

**Use of response surface methodology to optimise  
environmental stress conditions on *Penicillium glabrum*,  
a food spoilage mould**

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1 **Use of response surface methodology to optimise environmental stress conditions on**  
2 ***Penicillium glabrum*, a food spoilage mould**

3

4 **Laurent Nevarez, Valérie Vasseur<sup>\*</sup>, Stella Debaets, Georges Barbier**

5

6 *-Université Européenne de Bretagne, France*

7 *-Université de Brest, EA3882 Laboratoire Universitaire de Biodiversité et Ecologie Microbienne,*

8 *IFR148 ScInBioS, ESMISAB, Technopôle de Brest Iroise, 29280 Plouzané, France*

9

10 <sup>\*</sup> Corresponding author. Phone: +33 2 98 05 61 00; Fax: +33 2 98 05 61 01

11 *E-mail address:* [valerie.vasseur@univ-brest.fr](mailto:valerie.vasseur@univ-brest.fr)

12

13 **ABSTRACT**

14 Fungi are ubiquitous micro-organisms often associated with spoilage and biodeterioration of a large  
15 variety of foods and feedstuffs. Their growth may be influenced by temporary changes in intrinsic  
16 or environmental factors such as temperature, water activity, pH, preservatives, atmosphere  
17 composition, all of which may represent potential sources of stress. Molecular-based analyses of  
18 their physiological responses to environmental conditions would help to better manage the risk of  
19 alteration and potential toxicity of food products. However, before investigating molecular stress  
20 responses, appropriate experimental stress conditions must be precisely defined. *Penicillium*  
21 *glabrum* is a filamentous fungus widely present in the environment and frequently isolated in the  
22 food processing industry as a contaminant of numerous products. Using response surface  
23 methodology, the present study evaluated the influence of two environmental factors (temperature  
24 and pH) on *P. glabrum* growth to determine ‘optimised’ environmental stress conditions. For  
25 thermal and pH shocks, a large range of conditions was applied by varying factor intensity and  
26 exposure time according to a two-factorial central composite design. Temperature and exposure

27 duration varied from 30 to 50°C and from 10 min to 230 min, respectively. The effects of  
28 interaction between both variables were observed on fungal growth. For pH, the duration of  
29 exposure, from 10 to 230 min, had no significant effect on fungal growth. Experiments were thus  
30 carried out on a range of pH from 0.15 to 12.50 for a single exposure time of 240 min. Based on  
31 fungal growth results, a thermal shock of 120 min at 40°C or a pH shock of 240 min at 1.50 or 9.00  
32 may therefore be useful to investigate stress responses to non-optimal conditions.

33

34 *Keywords:* *Penicillium glabrum*, food spoilage, thermal shock, acid shock, alkali shock, mycelial  
35 growth, response surface methodology, central composite design.

## 36 **Introduction**

37 Fungi are ubiquitous micro-organisms often associated with spoilage and biodeterioration of a large  
38 variety of foods and feedstuffs. Various genera such as *Aspergillus*, *Cladosporium*, *Alternaria* or  
39 *Penicillium* are involved in different food spoilage (Pitt & Hocking 1997). *Penicillium* is one of the  
40 most widespread fungal genera isolated from food products and, in addition to the economic losses  
41 they cause, several *Penicillium* species may produce mycotoxins that represent a potential health  
42 risk for humans and animals (Pitt & Hocking 1997; Samson et al. 2004). Numerous intrinsic  
43 parameters in foods (water activity, pH, preservatives, *etc.*) or extrinsic ones (temperature,  
44 atmosphere composition, *etc.*) provide favourable conditions for moulds to develop. Changes in  
45 these parameters beyond the tolerated range may represent a potential source of stress that can  
46 affect germination, mycelial growth, conidiation or even synthesis of secondary metabolites as  
47 mycotoxins (Espeso et al. 1993; Calvo et al. 2002; Magan et al. 2002; Schmidt-Heydt et al. 2008).

48 Nevertheless, to our knowledge, little is known about the effects of shock conditions on the growth  
49 and physiology of food related filamentous fungi. Understanding these effects in spoilage moulds  
50 would be useful for a better risk management of alteration and toxicity of food products. In this  
51 study, investigations were conducted on *Penicillium glabrum*, which is very frequently encountered

52 in the food processing industry due to its ubiquitous presence in the environment and its capacity to  
53 disperse a large number of spores into the air (Pitt & Hocking 1997). This fungal contaminant was  
54 has been isolated in a large variety of products, including cheese (Hocking & Faedo 1992), nuts  
55 (Freire et al. 2000), bottled mineral water (Cabral & Fernandez Pinto 2002), *etc.* In our study, the  
56 effects of thermal and pH shock conditions were investigated in *P. glabrum* grown in liquid  
57 cultures. Temperature was chosen for this study because it is one of the most important factors that  
58 determine the ability of moulds to grow (Dantigny et al. 2005). pH was also investigated as this is a  
59 main environmental factor of physiological importance that can vary significantly depending on the  
60 food product. Shock conditions are usually defined by their intensity and their duration. To  
61 efficiently study the physiological effect of a large range of these two environmental shock  
62 conditions, we analysed *P. glabrum* growth using the response surface methodology (RSM) (Myers  
63 et al. 1989). A two-factorial central composite design (CCD) (Box et al. 1978) was applied to  
64 determine ‘optimised’ experimental shock conditions by combining different stress intensities and  
65 durations for each factor studied. Results from CCD were used to modelise the effects of shock  
66 intensity and duration on the measured response (fungal biomass growth).

67 This study brings a missing characterisation of the growth response of a fungal food contaminant to  
68 different thermal, acidic and alkaline conditions. Such results were needed to support the choice of  
69 temperature and pH shock conditions to investigate stress response of *P. glabrum*.

70

## 71 **Materials and methods**

### 72 **Fungal strain and culture medium**

73 The fungal strain used in this study was isolated from contaminated, aromatised mineral water. It  
74 was characterised as *Penicillium glabrum* (Wehmer) Westling according to the reference method  
75 for classifying *Penicillium* species based on morphological characteristics (Pitt, 1988). To confirm  
76 the morphological identification of the fungal strain, we sequenced the internal transcribed spacer  
77 region including the 5.8S of the ribosomal gene in both directions after PCR amplification. Species

78 identification was confirmed based on the results of BLAST (Basic Local Alignments Search Tool)  
79 searches against known sequences in the GenBank database using BLASTn. The strain has been  
80 registered as LMSA 1.01.421 in the Brittany Microbe Culture Collection (*Souchothèque de*  
81 *Bretagne*; University of Brest, France; www.ifremer.fr/souchotheque) and as LCP 08.5568 in the  
82 fungal collection of the Laboratory of Cryptogamy at the *Museum National d'Histoire Naturelle*  
83 (Paris, France; www.mnhn.fr). The cardinal (minimum, optimum and maximum) growth conditions  
84 of temperature and pH for this filamentous fungus have been estimated to be 6.6 °C, 24.3 °C and  
85 33.8 °C and pH 0.50 - 1.00, pH 5.50 and pH 11.20 (Nevarez et al. 2009).

86 For strain maintenance and collection of spores, the strain was cultured in tubes of potato dextrose  
87 agar medium (PDA, Difco Laboratories, Detroit, MI, USA) at 25 °C. The pH of this culture  
88 medium was 5.00.

89

#### 90 **Shock application on *P. glabrum* liquid cultures**

91 Spores were collected from seven-day-old mycelium by flooding each tube with 2 ml of sterile  
92 water containing 0.01 % of Tween 80 (Sigma-Aldrich, Saint Louis, MO, USA) under agitation at  
93 250 rpm. Mycelia were cultured from a suspension of  $5 \times 10^6$  *P. glabrum* spores inoculated into a  
94 250 ml Erlenmeyer flask containing 50 ml of potato dextrose broth medium (PDB, Difco  
95 Laboratories). Cultures of *P. glabrum* were first grown in standard PDB (pH = 5.0) at 25 °C,  
96 120 rpm for 48 h and then subjected to different experimental conditions. To test response to a  
97 range of thermal conditions, mycelia were aseptically filtered at room temperature and transferred  
98 immediately into 50 ml of standard PDB media and incubated at different temperature conditions at  
99 120 rpm. For efficient incubation, each inoculated medium had been previously warmed to its  
100 corresponding experimental temperature. To test response to pH conditions, mycelia grown for 48 h  
101 were aseptically filtered and transferred into modified PDB medium at different pH values. Each  
102 medium was prepared with PDB and to stabilise the pH, appropriate buffers were added: citrate-  
103 phosphate buffer for pH from 2.00 to 8.00, borate buffer for pH from 8.00 to 9.50, CAPS (N-

104 cyclohexyl-3-aminopropanesulfonic acid) buffer for pH 9.50 from to 11.50. For marginal pH  
105 conditions above pH 11.50 or below pH 2.00, buffer was not required for pH stability. The pH was  
106 then adjusted with NaOH and HCl (1, 2 or 10 M). The pH of each adjusted PDB was verified. The  
107 media were filtered through a 0.22 µm membrane and 50 ml was aseptically distributed into sterile  
108 250 ml Erlenmeyer flasks.

109 The pH of each adjusted medium was assessed after fungal culture and variation observed in each  
110 medium before inoculation was less than 0.5 pH units. These results confirmed the efficiency of the  
111 various buffers used to prepare the adjusted media.

112 After incubation under thermal or pH experimental conditions, the mycelia were aseptically filtered  
113 at room temperature and transferred into standard PDB medium (pH 5.00) for cultivation in optimal  
114 conditions for 48 h at 25 °C, 120 rpm. Mycelia were then filtered, washed thoroughly with distilled  
115 water, dried at 70 °C for 48 h and weighed with a precision balance. Mycelium growth was  
116 obtained by quantifying the mycelium dry mass.

117 Other than the experimental cultures of *P. glabrum* for each tested environmental factor  
118 (temperature, pH), two sets of control 'unstressed' mycelia were also cultured under optimum  
119 conditions at 25 °C. The first control, T48, was early-harvested after 48 h of culture and the dry  
120 biomass obtained was used as the minimum fungal growth reached by each culture before  
121 application of experimental thermal and pH conditions. A second control, T96, was harvested after  
122 96 h culture and corresponded to 48 h of culture under optimum conditions in standard PDB at 25  
123 °C and then aseptically filtered and transferred to standard PDB at 25°C for another 48 h. The dry  
124 fungal biomass obtained for this control was considered as the fungal growth after 96 h culture in  
125 optimum conditions. The growth results obtained for each temperature or pH condition were  
126 expressed as the relative growth rate, in percent. This is the ratio between the fungal dry weight  
127 obtained under a given experimental condition and the fungal dry weight obtained in the control  
128 T96.

129 **Experimental design and statistical analysis**

130 The thermal and pH stress experiments were conducted according to a two-factorial central  
131 composite design (Box et al. 1978) (Fig 1). For each of the three applied stresses (thermal, acid and  
132 alkali stress), the CCD (Factors/Blocks/Runs = 2/1/11) defined nine experimental conditions by  
133 varying the intensity of the tested environmental factor and its duration (Table 1). Given the  
134 biological variability of *P. glabrum* growth, each experimental point was replicated three times to  
135 increase precision (consequently, central conditions of each CCD were replicated nine times) and  
136 median values of those three replicates were considered. The growth results obtained for thermal,  
137 acid or alkali stress experiments were analysed separately using STATISTICA 8 (StatSoft) and a  
138 response surface was determined to model the effect of different stress on *P. glabrum* growth. The  
139 growth results of both controls (T48 and T96) were also reported in the response surface for  
140 comparison with CCD results.

141 For pH investigations, another experimental design was also implemented. Twenty pH values were  
142 studied in a large range from 0.15 to 12.50 with a single duration of 240 min. To obtain better  
143 estimates, four replicates were studied for each experimental condition. The results obtained were  
144 analysed with STATGRAPHICS 5.0 (Statistical Graphics Corp) using a one-way ANOVA and the  
145 LSD test to determine which fungal growths were significantly different depending on the tested pH  
146 condition. For each condition, means, (which were very close to median values) were plotted and  
147 LSD results were reported by assigning letters to the means. Means with the same letter are not  
148 statistically different ( $P > 0.05$ ).

149

## 150 **Results**

### 151 **Effect of thermal shock on fungal growth**

152 To investigate the effect of temperature on *Penicillium glabrum* growth, two-factorial CCD and  
153 RSM were used. Temperature and exposure duration varied respectively from 30 to 50 °C and from  
154 10 min to 230 min. The low, middle and high levels of both variables were determined from  
155 preliminary experiments on fungal growth (unpublished data).

156 *P. glabrum* growth results obtained using a CCD were statistically analysed and both parameters  
157 (temperature level and exposure time) and their interaction had significant effects ( $P < 0.05$ ) on  
158 fungal growth. A response surface was determined from the results (Fig 2) and the goodness-of-fit  
159 between the predicted values and the experimental data was very high (Fig 3), giving a coefficient  
160 of determination ( $R^2$ ) of 0.98, indicating a very good adjustment of the model with experimental  
161 data.

162 Inspection of the response surface showed that increasing temperature and exposure time affect *P.*  
163 *glabrum* growth in a gradual manner.

164 The adjusted surface response (Fig 2), showed three distinct response areas delimited by the results  
165 of both growth controls T96 and T48 (which was 48 % of T96). The first response area was  
166 observed for the less intense thermal shock conditions 30 °C:120 min to 40 °C:10 min and revealed  
167 an increase of fungal growth in comparison with the fungal biomass of the control T96. A clear  
168 fungal growth increase was measured for the lowest shock condition (30°C:120 min) at supra-  
169 optimal temperature (Nevarez et al. 2009). A second area was observed for intermediate thermal  
170 conditions (40°C:230 min, 40 °C:120 min or 47°C:42 min), which showed a moderate reduction in  
171 fungal biomass compared to the T96 control but still greater than the fungal growth of the T48  
172 control. The third area was observed for the highest thermal conditions, e.g. 47°C:198 min or 50  
173 °C:120 min, and revealed a strong decrease in *P. glabrum* growth. Fungal biomass values were  
174 even lower than those of the T48 control.

175 Considering the fungal growth results, thermal conditions as 40 °C:120 min, 40 °C:230 min or 47  
176 °C:42 min, appeared to affect moderately *P. glabrum* growth.

177

### 178 **Effect of pH shock on fungal growth**

179 Statistical analysis of the results obtained using a CCD for acid and alkali pH indicated that  
180 exposure time had no significant effect ( $P > 0.05$ ) on fungal growth for the range of values tested (10  
181 to 230 min) (data not shown). This result precluded 2D analysis and response surface modelling,



182 since only one of the two tested variables (pH value) had a significant influence. Consequently, we  
183 modified our experimental design by using a monofactorial experimental procedure on a large range  
184 of pH from 0.15 to 12.50 for a single exposure time of 240 min.

185 Global inspection of the results (Fig 4) shows that *P. glabrum* is able to grow relatively well after  
186 240 min of exposure in a very wide range of pH conditions, spanning pH 2.00 to 11.50.

187 Analysis of fungal growth clearly showed four distinct areas of response over the wide range of pH  
188 conditions tested. The first area was observed for pH 2.00 to 7.00, which had a very low influence  
189 on fungal growth (80-100 % of T96 growth). The second area was observed for alkaline conditions,  
190 from pH 8.00 to 11.50, which induced a decrease in fungal growth, showing a growth rate of 60-  
191 70 % of T96 growth.

192 The third and fourth response areas were observed for very acidic conditions (pH 0.15 to 1.00) or  
193 alkaline conditions (pH 12.00 to 12.50). These conditions induced a dramatic decrease in fungal  
194 growth after 240 min of exposition. The fungal biomass values were even lower than the control  
195 T48 value.

196 Considering the fungal growth results obtained, alkaline shock conditions between pH 8.00 to 11.50  
197 for 240 min duration affect significantly and moderately *P. glabrum* development. Concerning  
198 acidic shock, the transition from no detrimental to detrimental effect appears very steep.

199

## 200 **Discussion**

201 To investigate the effect of temperature and pH shocks on *Penicillium glabrum* growth, two-  
202 factorial CCD and RSM were employed. Using a CCD offers the possibility to assess the effect of a  
203 large range of conditions by testing a limited and optimised number of experimental points with a  
204 low number of replicates. This approach has been successfully employed in fungi to examine  
205 chitinase regulation (Lopes et al. 2008), to study the influence of environmental factors such as  
206 temperature and pH on the growth of the fermenting yeast *Pachysolen tannophilus* used in

207 industrial fermentation processes (Roebuck et al. 1995) or to investigate the influence of  
208 temperature, pH and  $a_w$  on yeast to study their antagonistic properties (Sinigaglia et al. 1998). RSM  
209 is a well-known method for optimising for example medium composition or other critical variables  
210 that affect for example enzyme production or microbial growth. This study used RSM to predict  
211 fungal growth anywhere within the limits of the experimentally tested environmental factors. The  
212 quadratic model obtained in those conditions permitted suitable predictions.

213 Regarding temperature, inspection of the response surface clearly shows that increasing temperature  
214 and exposure duration affect *P. glabrum* growth in a gradual manner. The influence of a given  
215 temperature in a wide range of exposure duration has been also observed in *Saccharomyces*  
216 *cerevisiae* exposed to 37 °C for 15, 30, 45, 60, 120, 240 and 480 min (Sakaki et al. 2003).

217 The less intense shock condition (30°C:120 min) did not reduce *P. glabrum* fungal growth, but  
218 showed an unexpected increase in fungal biomass. This result suggests that when a fungal  
219 contaminant such as *P. glabrum* is exposed to relatively weak stress conditions, its development on  
220 food products may be enhanced. This observation could be practically important for food industry.

221 The intermediate stress conditions (40°C:120 min, 40°C:230 min, 47°C:42 min) induced more or  
222 less moderate fungal biomass reductions which can be attributed simultaneously to growth  
223 reduction or increased lag time for growth due to thermal shock. This fungal growth reduction  
224 could be explained by a variety of cellular effects corresponding with thermal stress. It is known  
225 that elevated temperature can affect: (i) the structure of proteins, possibly modifying their biological  
226 activity and overall cellular functioning; (ii) the biosynthesis of a large number of ubiquitous  
227 proteins which decrease or are completely stopped (Plesofsky-Vig & Brambl 1987; Curle & Kapoor  
228 1988); and (iii) plasma membrane fluidity (Beney & Gervais 2001). As described in *S. cerevisiae*,  
229 an increase of plasma membrane permeability can affect cellular integrity and metabolism (Guyot et  
230 al. 2005).

231 Finally, in more drastic thermal conditions (47°C:198 min, 50°C:120 min), the fungal biomass  
232 decreased compared to the initial fungal biomass (T48) before applying the experimental stress

233 conditions. According to the literature, this decrease can be explained by fungal lysis (Emri et al.  
234 2004; Koutinas et al. 2005) or may be due to an ordered degradation of cellular reserves (McNeil et  
235 al. 1998). In yeast and filamentous fungi, autolysis can be characterised by a fungal biomass  
236 decline. Autolysis occurs in response to a wide range of extrinsic factors such as heat, chemical  
237 treatment, nutrient starvation, *etc.*, which may induce the loss of membrane function leading to a  
238 breakdown in intracellular compartmentalisation and the release of lytic enzymes responsible of  
239 macromolecular degradation (Hernawan & Fleet 1995; McNeil et al. 1998). Important reduction in  
240 fungal growth under high temperatures such as 50 °C has also been reported in *S. cerevisiae* (Seppa  
241 et al. 2004) and *Neurospora crassa* (Plesofsky-Vig & Brambl 1985), for which optimum growth  
242 conditions are 25°C and 30°C, respectively. The yeast *Candida albicans*, for which optimum  
243 growth temperature is 37°C, is also very affected by exposure at 55 °C (Zeuthen & Howard 1989).  
244 In our case, thermal conditions of 47°C:198 min or 50°C:120 min appeared to affect not only *P.*  
245 *glabrum* fungal growth but also proteins and mRNA integrity. In fact, subjecting total proteins to  
246 electrophoresis showed that most of the proteins observed in the T96 control condition were not  
247 visible in the highest stress conditions mentioned above (data not shown). Moreover, we analysed  
248 the total RNA using electrophoretic RNA separation on microfabricated chips to determine their  
249 quality. In the drastic growth conditions, this analysis revealed a decrease in the 18S and 28S  
250 ribosomal RNA peaks and an increase in smaller, intermediate RNA fragments, indicating  
251 substantial total RNA degradation (Nevarez et al. 2008).

252 Given the results on fungal growth, thermal conditions such as 40°C:120 min, 40°C:230 min or  
253 47°C:42 min, appeared to moderately, but significantly affect *P. glabrum* growth. These conditions  
254 may therefore be appropriate for further investigations on stress response. In addition, these thermal  
255 shock conditions (temperature level and exposure time) correspond pretty well with those used in  
256 other thermal stress studies conducted in fungi which are unfrequently justified. For example, a  
257 study was realised in *S. cerevisiae* in order to isolate heat shock proteins (HSP), HSP 82 and HSP  
258 104 by shifting optimal cultures from 25 °C to 39 °C for 1 h (Sanchez et al. 1993). Other

259 investigations conducted in *N. crassa* or *C. albicans* exposed fungi to experimental conditions from  
260 30 °C to 45 °C for 90 min and from 37 °C to 40 - 46 °C for 30 min, respectively (Plesofsky-Vig &  
261 Brambl 1985; Zeuthen & Howard 1989). Thermal stress was also investigated with a transcriptional  
262 approach using microarrays in yeasts *S. cerevisiae* or *Schizosaccharomyces pombe*, from 25°C to  
263 37°C for 2 h or 30°C to 39°C for 1 h, respectively (Causton et al. 2001; Chen et al. 2003). In our  
264 case, where physiological experimental stress conditions were being validated, the thermal  
265 condition of 40°C:120 min was employed as central condition to investigate heat shock response at  
266 the transcriptional level (Nevarez et al. 2008). A transcriptional study combining suppression-  
267 subtraction hybridisation and cDNA microarrays has been conducted in this fungus to isolate  
268 differentially expressed genes in response to thermal shock. Of the various isolated genes, a few are  
269 down-regulated and encode for proteins involved in general cellular metabolism. Given this thermal  
270 shock condition, gene down-regulation may explain the *P. glabrum* growth reduction as observed in  
271 this study.

272 The effect of a very large range of pH conditions was tested on *P. glabrum* growth for a single  
273 exposure time of 240 min. High pH tolerance has been already described in many filamentous fungi  
274 which appear to be little affected by changes in culture pH (Wheeler et al. 1991). Many *Penicillium*  
275 species including *P. chrysogenum*, *P. camemberti*, *P. aurantiogriseum*, *P. marneffeii*, *P. crustosum*  
276 or *P. islandicum* are able to grow from pH 3.00 - 4.00 to 9.00 - 10.00 (Wheeler et al. 1991;  
277 Thompson et al. 1993; Cao et al. 2007). The ability of fungi to develop in a wide range of pH is  
278 partially due to adaptation associated with a genetic regulatory system that tailors gene expression  
279 to the ambient pH (Arst & Penalva 2003). Considerable progress has been made in characterising  
280 fungal genetic pH regulatory systems because they are important for major processes including  
281 pathogenesis and the production of extracellular enzymes, penicillin or mycotoxins (Espeso et al.  
282 1993; Denison 2000). Various pH regulatory systems have been described in fungi (*Aspergillus*  
283 *nidulans*, *A. niger*, *Penicillium chrysogenum*, *S. cerevisiae*, *C. albicans*, *Yarrowia lipolytica*;  
284 Denison 2000; Arst & Penalva 2003).

285 In acidic conditions (pH 2.00 to 7.00), fungal growth was only slightly affected. This observation is  
286 in accordance with reports that most filamentous fungi including *Aspergillus* spp., *Fusarium* spp.  
287 and *Penicillium* spp. show high tolerance to acidic media and their optimum growth is around pH  
288 5.50 - 6.00 (Deacon 2006). Similar observations have been made on several *Penicillium* species  
289 such as *P. citreonigrum*, *P. jensenii* or *P. roqueforti* (Sacks et al. 1986; Wheeler et al. 1991; Gock et  
290 al. 2003). It has also been shown in *S. cerevisiae* that yeast cells grow more rapidly in acidic media  
291 than in neutral or alkaline media (Lamb et al. 2001). An important factor for the maintenance of an  
292 acidic environment is the yeast plasma membrane H<sup>+</sup>-ATPase, which actively extrudes protons and  
293 imports many nutrients and cations (Serrano et al. 2002).

294 The alkaline pH conditions (pH 8.00 to 11.50) induced a greater growth decrease area than  
295 observed in acidic conditions. This effect may be explained by some cellular modifications caused  
296 by alkaline pH. In *S. cerevisiae* for example, it has been shown that alkaline media induce  
297 disruption of membrane proton gradients that normally supply energy for nutrient and ion transport  
298 essential for fungal development (Lamb et al. 2001). Some authors also report in *S. cerevisiae* a  
299 significant repression of genes involved in amino acid or purine biosynthesis and in carbohydrate  
300 metabolism that could explain limitation of yeast growth (Serrano et al. 2002). These authors  
301 suggest that copper or iron availability and solubility can be reduced by alkaline pH, which could  
302 affect some enzymatic activities. Thus, highly alkaline environments can be considered as stressing  
303 conditions.

304 Given the results on fungal growth, alkaline stress conditions between pH 8.00 to 11.50 for 240 min  
305 may be appropriate for further investigations on stress response as they seemed to moderately, but  
306 significantly affect fungal development. On the basis of our results, the experimental point pH  
307 9.00:240 min may be employed as central condition for further studies in *P. glabrum*. Comparable  
308 alkaline pH conditions have also been used in several studies on fungi as, for example, in *C.*  
309 *glabrata* to analyse its pH response by transferring cultures from pH 4.00 to pH 8.00 (Schmidt et al.  
310 2008). Some molecular studies have been also conducted at pH 4.00 to 8.00 in *C. albicans* or *A.*

311 *nidulans* to investigate the role of pH transcription factors (Rim13p and PacC respectively) (Espeso  
312 & Arst 2000; Li et al. 2004). A transcriptional approach using microarrays has been employed in *S.*  
313 *cerevisiae* at pH ranging from 6.00 to 7.90 (Causton et al. 2001).

314 Finally, in extreme pH conditions (pH 0.15 to 1.00 and pH 12.00 to 12.50), a great decrease in  
315 fungal biomass was induced. These drastic conditions severely affected cellular metabolism. As  
316 suggested for thermal stress, the effect induced by these conditions on *P. glabrum* growth may be  
317 also explained by fungal autolysis (McNeil et al. 1998).

318 In summary, the present work investigated a wide range of temperature and pH conditions to  
319 analyse their effect on *P. glabrum* growth to determine ‘optimised’ experimental shock conditions.  
320 To our knowledge, this approach has not been previously reported in other fungal stress studies,  
321 which generally use a limited number of experimental conditions. The results obtained here made it  
322 possible to determine experimental conditions that may be potentially appropriate for further  
323 investigations on stress response. Based on our results, we propose that conditions such as  
324 40°C:120 min, pH 1.50:240 min or pH 9.00:240 min, can be used to produce a physiological stress  
325 response because they moderately, but significantly affect growth in *P. glabrum*.

326

327

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330

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