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► To cite this version:

Laurent Nevarez, Valérie Vasseur, A. Le Madec, M. A. Le Bras, Louis Coroller, et al.. Physiological traits of *Penicillium glabrum* strain LCP 08.5568, a filamentous fungus isolated from bottled aromatised mineral water. *International Journal of Food Microbiology*, 2009, 130 (3), pp.166-171. 10.1016/j.ijfoodmicro.2009.01.013 . hal-00551244

HAL Id: hal-00551244

<https://hal.univ-brest.fr/hal-00551244v1>

Submitted on 3 Jan 2011

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1 **Physiological traits of *Penicillium glabrum* strain LCP 08.5568,**
2 **a filamentous fungus isolated from bottled aromatised mineral**
3 **water**

4
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31 **Abstract**

32

33 *Penicillium glabrum* is an ubiquitous fungus distributed world wide. This fungus is a frequent
34 contaminant in the food manufacturing industry. Environmental factors such as temperature, water
35 activity and pH have a great influence on fungal development. In this study, a strain of *P. glabrum*
36 referenced to as LCP 08.5568, has been isolated from a bottle of aromatised mineral water. The
37 effects of temperature, a_w and pH on radial growth rate were assessed on Czapeck Yeast Agar
38 (CYA) medium. Models derived from the cardinal model with inflection (Rosso et al., 1993 An
39 unexpected correlation between cardinal temperatures of microbial growth highlighted by a new
40 model. J Theor. Bio. 162, 447-463) were used to fit the experimental data and determine for each
41 factor, the cardinal parameters (minimum, optimum and maximum). Precise characterisation of the
42 growth conditions for such a fungal contaminant, has an evident interest to understand and to
43 prevent spoilage of food products.

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55 Keywords: *Penicillium glabrum*, predictive mycology, food spoilage, fungal growth, temperature, water activity, pH,
56 cardinal values, mineral water

58 1. Introduction

59

60 Filamentous fungi are widely distributed in the environment and responsible for numerous
61 spoilage of food products (Pitt and Hocking, 1997; Samson et al., 2004). In addition to the
62 economic losses associated to their visual appearance, another concern is the possibility of off-
63 flavours and mycotoxins production. The most widespread and frequent mould spoilages of food
64 products are caused by several genera such as *Aspergillus*, *Fusarium* or *Penicillium*. Among this
65 last genus, *Penicillium glabrum* is an ubiquitous and cosmopolitan fungus, frequently encountered
66 in food manufacturing industry, due to its wide presence and its important conidiation (Pitt and
67 Hocking, 1997). This filamentous fungus has been previously isolated in a large variety of products
68 as cheese (Northolt et al., 1980; Hocking and Faedo, 1992), maize (Mislivec and Tuite, 1970),
69 commercially marketed chestnuts (Overy et al., 2003), rice (Kurata et al., 1968), jam (Udagawa et
70 al., 1977) and bottled water (Cabral and Fernandez Pinto, 2002; Ancasi et al., 2006). To our
71 knowledge, this fungal contaminant does not seem to produce any known mycotoxin that could
72 threaten the food safety and the consumer health (Pitt and Hocking, 1997). Nevertheless, no precise
73 affirmation can be formulated due to inherent differences which could be observed among several
74 strains of the same species. Despite its large implication in food contamination, to our knowledge,
75 very few studies have been conducted to characterise precisely growth conditions of this species.

76 Growth of filamentous fungi is influenced by a variety of environmental or intrinsic factors.
77 Temperature and water activity (a_w), for example, are recognised as the most important ones that
78 determine the ability of moulds to grow (Dantigny et al., 2005). Other factors such as the
79 composition and intrinsic factors of the product, especially pH, potentially influence the fungal
80 development.

81 In order to analyse the physiological traits of a strain of *P. glabrum* isolated from a
82 polyethylene terephthalate (PET) bottled aromatised mineral water, the present study aims at
83 determining the cardinal values of this strain for temperature, a_w and pH. After investigating in solid

84 medium, its mycelial growth response towards different factors: temperature, a_w and pH, the
85 development of this strain was studied by using a predictive mycology approach.

86

87 For over 20 years, predictive microbiology was focused mainly on food-pathogenic bacteria
88 (Buchanan, 1993) and despite a similar interest, modelling filamentous fungal growth has not
89 received the same level of attention. Actually, quantification of fungal growth is more complicated
90 because, whereas bacteria reproduce by fission and grow homogeneously through a liquid medium,
91 filamentous fungal growth implicated the development of tree-dimensional ramified hyphae with
92 apical growth (Gibson et al., 1994; Gibson and Hocking, 1997). Taking account of these
93 difficulties, the predictive mycology has been developed in several studies (Dantigny et al., 2005)
94 by adapting different models used for bacterial investigations (Ratkowsky et al., 1983; Davey,
95 1989; Rosso et al., 1993; Baranyi et al., 1993; Miles et al., 1997). It appears that cardinal models
96 with inflection (CMI) are suitable for modelling the effect of environmental factors on fungal
97 growth (Rosso and Robinson, 2001). This kind of model originally developed for bacteria (Rosso et
98 al., 1993; Rosso et al., 1995) has been successfully used to the effect of a_w on growth of several
99 filamentous fungi such as *P. chrysogenum* or *Aspergillus flavus* (Sautour et al., 2001a).

100

101 In the present study, CMI were used to model the effects of temperature, a_w and pH on the
102 radial growth rate of *P. glabrum*. This method allows the estimation of the cardinal values of this
103 filamentous fungus for each tested factor. These results define the eco-physiological requirements
104 of this fungal contaminant and has an evident interest to understand its contamination abilities in
105 food manufacturing industry.

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109 **2. Materials and methods**

110

111 **2.1. Isolation and identification of the mould**

112

113 Visible pellets were observed in a sealed PET bottle of aromatised mineral water. Three
114 samples of 100 mL were shaken and filtered through sterile membrane porosity 0.45 µm (Millipore,
115 Guyancourt, France). Visible hyphae were then transferred on Potato Dextrose Agar medium (PDA,
116 Difco Laboratories, Detroit, MI, USA) and incubated for 7 days at 25 °C. A loopfull taken from a
117 visible colony was examined under a microscope for morphological visualisation. Microscopic
118 evaluation of the filamentous fungi isolated, indicated morphology similar to the description given
119 by Pitt and Hocking for the genus *Penicillium* (phialides bearing chains of conidies) (Pitt and
120 Hocking, 1997; Samson et al., 2004). The phialides were attached to the stipe directly, so the
121 species produces monoverticillate penicilli and was classified in the subgenus *Aspergilloïdes*.
122 Identification of the mould was further completed with inoculation of different media incubated at
123 different temperatures following the reference method (Pitt, 1988). Observations were made on the
124 morphology and diameters of the colonies and this filamentous fungus was characterised as
125 *Penicillium glabrum* (Wehmer) Westling. This strain was registered as LMSA 1.01.421 in
126 “Souchothèque de Bretagne” (University of Brest, France / www.ifremer.fr/souchotheque) and
127 LCP 08.5568 in the fungal collection of Laboratory of cryptogamy, Museun National d’Histoire
128 Naturelle (Paris, France / www.mnhn.fr).

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130

131 **2.2. Media preparation and culture conditions**

132

133 The effect of each factor tested experimentally on the growth of this strain of *P. glabrum*, was
134 studied in solid cultures using inoculum consisted in conidia harvested from 7 days-old grown in
135 PDA medium at 25 °C, 0.99 a_w and pH 5.5. Conidia were suspended in 1 mL of sterile water with

136 0,01% Tween 80 (Sigma-Aldrich, Saint Louis, MO, USA). One drop of inoculum containing 10^4
137 spores /ml, was applied with thin pipette, on two points equidistant from the center and the edge of
138 Petri dish that contained the Czapeck Yeast Agar medium (CYA).

139

140 Temperature investigations: standard CYA medium was used and contained 3 % sucrose, 0.5
141 % yeast extract, 0.1 % K_2HPO_4 , 1.5 % agar and 1 % Czapek concentrate (5 % KCl, 30 % $NaNO_3$, 5
142 % $MgSO_4 \cdot 7 H_2O$, 0.01 % $FeSO_4 \cdot 7 H_2O$ and 0.01 % $CuSO_4 \cdot 7 H_2O$). pH and a_w were respectively
143 measured at 6.8 and 0.99. After inoculation of 12 replicates (6 plates), for each condition tested,
144 media were then incubated for 7 days at temperatures in the ranges 5-45 °C.

145 Water activity investigations: CYA media were adjusted to various a_w from 0.79 to 0.99 by
146 substituting a part of water by glycerol (w/w) according to the relation of Langmuir (Lerici et al.,
147 1996): $M (\text{water(g)} / \text{glycerol (g)}) = 0.236 a_w / (1 - 0.99 a_w)$. Inoculations were realised, as described
148 previously except that inoculum was only applied in one point per plate. Triplicate plates were
149 inoculated for most a_w tested (0.79, 0.81, 0.83, 0.85, 0.87, 0.89, 0.91, 0.92, 0.93, 0.94) and for
150 highest values (0.95, 0.96, 0.97, 0.98 and 0.99), 8 replicated plates were realised. The different
151 media were incubated at 25 °C for 7 days. During the experiments, a_w of each medium was
152 stabilised by placing Petri dishes in 1,5 l closed boxes with a glycerol-water solution of the same a_w
153 as the medium (Sautour et al., 2001b). Stability of the different media was also controlled by
154 assessing a_w with FA-st/1 (CBX Scientific Instruments, Romans, France).

155 pH investigations: cultures of *P. glabrum* strain LCP 08.5568 were realised in different CYA
156 media with pH adjusted to each experimental condition. Precise volumes of sterile H_3PO_4 5M,
157 H_3PO_4 2M and NaOH 1M, were added respectively for pH 0.5-2.0, pH 3.0-7.0 and for pH 8.0-11.0
158 (Table 1). The adjusted media from pH 0.5 to 11.0 were inoculated as previously described using 8
159 replicates (4 plates) for each conditions tested. The different media were then incubated at 25 °C for
160 7 days. The pH values of each medium used, was also measured after 7 days of culture in order to
161 confirm their stability.

162

163

164 2.3. Growth rate calculation

165

166 Each factor was studied individually at 5 levels of temperature, 12 levels of a_w and 11 levels
167 of pH containing for each level 12, 3 or 8 and 8 replicates respectively. The radius of the colony
168 (mm) was measured in two directions at right angle and the mean was plotted against time (d). The
169 radial growth rate μ (mm d^{-1}) was defined as the slope of the straight line.

170

171 2.4. Model equations

172

173 The relationship between the growth rate (μ) and the 3 environmental factors tested
174 (temperature, a_w and pH) were assessed using the equations described below. The equations are
175 based on the cardinal model with inflection (CMI) approach. For temperature the CMI originally
176 developed by Rosso et al. (1993) was used

177

$$178 \mu(T) = \frac{\mu_{opt} (T - T_{max})(T - T_{min})^2}{(T_{opt} - T_{min})[(T_{opt} - T_{min})(T - T_{opt}) - (T_{opt} - T_{max})(T_{opt} + T_{min} - 2T)]} \quad (1)$$

179

180 The CMI modified by Sautour et al. (2001a) was used for a_w

181

182

$$183 \mu(a_w) = \frac{\mu_{opt} (a_w - 1)(a_w - a_{wmin})^2}{(a_{wopt} - a_{wmin})[(a_{wopt} - a_{wmin})(a_w - a_{wopt}) - (a_{wopt} - 1)(a_{wopt} + a_{wmin} - 2a_w)]} \quad (2)$$

184

185

186

187 For pH the CMI described by Rosso et al. (1995) was used

188

189

$$190 \mu(pH) = \frac{\mu_{opt} (pH - pH_{min})(pH - pH_{max})}{(pH - pH_{min})(pH - pH_{max}) - (pH - pH_{opt})^2} \quad (3)$$

191

2.5. Model fitting and determination of cardinal conditions

Before fitting, a square-root transformation was performed to homogenise the variance of the experimental growth rate (Dantigny and Bensoussan, 2008). Cardinal values were determined by iterative calculation based on minimising the sum of squares of the residual values (SSR) with NLINFIT function of MATLAB R2008A (The Math-works). 95 % confidence intervals were obtained by using traditional methods based on a linear approximation with NLPARCI function in MATLAB. For each factor modeled the Root Mean Square Error (RMSE) was calculated in order to measure the goodness of fit of each model. According to Ratkowsky (2004), this criterion should be preferred to the regression coefficient r^2 for non-linear models.

3. Results and discussion

3.1. Effect of temperature

The experimental growth results obtained in different conditions of temperature after 7 days of culture in CYA medium, were used to model the growth of this strain according to equation 1 of the CMI (Fig. 1). The minimal, optimal and maximal temperatures were estimated to 6.6, 24.3 and 33.8 °C respectively (Table 2). A good quality of fit was obtained as suggested by the low RMSE value of 0.077.

The optimal temperature around 24 °C for this strain of *P. glabrum*, is in accordance with literature data for this species that describes also an optimum around 25° C (Pitt and Hocking, 1997; Sinigaglia et al., 1998). Similar results were also reported in studies related to *P. chrysogenum* (Gonzalez et al., 1988), *P. expansum* (Lahlali et al., 2005), *P. digitatum* and *P. italicum* (Plaza et al., 2003). Meanwhile, optimal temperature varied slightly from 20 °C for *P. polonicum* (Nunez et al., 2000) to 30 °C for *P. citrinum* (Gonzalez et al., 1988; Montani et al., 1988). The range of

219 temperatures from 20 to 30 °C is frequently encountered in food manufacturing industries and may
220 be also reached in non-refrigerated storage of some products as bottles of aromatised mineral water.

221 The maximal temperature condition for this filamentous fungus was close to 34 °C which is in
222 accordance with some data reporting the absence of growth above 37 °C (Pitt and Hocking, 1997)
223 but differs from others reporting a fungal growth up to 40 °C (Sinigaglia et al., 1998). Results
224 obtained for this strain of *P. glabrum* also showed the minimal temperature condition of 7 °C
225 which may differ from literature data, reporting a slight development of microcolonies up to 4 mm
226 after several days at 5.0 °C (Pitt, 1988).

227

228 **3.2. Effect of water activity**

229

230 As reported previously (Sautour et al., 2001a), a gradual increase in the radial growth rate was
231 exhibited at sub optimal water activities. In contrast a sharp decrease in the growth rate was
232 observed was noticed between the optimum and 1 (Fig 2). The minimal and the optimal a_w were
233 estimated to 0.820 and 0.983 respectively (Table 2). A good quality of fit was obtained as suggested
234 by the low RMSE value of 0.078.

235 The minimal a_w for this stain 0.82 was less than the minimal value 0.88 a_w reported
236 previously in another study for this species (Sinigaglia et al., 1998). Filamentous fungi are among
237 the organisms capable of growing below 0.90 (Pitt and Hocking, 1997) and most *Penicillium*
238 species presented a minimal a_w between 0.82 and 0.86 (Northolt et al., 1995). Similar a_w conditions
239 are tolerated by some xerophilic *Penicillium* species as *P. chrysogenum* growing above 0.78-0.81
240 (Hocking and Pitt, 1979; Sautour et al., 2001b) or *P. roqueforti* growing from 0.82 (Gock et al.,
241 2003). The minimal a_w for growth obtained in our study was lower than results obtained from *P.*
242 *hordei*, *P.aurantiigriseum* (Marin et al., 1998) and *P. olsonii* (Lopez-Diaz et al., 2002). Several
243 other *Penicillium* species showed minimal a_w around 0.90 as *P. expansum* (Lahlali et al., 2005), *P.*
244 *verrucosum* (Cairns-Fuller et al., 2005) or *P. italicum* and *P. digitatum* (Lahlali et al., 2006).

245 The estimated optimal a_w condition was 0.98 which is in accordance with literature data on
246 this species, reporting also the same value (Sinigaglia et al., 1998). Most *Penicillium* species also
247 showed similar response to medium a_w and optimal conditions around 0.97-0.98 (Hocking and Pitt,
248 1979). For example, the optimal a_w for growth was estimated to 0.98 for *P. chrysogenum* using the
249 same CMI than that described by eq (2) in this study (Sautour et al., 2001a).

250

251

252 3.3. Effect of pH

253

254 Radial growth rate was almost constant in the pH range 2.0-7.0 (Fig. 3). Experimental data
255 were fitted by the model eq (3) rather satisfactorily, as suggested by the low RMSE value, 0.089
256 (Table 2). The optimal and the maximal pH values were 5.5 and 11.2 respectively but the minimal
257 pH was estimated in the negative range at -2.1. Application of another model (Zwietering et al.,
258 1992), gave with even a higher RMSE, aberrant minimal pH when applied to the same data (results
259 not shown).

260 These results obtained showed the difficulty to model the growth response of this strain under
261 very acidic conditions. Future studies should be directed to find a convenient model that fits
262 correctly the pH growth response of this filamentous fungus. Nevertheless, the experimental data
263 obtained gave some precious information as no fungal growth was observed at pH 0.5 which
264 indicate that the minimal pH conditions seemed to be between 0.5 and 1.0. It differs from previous
265 description of this species reporting a minimal pH value of 2.0 (Sinigaglia et al., 1998).

266 From the modeling of the pH response, the optimal pH condition of 5.5 and the large
267 tolerance observed for this filamentous fungus towards a large range of pH conditions, were in
268 accordance with literature describing optimal growth rate of many filamentous fungi around pH 5.0
269 (Pitt and Hocking, 1997) and in the pH range 3.0 to 8.0 (Wheeler et al., 1991). As reported in
270 literature, sensibility of this strain of *P. glabrum* towards alkaline conditions appeared higher than

271 acidic ones. The pH response observed for this strain could be compared with other pH studies on
272 several *Penicillium* species conducted in solid medium (Wheeler et al., 1991). From these results, *P.*
273 *citreonigrum* seemed to present a similar response than *P. glabrum* and its optimum was defined at
274 pH 4.4-6.3. The results obtained in our study were also similar to those observed for *P. jensenii*
275 (Sacks et al., 1986) as this filamentous fungi seemed not very sensitive to pH range from 3.5 to 7.1
276 but showed an important fungal growth decrease just below at pH 3.3. *P. roqueforti* also showed a
277 large tolerance to several pH values tested from 4.5 to 7.5 (Gock et al., 2003). In a large range of
278 values, the medium pH seems to have a very low influence on the growth of this fungus as reported
279 also for several *Penicillium* species between pH 4.0-10.0 (Thompson et al., 1993). The tolerance
280 observed here for *P. glabrum* towards a large acid pH range may explain the presence of this
281 species on a large variety of food products of different pH. The pH sensibility increase in the
282 alkaline range until the estimated maximal pH value of 11.18. This value seemed coherent with the
283 results previously obtained on different *Penicillium* species (Wheeler et al., 1991).

284

285 Considering the good fit of the temperature and a_w models (RMSE of 0.077 and 0.078
286 respectively) and the estimated cardinal values, the method of CMI developed by Rosso *et al.*,
287 seemed well adapted to analyse the effect of both factors on the growth of this strain of *P. glabrum*.
288 The robustness of the approach of Rosso *et al.* of has been reported in a study on the effects of
289 temperature and a_w on *Aspergillus carbonarius* growth (Tassou et al., 2007). Analysis of the results
290 obtained with other predictive mycology methods, showed that Rosso *et al.* approach was the most
291 adapted to model the growth of this filamentous fungus in different conditions. This method has
292 been successfully used, for example, in *P. chrysogenum*, *Aspergillus flavus*, *A. parasiticus*, *A.*
293 *oryzae* to model the effect of a_w on fungal growth (Rosso and Robinson, 2001; Sautour et al.,
294 2001a). This method has also the advantage to define fungal growth rate (μ), by 4 parameters with
295 concrete physiological meaning: optimal growth (μ_{opt}) and minimal, optimal and maximal
296 conditions for each factor tested. Thus application and fitting of these models allowed to calculate

297 these parameters for each factor tested. For this reason, the use of CMI method has been well
298 adapted to provide physiological characteristics of this strain of *P. glabrum* for temperature and a_w .
299 Nevertheless some difficulties were shown to fit the experimental data with the CMI in very acidic
300 conditions. Cardinal models are versatile tools that can adapt to the different shapes of the curves μ
301 vs temperature and μ vs a_w . There are no reason that could prevent the CMI from fitting data pH vs
302 pH with a good accuracy. The lack of fit that was demonstrated under acidic pH may be due to no
303 data were available between pH 0.5 and 1, but this should be verified.

304

305 The different results obtained in this study provide useful background to improve
306 characterisation of the strain of *P. glabrum* isolated from PET bottled aromatised mineral water.
307 The microbiological quality of bottled mineral water is of great interest but has not been very
308 largely investigated. In addition to indigenous bacteria that do not induce any risk to public health,
309 mineral water may sometimes contain contaminants as bacteria or filamentous fungi. Some authors
310 described that the fungal foreign bodies visible in the mineral water samples, were made up of
311 pellets with a diameter of 3 to 20 mm (Fujikawa et al., 1997). The most frequent fungal genera
312 isolated from mineral water were *Penicillium* followed by *Cladosporium*, *Trichoderma*,
313 *Aspergillus*, *Alternaria* and *Acremonium* (Fujikawa et al., 1997; Liceaga-Gesualdo et al., 2001;
314 Hageskal et al., 2006). Among the genus *Penicillium*, *P. citrinum* and *P. glabrum* were the 2 most
315 isolated species (Cabral and Fernandez Pinto, 2002). Although filamentous fungi in water usually
316 do not generate public health problems, nevertheless some of the fungi isolated from bottled
317 mineral water as *Alternaria alternata* and *P. citrinum* have some toxigenic potential which could
318 determine some health risk (Cabral and Fernandez Pinto, 2002).

319 The contamination of these products may be explained by microbial presence from the
320 surrounding environment when filling and capping bottles of mineral water (Fujikawa et al., 1997).
321 This last hypothesis was supplied by the fact that many filamentous fungi as some *Penicillium*
322 species disperse a large number of spores in the environment.

323 In our study, the strain of *P. glabrum* isolated from aromatised mineral water, seemed to have
324 very low nutritional requirements as it can develop in visible pellets in such a poor nutritive
325 environment with slight carbohydrate concentrations, various salts and limited oxygen
326 concentration [as only a small fraction of air is enclosed in tight sealed bottles](#). In literature, it was
327 also shown that sometimes, fungal contaminants could use as nutriments, organic compounds
328 releases during storage, from PET (Criado *et al.*, 2005), a beverage bottling material used for
329 conditioning a large variety of commercialised water as the one which is studied here. This
330 aromatised bottled mineral water presented a very high a_w , a pH at 7.0 and the storage of this
331 product was often made at room temperature (around 18-25 °C). The characteristics of this
332 aromatised mineral water may be favourable for the growth of this strain of *P. glabrum* by
333 extrapolating its physiological requirements obtained in solid medium. Several authors have
334 previously reported the presence of this species in commercialised water (Cabral and Fernandez
335 Pinto, 2002; Ancasi *et al.*, 2006). The contamination of this product by this filamentous fungus was
336 also explained by its ubiquitous presence in the environment and its large conidiation in the
337 atmosphere. Moreover, the physiological characteristics of this strain of *P. glabrum* seemed to
338 present important similarities with the temperature, a_w and pH requirements of another frequent
339 fungal contaminant of water products such as *P. citrinum* (Hocking and Pitt, 1979; Gonzalez *et al.*,
340 1988; Montani *et al.*, 1988; Wheeler *et al.*, 1991; Comerio *et al.*, 1998).

341

342 Precise characterisation of growth conditions of this strain of *P. glabrum* has an evident
343 interest to understand its contamination abilities in food manufacturing industry. The influence of
344 temperature, a_w and pH on fungal growth could be taken into account to maintain good conditions
345 on stored product. Nevertheless these results should be considered carefully as fungal
346 contamination of different products could also concern several other species than *P. glabrum* and
347 may interact in a competitive or associative way.

348

349 **Acknowledgements**

350

351 This entire work was realised with the financial support of the Brittany region.

352

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