratories with activities in microbiology was organised. They were asked to include in their routine analyses a few TCB strains supplied by BCCM for evaluation. They evaluated these strains following the evaluation form presented in the following pages.

A coefficient has been assigned to each level of satisfaction: very satisfied: 10 points, satisfied: 3 points, neutral: 0 point, dissatisfied: -3 points, very dissatisfied: -10 points. For each criterion, the number of replies has been multiplied by the quoted coefficient. The satisfaction mark by criterion was obtained in dividing this amount by the number of replies. A negative mark emphasizes more spontaneously a lack, a failing (Détrie, 2001).

Address

EVALUATION FORM

We thank you for taking part in this test phase of this project. Completing this evaluation form is assisting BCCM as supplier of microbial reference strains in meeting your needs as end user.

General information

Your laboratory is

❑ accredited ❑ certified ❑ working towards an accredition ❑ working towards a certification ❑ none of these

Evaluation of the BCCM microbial reference strains

We would appreciate it if you could quote your satisfaction for several aspects and if you could add some comments or specify your answer, especially when dissatisfied or very dissatisfied.

	Very dissatisfied	Dissatisfied	Neutral	Satisfied	Very satisfied	Comments and/or specification
1. The condition of the parcel and reference strains upon receipt (incl. packaging)						
2. The strain information given on the delivery note						
3. The instructions for use of the cryovials with cryobeads						
4. The information given on the certificate						If there are more/less aspects you want to see on the certificate, please specify them.
5. The value of the certificate in the frame of your quality system						
6. The userfriendliness of the cryovials with cryobeads (manipulation, ready to use facility…)						
7. The recovery of the reference strains from the cryobeads						
8. The purity of the reference strains						

9. The performance of the reference strains (specific characteristics in relation to the norms/standards)						If a strain did not react as expected, please indicate for which analysis/norm and give a description of the aberrant characteristic(s).
10. The manner of preservation of the reference strains on cryobeads (cryopreservation at –20°C)						
11. The selection of reference strains available						Please mention which additional reference strain you would like to see on the BCCM list.
12. The selection of (inter)national norms/standards used as basis to test the specific characteristics of the BCCM microbial reference strains						Please mention which additional norms/standards you would like to see on the BCCM list.

Please scale your satisfaction of the BCCM microbial reference strains.

Comparison of BCCM reference strains with other reference strains available on the market concerning their:	Very dissatisfied 1	2	3	4	5	6	7	8	9	Very satisfied 10
13. Userfriendliness										
14. Recovery										
15. Performance (specific characteristics in relation to the norms / standards)										

Final evaluation

Please scale your satisfaction.

16. General satisfaction of the BCCM microbial reference strains										

Do you consider yourself as potential user of this kind of BCCM reference strains ? ❑ YES ❑ NO
Would you recommend the BCCM microbial reference strains to other potential users ? ❑ YES ❑ NO

What do you find a reasonable price **for one BCCM microbial reference strain** delivered in a vial with **25 cryobeads** in frozen condition (incl. certificate, excl. transport costs)? .....

General suggestions and remarks

Name: Date:

5.2 Results

All the contacted laboratories cooperated to the test phase. The results of the test phase were analysed and synthesized in the Figures 28 and 29.

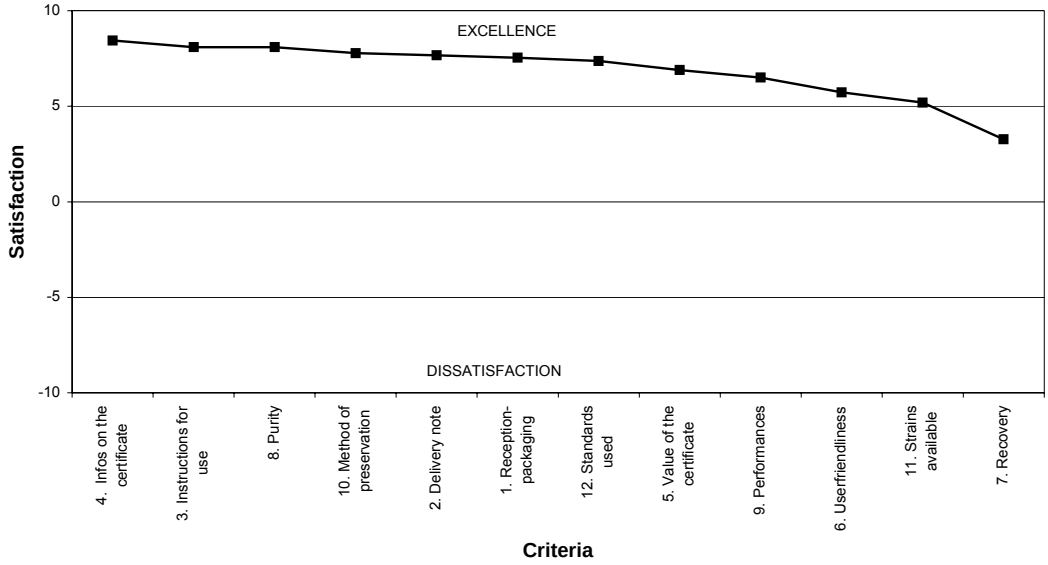


Figure 28: evaluation of the BCCM TCB strains.

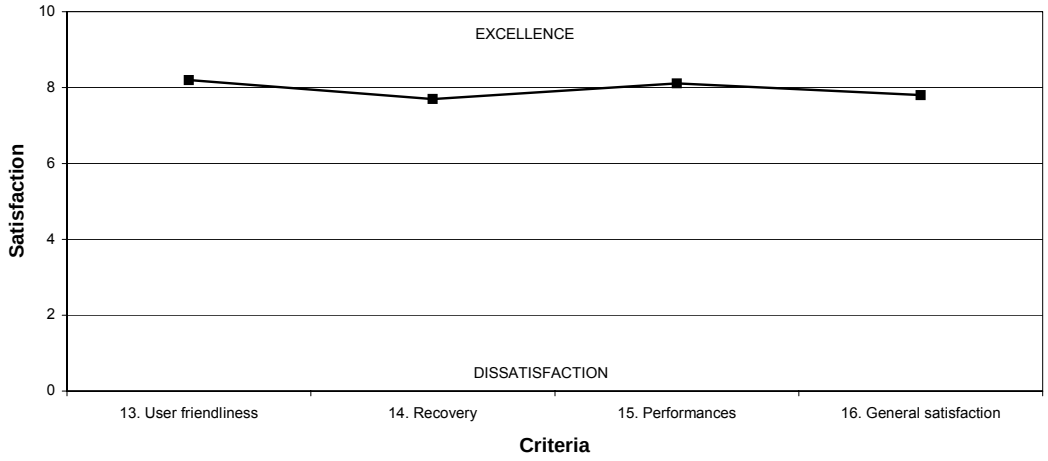


Figure 29: comparison of the BCCM TCB strains with other TCB strains available on the market.

In the Figure 28, the results were sorted in descending order. The laboratories are satisfied for all criteria. The recovery is the worst result. The explanation therefore is that some strains sent by BCCM/LMG were strains with a bad recovery on the cryovials and which will not be supplied on cryovials in the future.

Concerning the evaluation of the BCCM TCB strains compared with other TCB strains available on the market, the laboratories are satisfied with the user friendliness, the recovery and the performances. The degree of general satisfaction is high.

Average price suggested by the laboratories for BCCM TCB strain delivered in a cryovial in frozen conditions is 66 EUR.

General suggestions and remarks from the laboratories

- Price must be realistic for use in routine. A too expensive price from other firms is the reason why we are making our own reference material starting from your cultures. (this laboratory suggested a price of max. 10 EUR);
- Subcultures used in the laboratory routine have a low live expectancy. New working stocks need to be supplied within 4-5 days or the culture will be lost;
- We use for the moment reference material from the Keuringdienst Van Waren. They are very cheap and it is able to enumerate the colonies which gives an extra value to the material;
- The need on the market, are the quantitative capsules and not so much the qualitative.

The test phase is thus very positive. The laboratories are satisfied with the TCB strains delivered under frozen conditions in cryovial.

6 DIFFUSION AND VALORISATION

This study results mainly in two concrete documents. On one hand, a letter will be transmitted to the Belgian normalisation body (IBN – BIN) and to the Belgian accreditation body (Beltest). It will content remarks about the normative texts in the different fields considered and suggestions to improve them thanks to the expertise acquired during this project.

On the other hand, we prepared a catalogue focused on the TCB strains. This "Catalogue of test, control or bioassay strains from the BCCM culture collections" puts together not only strains listed in the norms, but proposes as well strains convenient for normative method that do not impose accurate strains and alternative efficient strains if those set in the norm do not work properly. In some cases, performances of the strains are mentioned.

This project will allow the clients from the BCCM/LMG collection to receive their TCB strains with a certificate of proven viability, purity, identity and performances. Most of these strains will be available as a user-friendly, ready-to-use working stock with a guaranteed storage life (cryovials with 25 beads to be stored at -20°C).

7 CONCLUSION AND PERSPECTIVES

At the end of this project, the conclusion is rather different for the three participants. As noticed in the introduction, we observed that bacterial TCB strains are much more used than fungi or yeasts. This is probably also partly reflected in the higher number of strains supplied each year by BCCM/LMG compared to those of BCCM/IHEM or BCCM/MUCL.

For BCCM/IHEM, culture collection specialised in fungi and yeasts from medical importance, the project will have very few practical consequences: norms necessitating reference fungal strains, and consequently reference strains, are not numerous. It is thus not possible to satisfy clients asking a "user friendly" form of preservation and to sell strains preserved on cryobeads on dry ice, the corresponding invoice being much too expensive.

For the BCCM/LMG collection, this project has a more important impact. As noted above considerably more bacterial strains are used as reference strains, most of which can now be distributed by BCCM/LMG with a certificate of proven performance. Furthermore, the clients will be able now to receive many of these strains under a more user-friendly ready-to-use working stock, a need expressed in the inquiry. Moreover the BCCM/LMG bacterial collection has gained during the project more experience about the TCB strains used in the food sector and new tests were performed to determine their most important characteristics. This will allow to give more detailed information to the clients about the reference strains studied. The intern quality system of the collection was also further developed and implemented: specific technical procedures for testing the performance of the TCB strains and a quality plan for the TCB strains were drawn up during the project.

For BCCM/MUCL, agro-industrial fungi and yeasts collection, the tests carried out in this study are all related to European standards used to test the durability and the protection of wood and wood-based products, and to standard EN 113 in particular. In these tests, the level of virulence of the strains used but also its stability under different preservation methods is key factor. For the EN 113, only one out of the four obligatory strains and nine out of the 16 alternative strains have shown the required virulence. As consequence, a re-evaluation of the preserved material seems necessary to control the evolution of the fungal material during the storage (mutation, metabolic dormancy, …).
Parallel to this work, a quality system for the BCCM/MUCL collection has been developed to check the viability, purity and identity of the biological material.

ACKNOWLEDGEMENTS

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Special thank to Mrs Vanhonacker, ex-colleague from the BCCM/LMG Bacteria Collection, who was in charge of the project coordination from the layout of the project until the end of the year 2002.

We express our gratitude to people from the laboratories and companies who spent time to answer our questionnaire, and to participate to the test phase.

We wish to thank Mr Croon, Mr Voorhof, Mr Janssens, Mr Dinant and Mr Dellow from the Belgian Institute for Standardisation for their advice and assistance in collecting information on norms.

We are also grateful to Mrs Vanlaethem and Mrs Dierick from Beltest for their help in listing companies and laboratories working in the field of this project and for advice about the interpretation of performance tests.

We are indebted to Prof. De Zutter (Diergeneeskundig toezicht op eetwaren, Faculteit Diergeneeskunde, Universiteit Gent), Mrs De Loy (Levensmiddelentechnologie en voeding, Landbouwfaculteit, Universiteit Gent), Mrs De Roose (Stadslabo Gent), Prof. Nelis (Farmaceutische analyse, Faculteit Farmaceutische Wetenschappen, Universiteit Gent), Mr Iliano (Labo Iliano), Dr Dierick (Dienst Voedingswaren, Institute of Public Health, Brussels), Prof. Daube and Dr Ghafir, (Département des sciences des denrées alimentaires, Faculté de Médecine Vétérinaire, Université de Liège), Mrs Thomassin and Le Bayon (Centre Technique du Bois et de l'Ameublement, Bordeaux, France) for their help in collecting specific information in their respective areas of competence.

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