J Ind Microbiol Biotechnol (2004) 31: 199–203
DOI 10.1007/s10295-004-0133-x

ORIGINAL PAPER

**Sonia Savluchinske Feio · Ana Barbosa
Manuela Cabrita · Lina Nunes · Alexandra Esteves
José Carlos Roseiro · Maria João Marcelo Curto**

Antifungal activity of *Bacillus subtilis* 355 against wood-surface contaminant fungi

Received: 30 September 2003 / Accepted: 8 March 2004 / Published online: 12 June 2004

Abstract A strain of *Bacillus subtilis* was examined for antifungal activity against phytopathogenic and wood-surface contaminant fungi. The bacterium was grown in five culture media with different incubation times in order to study cell development, sporulation, and the production of metabolites with antifungal activity. The anti-sapstain and anti-mould activity of the bacterium grown in yeast extract glucose broth (YGB) medium in wood was also evaluated. In YGB, the bacterium inhibited the growth of several fungi and displayed a broader spectrum of activity than in the other media tested. A relationship between bacterial spore production and the formation of metabolites with antifungal activity was detected. YGB medium displayed effective control in wood block tests. YGB medium was extracted with solvents of increasing polarity and the dry residues were applied to silicagel plates, resolved with the appropriate solvent and sprayed with different solutions, detecting the presence, of amines, and higher alcohols. The bioautographic method revealed the presence of at least two active compounds against the blue-stain fungus *Cladosporium cucumerinum*.

Keywords Antifungal activity · *Bacillus subtilis* · Bioautographic method · Wood preservation

S. S. Feio (✉) · A. Barbosa · M. Cabrita · A. Esteves
J. C. Roseiro · M. J. M. Curto
Departamento de Tecnologia de Indústrias Químicas,
Instituto Nacional de Engenharia e Tecnologia Industrial,
Estrada ao Paço do Lumiar, 1699-038 Lisbon, Portugal
E-mail: sonia.feio@ineti.pt
Tel.: +351-21-7162712
Fax: +351-21-7166966

L. Nunes
Laboratório Nacional de Engenharia Civil,
Av. do Brasil, 101, 1700-066 Lisbon, Portugal

Introduction

The literature describes the antagonistic effects of several microorganisms, often referred to as potential biocontrol agents, against a variety of phytopathogenic fungi [14, 18]. Among microorganisms producing metabolites with antifungal activity, bacteria of the genera *Pseudomonas*, *Streptomyces* and *Bacillus* have been shown to be effective against wood blue-stain fungi [8, 21]. Secondary metabolites produced by certain species and strains of *Bacillus* show antifungal activity against phytopathogenic microorganisms [11] . Some authors have suggested that the use of such strains or species, or their metabolites, may be an alternative to agrochemical plant protection [3, 14]. Other metabolites, such as biosurfactants and enzymes that degrade the cell wall, are also produced by *Bacillus* species [14]. Antibiotics produced by *Pseudomonas* and *Streptomyces* species have also been described; Benko [1] tested more than 200 bacteria of different genera for antibiosis and observed *Pseudomonas* and *Streptomyces* spp. to be the most active.

Sapstain and mould growth on lumber are serious problems in the forest industry. Structural damage to timber is minimal, but the discolourations are objectionable to buyers and highly detrimental to the pulp and paper industry. Protective solutions containing toxic chemicals that may accumulate in animal tissues [14] face increasingly strict restrictions, and biological control [9, 12] is being considered for wood protection against both sapstain and decay fungi [4]. However, biological protection of wood has not been widely used. In contrast, there are some well-known examples of biological forest protection, such as the treatment of pine stumps with *Peniophora gigantea* against *Heterobasidium annosus* [19].

In the present work, *Bacillus subtilis* was grown under different conditions in a search for metabolites with antifungal properties as potential substitutes for conventional fungicides in the protection of wood.

Materials and methods

Microorganisms

All microorganisms were obtained from the culture collection of industrial microorganisms (CCMI), LMI-INETI, Lisbon, Portugal. Bacterium: *B. subtilis* CCMI 355; blue-stain fungus: *Cladosporium cucumerinum* CCMI 206; phytopathogenic fungi: *Botrytis cinerea* CCMI 899, *Fusarium oxysporum* CCMI 898; moulds: *Apergillus niger* CCMI 296, *Penicillium expansum* CCMI 625,*Trichoderma* sp., *T. harzianum* CCMI 783, *T. koningii* CCMI 877, *T. virgatum* CCMI 303.

Wood

Blocks (40×40×8 mm) of maritime pine (*Pinus pinaster*) sapwood were cut from green timber without visible stain. The timber had been stored in a deep freeze for 6 months.

Growth and sporulation in different culture media

Nutrient agar (NA; Difco, Detroit, Mich.) was used for *B. subtilis*. Fungi were incubated in malt extract agar (MEA; Difco). Other culture media were selected according to Benko and Highley [2] and Leifert et al. [14]. Brain heart infusion (BHI; Difco); MYDT (g/l deionised water: meat extract 1, yeast extract 1, dextrose 10, tryptone 2); PYB (g/l deionised water: yeast extract 5, peptone 10, NaCl 5); YGB (g/l deionised water: yeast extract 4, glucose 2, NaCl 5, K_2HPO_4 2.5, KH_2PO_4 0.5); YGB + Mg (YGB + 1% $MgSO_4$). *B. subtilis* was cultured on NA slants for 24 h at 30°C.

BHI, MYDT, PYB, YGB and YGB + Mg were inoculated with *B. subtilis* as follows: 100 ml of each medium was transferred to sterilised 500 ml Erlenmeyer flasks, which were inoculated with 0.1 ml *B. subtilis* suspension and the turbidity adjusted to give an absorbance of 1.0 at 620 nm. The flasks were incubated at 30°C, 110 rpm for 14 days. To follow cell growth, spore formation and antifungal metabolite production, samples of culture medium were collected on days 0, 1, 2, 3, 4, 7, 11 and 14. For *B. subtilis* spore counts, samples of culture medium were heated at 77°C for 12 min in a water bath. A 1 ml aliquot of each sample was incorporated into NA in Petri dishes, and incubated at 30°C for 48 h, after which colony counts were carried out. Samples (4 ml) of each culture medium were centrifuged at 500 *g* for 30 min and the supernatants were kept at −20°C for further analysis.

Bioassays of antifungal activity

Fungal suspensions (10^8 cfu ml^{-1}), obtained by dilution and counting in a haemocytometer, were incorporated into molten MEA at 45°C in Petri dishes. Filter paper discs impregnated with 0.02 ml of each culture medium were placed on the agar. After 48 h incubation, plates were observed for antifungal activity, indicated by the formation of a clear zone around the discs. The minimal inhibition zone was defined as 2 mm of clear inhibition around the disc.

YGB medium inoculated with *B. subtilis* was extracted successively with *n*-hexane, chloroform, diethyl ether and *n*-butanol. All solvents were purified by standard methods and anhydrous solvents were dried and freshly distilled. Each organic extract was dried over anhydrous sodium sulphate, filtered, concentrated by rotoevaporation and the residue obtained was dried under vacuum. The dry residues were dissolved in dichloromethane, applied to silicagel plates (silica gel 60 F254; Merck, Darmstadt, Germany) and resolved with a mixture of *n*-butanol:acetic acid (5:1, v/v). Plates were observed at 254 and 366 nm and each was sprayed with a different dyeing reagent solution, namely Draggendorff reagent, vanillin-sulphuric acid, Schweppe reagent, fluorescein, trifluoracetic acid, and tungstophosphoric acid, to detect the presence of amines, higher alcohols, acids, lipids, steroids, and sterols, respectively. Another set of silicagel plates was tested for antifungal activity with *C. cucumerinum*, according to the procedure described in [10]. A dichloromethane solution (0.02 ml) containing 200 µg extract was applied to a silicagel plate, dried and resolved with a mixture *n*-butanol:acetic acid (5:1, v/v). The plate was dried and sprayed with a suspension of *C. cucumerinum* and incubated in a moist chamber for 72 h. The presence of antifungal activity was indicated by zones of fungal growth inhibition, contrasting with the dark background of fungal growth, in spots corresponding to metabolites.

Anti-sapstain and anti-mould activity of YGB culture medium in wood

The test was based on methodology commonly used to test wood preservatives and other anti-stain and anti-mould materials or organisms under laboratory conditions [15–17].

Blocks (15 replicates) were brushed with 2.5 ml YGB inoculated with 11-day-old *B. subtilis* culture and with the same amount of a 50% solution of the same culture medium in sterile distilled water. The blocks were left to stand on plastic racks for 1 h and then placed in plastic bags to keep the moisture levels high during the test period. A third group of 15 replicates of untreated maritime pine was used as the control. The wood was used without any sterilisation and/or inoculation. The test blocks were conditioned at 22°C and 70% relative humidity for 3 weeks, after which the extent of fungal colonisation was evaluated according to the following scale: 0 = no growth; 1 = traces of visible fungi apparent; 2 = fungi established; 3 = sporulation becoming heavy; 4 = full growth, underlying wood obscured.

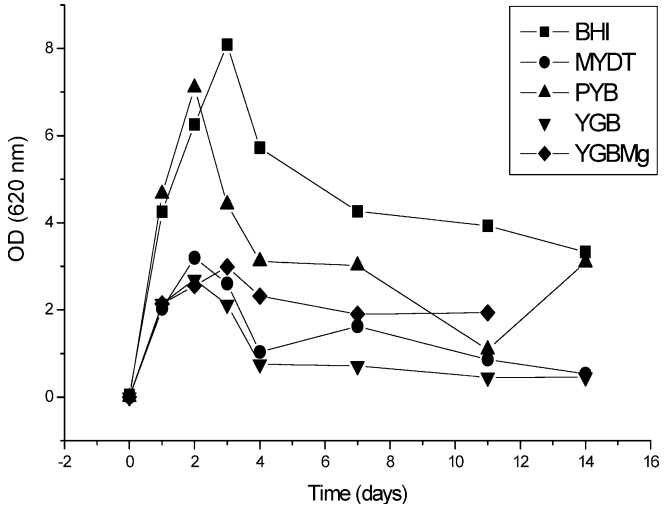

Fig. 1 *Bacillus subtilis* growth on BHI, MYDT, PYB, YGB, YGB + Mg culture media over a 14-day incubation (see Materials and methods for media composition)

Results

Growth and sporulation of *B. subtilis* in different culture media

BHI culture medium stimulated bacterial growth (Fig. 1), whereas sporulation (Table 1) started only on day 3 of incubation, and reached a maximum value after 7 days (3×10^5 cfu ml^{-1}). A similar pattern was observed for PYB. MYDT medium did not promote bacterial growth, which reached a maximum on day 2 of incubation before decreasing, and stimulated spore formation only weakly. YGB and YGB + Mg promoted microbial growth poorly. In YGB + Mg, sporulation was impaired on day 11 compared to results obtained on days 10 and 14. Sporulation in *B. subtilis* can be triggered by carbon catabolite limitation; conversely, excess catabolites inhibit sporulation. Spore production was stimulated in YGB medium, with an augmentation of spore production between days 7 and 11 (33×10^6 cfu ml^{-1}). The profile of sporulation of *B. subtilis* grown in YGB + Mg shows that sporulation started on day 3 and reached a maximum on day 7 (24×10^5 cfu ml^{-1}).

Table 1 Sporulation of *Bacillus subtilis* on BHI, MYDT, PYB; YGB, YGB + Mg culture media over a 14-day incubation (10^3 cfu ml^{-1})

Day	Culture medium				
	BHI	MYDT	PYB	YGB	YGB + Mg
0	42	30	5	5	0.1
1	8	10	20	1	1.5
2	10	20	140	240	1.35
3	11	30	80	650	20,600
4	244	30	420	1,800	22,400
7	300	13	680	2,160	24,066
11	106	400	100	33,000	4,000
14	130	2,300	100	28,000	12,300

Bioassay of antifungal activity

The antifungal activity of *B. subtilis* grown on five different media was evaluated towards phytopathogenic and wood-surface contaminant fungi. Figure 2 shows the results obtained with YGB culture medium. The growth of *Aspergillus* sp., *A. niger, C. cucumerinum* and *F. oxysporum* was inhibited on days 11 and 14, whereas *B. cinerea* and *P. expansum* were inhibited only on day 11 of incubation. Among the *Trichoderma* species, *T. virgatum* was sensitive to metabolites produced by *B. subtilis* in YGB on days 11 and 14, whereas *Trichoderma* sp. was inhibited by metabolites present on days 4, 7 and 11. The results obtained with YGB + Mg are shown in Fig. 3. Growth of *C. cucumerinum* and *F. oxysporum* was inhibited by metabolites produced over the whole incubation period after day 1. *Aspergillus* sp. was inhibited on days 11 and 14, whereas growth of *A. niger* was poorly inhibited, and only on day 3. Mg increased antifungal activity towards *T. virgatum*, especially on days 0, 7, 11 and 14, and towards *T. harzianum* on days 7, 11 and 14. PYB displayed a narrow antifungal range, inhibiting the growth of only *T. koningii*

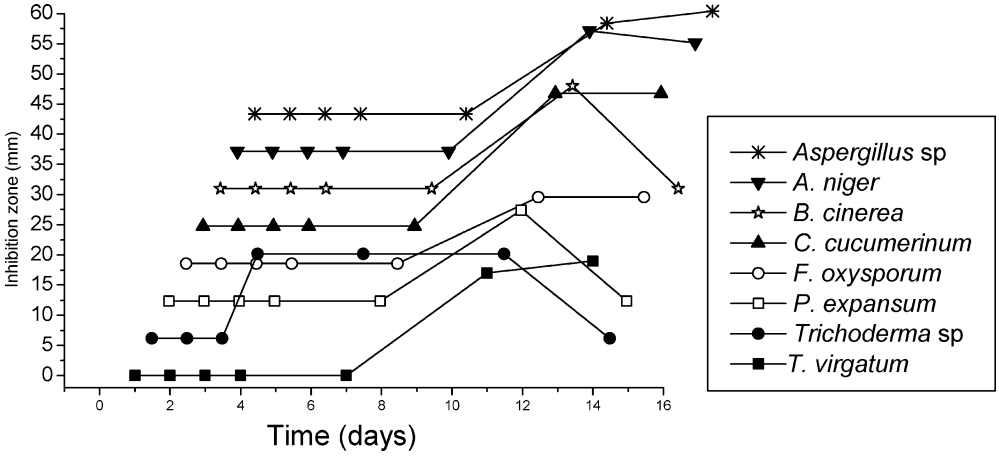

Fig. 2 Antifungal activity of *B. subtilis* on YGB medium against *Aspergillus* sp. *Aspergillus niger*, *Botrytis cinerea*, *Cladosporium cucumerinum* 206, *Fusarium oxysporum*, *Penicillium expansum*, *Trichoderma virgatum*, and *Trichoderma* sp

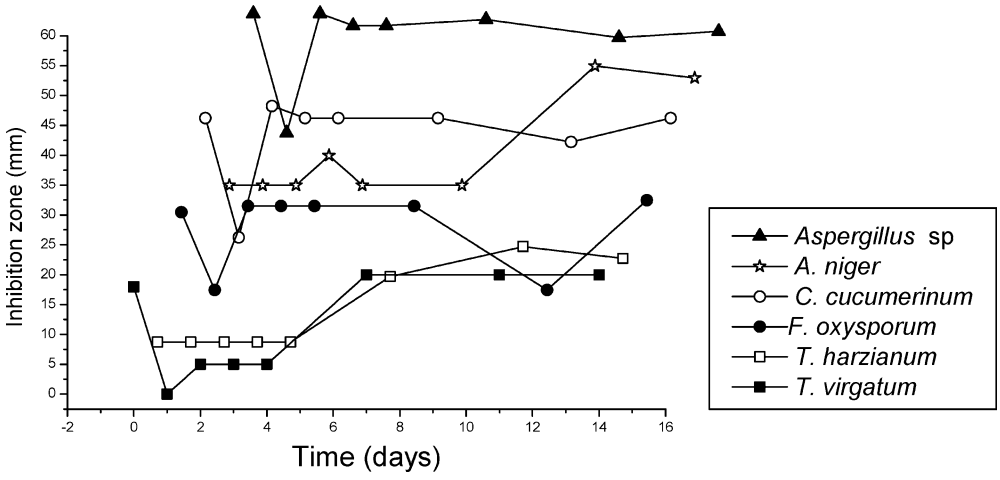

Fig. 3 Antifungal activity of *B. subtilis* on YGB + Mg medium against *A. niger*, *B. cinerea*, *C. cucumerinum*, *F. oxysporum*, *T. harzianum*, and *T. virgatum*

and *T. harzianum* (results not shown). Comparison of Figs. 2 and 3 shows that the formation of metabolites in YGB occurred preferentially on days 11 and 14, whereas in YGB + Mg it occurred on almost all days.

Extraction of YGB culture medium inoculated with *B. subtilis* with *n*-hexane, chloroform, diethyl ether and *n*-butanol yielded residues in which the presence of amines and higher alcohols was detected.

The bioautographic method with *C. cucumerinum* revealed the presence of at least two compounds with antifungal activity. In a preliminary identification they appear to be amines.

Anti-sapstain and anti-mould activity of YGB culture medium in wood

At the end of the test period all untreated controls had reached grade 4 (full growth) and the result obtained with the culture medium (average grade = 3.8 ± 0.4) was not statistically different from the untreated controls (Newman-Keuls test, $P \leq 0.05$).

However, the result obtained with the diluted solution of culture medium (average grade = 3.1 ± 0.9) was significantly lower than the untreated control (Newman-Keuls test, $P \leq 0.01$) and the culture medium (Newman-Keuls test, $P \leq 0.01$), showing some evidence of control of the natural sapstain and mould inoculum.

Discussion

In YGB and YGB + Mg media, growth of *B. subtilis* was weakly stimulated whereas the production of active antifungal metabolites was strongly stimulated. Active metabolite production cannot merely be associated with higher bacterial growth; although some medium supplements that stimulate high antifungal activity also promote a significant increase in growth, others had an inhibitory effect without enhancing bacterial growth [7].

In the present work, antifungal activity was dependent on nutrients in the assay medium and/or incubation time. These observations agree with those of Katz and Demain [13], who reported that antibiotic production was dependent on the composition of the growth medium, and with those of Benko and Highley [2], who observed that small deviations in medium components can make a significant difference in the production of secondary metabolites, particularly antibiotics. Culture age is also an important factor in the production of metabolites with antifungal activity. In the present work, production of active metabolites in different culture media took place at different incubation times. YGB + Mg medium inhibited the growth of some fungi at the start of the incubation, probably due to production of active metabolites by *B. subtilis* inoculated on NA slants in the first 24 h incubation prior to the incubation on YGB + Mg. Those metabolites were not present on day 1, indicating some kind of inactivation. The production of metabolites after the exponential growth phase observed in the present work agrees with reports that synthesis of antibiotics, such as mycobacilin and bacilysin, occurs after the exponential phase of development in *B. subtilis* [13]. The enzymes required for production of these metabolites are synthesised after the exponential phase of *B. subtilis* and, after synthesis of the antibiotics, the enzymatic activity can disappear in few hours. However, this is not a general rule, as several antibiotics are synthesised during the growth of the bacteria, and nutritional or genetic modifications can determine the exact time of antibiotic synthesis in relation to the growth phase [13].

In the present work, sporulation seems to be related to production of active metabolites by *B. subtilis*, in agreement with observations in which the production of antibiotics by *B. subtilis* seems to take place during sporulation [13]. Metabolites produced by *B. subtilis* possess activity against fungi from different taxonomic groups and are even capable of partially controlling a natural infection of wood. Different fungi may respond to different components in the metabolite mixture or variation in antifungal activity may reflect differences in sites of action or the abilities of fungi to detoxify the metabolites [7].

Recently, Cho et al. [6] detected and characterised an iturin A-like compound produced by *B. subtilis* KSO3, which inhibits growth of the phytopathogenic fungus *Gloeosporium gloeosporioides*. Chitarra et al. [5] detected an antifungal compound produced by a strain of *B. subtilis* that permeabilises fungal spores and blocks germination of *Penicillium roqueforti*.

YGB medium displayed antifungal activity against sapstain and mould inoculum of wood. In the present work, diluted culture medium had a stronger protective effect than undiluted medium. In other experiments (data not shown) we observed this antifungal activity effect in wood. We attribute this effect to easier penetration of the diluted medium into the wood. A protective effect of *B. subtilis* culture medium against stain or mould growth on pine blocks was observed by Seifert et al. [20]. Payne et al. [17] observed that some bacterial isolates gave effective protection in wood block tests against mould and stain fungi.

In the present work, at least two compounds were found to have antifungal activity, which agrees with the results of Katz and Demain [13], in which a family of closely related peptides rather than a single substance is produced by an organism. Preliminary studies indicate that the active metabolites contain amine functions; however, further studies will be necessary to determine the structures of the active compounds.

Acknowledgements This work was supported by Fundação de Ciência e Tecnologia and by FEDER. Project 391647BSE/2001.

References

1. Benko R (1988) Bacteria as possible organisms for biological control of blue stain. International Research Group on Wood Preservation. Doc. No IRG/WP/1339, pp 1–12
2. Benko R, Highley TL (1990) Selection of media for screening interaction of wood-attacking fungi and antagonistic bacteria. Mater Org 25:173–180
3. Berger F, Li H, White D, Frazer R, Leifert C (1996) Effect of pathogen inoculum, antagonist density, and plant species on biological control of *Phytophthora* and *Pythium* damping-off by *Bacillus subtilis* Cot1 in high-humidity fogging glasshouse. Phytopathology 86:428–433
4. Bruce A (1998) Biological control of wood decay. In: Bruce A, Palfreyman JW (eds) Forest products biotechnology. Taylor and Francis, London, pp 251–266
5. Chitarra SG, Breeuwer P, Nout RJM, Van Aelst CA, Rombouts MF, Abee T (2002) An antifungal compound produced by *Bacillus subtilis* YM 10-20 inhibits germination of *Penicillium roqueforti* conidiospores. J Appl Microbiol 96:159–166
6. Cho SJ, Lee SK, Cha BJ, Kim YH, Shin KS (2003) Detection and characterization of the *Gloeosporium gloeosporioides* growth inhibitory compound iturin A from *Bacillus subtilis* strain KSO3. FEMS Microbiol Lett 223:47–51
7. Fiddaman PJ, Rossal S (1994) Effect of substrate on the production of antifungal volatiles from *Bacillus subtilis*. J Appl Bacteriol 76:395–405
8. Freitas JR, Germida JJ (1991) *Pseudomonas cepacea* and *Pseudomonas putida* as winter wheat inoculants for biocontrol of *Rhizoctonia solani*. Can J Microbiol 37:780–784
9. Greaves H (1970) The effects of selected bacteria and actinomycetes on the decay capacity of some wood-rotting fungi. Mater Org 5:265–279
10. Homans LA, Fuchs A (1970) Direct bioautography on thin-layer chromatograms as a method for detecting fungitoxic substances. J Chromatogr 51:323–327
11. Jack R, Tagg WRJ, Ray B (1995) Bacteriocins of Gram-positive bacteria. Microbiol Rev 59:171–200
12. Jacquiot C (1968) Antagonistic action of bacteria against fungi and its role in the preservation of pulpwood chips. BWPA Annual Convention 1468:1–3
13. Katz E, Demain A (1977) The peptide antibiotics of *Bacillus*: chemistry, biogenesis and possible functions. Bacteriol Rev 41:449–474
14. Leifert C, Li H, Chidburee S, Hampson S, Workman S, Sigee D, Epton HAS, Harbour A (1995) Antibiotic production and biocontrol activity by *Bacillus subtilis* CL27 and *Bacillus pumillus* CL45. J Appl Bacteriol 78:97–108
15. Morales L, Dickinson DJD (1998) A study of the factors governing the performance of preservatives used for the prevention of sapstain on seasoning wood with regard to the establishment of European standards: overview of co-operative project and development of laboratory test methods. In: Biology and prevention of Sapstain. Forest Products Society, Madison, Wis., pp 77–85
16. Nunes L, Silva A, Nobre T (2001) FAIR CT98-3689. Sustainable wood; new clean technology methods for the control of fungal stain in wood. Periodic report III. LNEC, Technical Report 26/2001. NM, Lisbon
17. Payne C, Bruce A, Staines H (2000) Yeast and bacteria as biological control agents against fungal discolouration of *Pinus sylvestris* blocks in laboratory-based tests and the role of antifungal volatiles. Holzforschung 54:563–569
18. Podile A, Prakash A (1996) Lysis and biological control of *Aspergillus niger* by *Bacillus subtilis* AF1. Can J Microbiol 42:533–538
19. Rishbeth J (1963) Stump protection against *Fomes annosus* III. Inoculation with *Peniophora gigantea*. Ann Appl Biol 52:63–77
20. Seifert K, Hamilton EW, Breuil C, Best M (1987) Evaluation of *Bacillus subtilis* C186 as a potential biological control of sapstain and mould on unseasoned lumber. Can J Microbiol 33:1102–1107
21. Silva AA, Morrell JJ (1998) Inhibition of wood-staining *Ophiostoma picea* by *Bacillus subtilis* on *Pinus ponderosa* sapwood. Mater Org 32:241–252