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# Water activity, solute and temperature modify growth and spore production of wild type and genetically engineered *Aspergillus niger* strains

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Key words: water activity, temperature, growth, sporulation, genetically modified, *Aspergillus niger*

**Abstract**

The effect of interactions of water activity ($a_w$) (0.99-0.90), temperature (20, 30 and 35°C) and modifying aw solute (glycerol, NaCl) on growth and sporulation of a wild type strain of *Aspergillus niger* (W) and two genetically engineered lysozyme producing strains (L11, B1) was examined for the first time. Maximum growth rates were achieved for both strains (L11 and B1) under moderate $a_W$ levels. L11 showed a higher growth rate than B1. Fastest growth was achieved at 30$^o$C, using glycerol as solute. Optimum conditions for growth of strain L11 were estimated by means of contour plot surfaces and found to be 0.965 $a_w$ with glycerol as solute at 35ºC. The predicted value of the optimum growth rate was 10.5 mm/day. A value of 10.85 mm/day was obtained experimentally giving a good correlation between the estimated and the measured results. Sporulation was optimum for the W strain at 0.99-0.95 by B1 at 35$^o$C. However, a significant higher production of conidia by L11 at 0.97-0.93 $a_w$ and at 0.97 $a_w$ and 35$^o$C for B1 strain. Optimum conditions for spore production were different from those for growth. Under similar ecological conditions the W and both the genetically engineered strains had a different growth and sporulation pattern.

**Introduction**

Filamentous fungi, especially *Aspergillus* species, are attractive hosts for the production of foreign proteins because of their high secretory capability [1,2,3,4,5,6]. This has been demonstrated for a number products from *Aspergillus nidulans* and *A.niger* [7,8,9,10]. *A.niger* is a xerophilic species which can normally tolerate quite dry environments [11,12,13]. Water activity ($a_w$) and temperature are critical factors affecting the growth and metabolism of fungi [14,15]. From an ecological point of view little information is available on whether genetically modified strains will behave in a similar manner to wild type strains or not. No studies have attempted to evaluate this, although it has been shown that in wild type strains of pharmaceutically useful fungi, subtle changes in water stress can result in a significant stimulation of secondary metabolite production [13,16]. Thus, the objective of this study was to evaluate the effects of water availability (water activity, $a_w$), solute used to modify $a_w$, and temperature on growth and spore production by a wild (W) and two genetically-engineered strains (B1, L11) of *Aspergillus niger*.

## Materials and Methods

*Fungal strains*

*A.niger wild type* IMI 149007 (W), and two transformants B1 and L11 of strain AB4.1 [17] containing the full-length hen egg white lysozyme (HEWL) cDNA under the control of the *A.niger* var. *awamori* glucoamylase promoter were used in this study [18]

*Media preparation, incubation and growth rate assessment*

The water activity of 4.8 % w/v malt extract agar (MEA) (Oxoid) was modified with calculated amounts of the non-ionic solute glycerol and the ionic solute NaCl to 0.99-0.90 $a_w$. A Novasina Humidat-IC-II (Switzerland) was used to check the $a_w$ levels obtained and found to be within 0.005 of the desired $a_w$ level.

Actively growing 8-day-old colonies of W, L11, B1 on MEA were used to prepare a spore suspension (1.8 E+007 spores/ml ± 2%). Fungal spore suspensions were prepared in a solution of Tween 80 (100µl/l). Petri plates with glycerol or NaCl- modified MEA were inoculated with 5 µl of the spore suspension and incubated at 25, 30 and 35ºC.

The temporal mycelial extensions of treatments and replicates were measured in two directions at right angles to each other. Measurements were recorded on alternate days during the growth until the Petri plate were completed colonised [13,16,19]. A linear regression of the data was performed in order to calculate the growth rate. The calculated growth rate was used for statistical analysis. It has been previously shown by Trinci [20,21] that the linear growth rate on a solid substrate, e.g. agar medium, is a good approximation of biomass increase in liquid culture.

Spores were recovered from Petri plates by agitating the surface with 10 mls of sterile water (+ a drop of Tween 80) twice and decanting into a Universal bottle. The number of spores was determined using a haemocytometer and microscope (Olympus ABHZ, Olympus UK) . Spore number were recorded when the treatment strain had colonised the surface of the Petri plate fully up to a maximum of 28 days.

*Experimental design and data treatment*

For growth experiments a fully randomised factorial design ($3^3$) run in quadruplicate was used to describe the growth rate of *A. niger* in relation to the three strains, three temperatures, five $a_w$ levels and two solute types. In all cases linear regression of increase in radial extension against time was used to obtain the growth rates under each set of treatment conditions.

A fully randomised factorial design ($3^3$) in triplicate was used to describe the spore production of *A. niger* in relation to the same treatments used for growth. The number of spores produced at the end of each experimental run was recorded and used in the statistical analysis. Orthogonal experiment optimization was computed in order to calculate the optimal conditions of growth rate and contour surface plots were used to find the maximum values of growth and sporulation [22].

## Results

### Effect of environmental factors on growth

Figure 1 shows an example of the temporal radial extension of colonies of *A. niger* (L11) on media modified with the non-ionic solute glycerol or the ionic solute NaCl at three different temperatures. This shows that mycelial extension was faster at 0.95 $a_w$ than on unmodified media with freely available water. This information was used to compare growth rates *of A. niger* in the different treatments.

Figure 2 compares growth of all three strains of *A.niger* at different aw levels at 35$^o$C, the optimum temperature for growth using both solutes. This shows that the wild-type strain grew optimally at 0.95-0.93 $a_w$ on glycerol-modified media, but optimally at 0.99 $a_w$ when NaCl was used. For the two genetically-modified strains growth was optimal at 0.97 $a_w$, regardless of solute used. Overall, strain L11 grew faster than the others examined. The overall growth pattern of the two genetically modified strains was similar, and different from the wild-type strain. In unmodified medium (0.99 $a_w$) growth of the wild type was faster than the two modified strains. Statistical analysis of the data showed that all factors and interactions were significant ($P<0.001$) for growth rates (Table 1).

The most important factors overall were $a_w$, solute and their interactions. A contour surface plot summarises the optimum conditions in relation to aw and temperature for all three strains (Figure 3). The data from the experiments were used to find the optimal conditions using orthogonal optimization to determine the highest growth rates (Table 2). To confirm the accuracy of the prediction, an experiment using these conditions was carried out in triplicate. A value of 10.85 mm/day was obtained verifying a good correlation between the predicted and the measured results.

**Effects of environmental conditions on sporulation of *A.niger* strains**

Figure 4 shows the effect of temperature and aw on sporulation of *A. niger* strains on glycerol-amended media. This shows that the wild type strain (W) produced most spores at 0.97 $a_w$, and at 35$^o$C. There were significant differences between strains with L11 producing a 10-fold higher amount of spores, especially at 35$^o$C between 0.97-0.93 $a_w$. Thus L11 has markedly different sporulation capacity compared to the other two strains examined. Table 3 shows the statistically significant factors were $a_w$, strain, temperature, solute and $a_w$ x strain, $a_w$ x temperature and strain x temperature.

The contour surface plot of the impact of $a_w$ x temperature on sporulation capacity of L11 is shown in Figure 5. Optimum conditions for spore production are different from that for growth. High production of spores was found with L11 at 0.95 $a_w$ modified with glycerol at 35ºC. The range of significant spore production can be defined between 0.97-0.93 $a_w$ and 33-35$^o$C. This suggest that higher temperatures are more suitable for sporulation and spore production.

**Discussion**

This is the first time that ecological comparisons have been made between wild-type and genetically modified strains of xerophilic fungi. We conclude that genetic modification can affect both water and temperature relations for growth, and particularly for sporulation. The optimal growth of the three strains of *A. niger* examined was found to be in the range 0.97-0.95 $a_w$. The growth rate of the genetically engineered strains were similar to each other, and different from the wild-type strain. Strains L11 and B1 had a higher tolerance to lower water activity than the wild type when modified with the ionic solute, NaCl. In all cases the $a_w$ range of 0.97-0.95 resulted in a faster growth rate when compared to the control (0.99 $a_w$).

The use of glycerol to modify media water availability produced a higher growth rate than with NaCl, probably because it can be utilised as a carbon and energy source and can act directly as a compatible solute. In contrast, high concentrations of NaCl can be toxic and this may explain the differential growth patterns observed. The effect of the temperature and $a_w$ were significant and both affected the growth rate. Growth patterns of both transgenic strains were similar. Growth of filamentous fungi has been previously shown to be dependent on thermodynamic factors such as water availability and temperature [14,15].

Previous studies on *A.niger* suggest a range of 10-40$^o$C and 0.77-0.99 optimum at 35$^o$C and 0.99 $a_w$ [23]. However, sporulation ranges were not considered previously and this early work was carried out when no information on related *A. niger* group species (e.g. *A carbonarius*) was known. Recent studies on the *Aspergillus* section nigri group isolated from grapevine suggest optima for growth of 0.95 $a_w$ at 30-35$^o$C [24]

The relative effects of the environmental factors on sporulation were more dramatic than on growth. The genetically modified strain L11 behaved in a completely different manner compared to the other two strains examined, producing substantially more conidia and over a wider aw range, especially at the optimum temperature, 35$^o$C. Very few studies have examined the impact of environmental factors such as $a_w$ or temperature on sporulation of wild type strains of fungi and none on genetically modified ones [25,26,27]. For example, Gervais et al. [26,27] showed that *P.roquefortii* strains from cheese grew optimally at 0.97-0.98 $a_w$, while maximum spore production was at 0.96 $a_w$. The present study suggests that genetic manipulation can alter the physiology of a fungus in an unpredictable way. The two GM-strains have multiple gene copies integrated into the genome at more than one locus, and this may have caused the changes observed.

**Conclusions**

From this study of the growth rate of genetically engineered *Aspergillus niger*, several parameters important for maximizing the growth rate have been identified. A combination of $a_w$, temperature and solute used to modify the $a_w$ had the greatest effect on growth rate. L11 grew significantly better than the other strains examined. Thus, such screening criteria based on these environmental parameters ($a_w$, temperature, solute type) can facilitate the selection of appropriate transformed strains for studying secondary metabolite or heterologous protein production systems.

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Table 1. Analysis of variance of water activity ($a_w$), solutes type, and temperatures in the growth rate of three strains of *Aspergillus niger* (L11, B1 and native strain W)

| Factor | Df | MS | F |
|---|---|---|---|
| $a_w$ | 4 | 375.01 | 1060.29** |
| Strain | 2 | 41.59 | 235.19** |
| Temperature | 2 | 20.98 | 118.62** |
| Solute | 2 | 209.73 | 1185.94** |
| Factor interactions | | | |
| $a_w$ x Strain | 8 | 12.21 | 17.26** |
| $a_w$ x Solute | 8 | 65.09 | 92.02** |
| Strain x Solute | 4 | 40.94 | 115.76** |
| Temperature x Solute | 4 | 17.85 | 50.48** |
| Residual | 277 | 3.04 | |

** Significant at the level $p<0.001$

Table 2. Optimal combination of factor and levels found with orthogonal design optimization.

| Optimum Conditions | |
|---|---|
| Factors | Level |
| $a_w$ | 0.965 |
| Strain | L11 |
| Temperature (ºC) | 35ºC |
| Solute | Glycerol |
| Expected | 10.514 mm/day |

Table 3. Analysis of variance of water activity ($a_w$), solutes type, and temperatures in the spore production of three strains of *Aspergillus niger* (L11, B1 and native strain W)

| Factor | df | MS | F |
|---|---|---|---|
| $a_w$ | 4 | 2.0E+15 | 2.68** |
| Strain | 2 | 5.6E+15 | 15.22** |
| Temperatura | 2 | 5.4E+15 | 14.69** |
| Solute | 2 | 2.5E+15 | 6.81** |
| $a_w$ x Strain | 8 | 8.8E+14 | 0.60* |
| $a_w$ x Temperature | 8 | 5.9E+14 | 0.40* |
| $a_w$ x Solute | 8 | 7.4E+14 | 0.50 |
| Strain x Temperature | 4 | 2.1E+15 | 2.82* |
| Strain x Solute | 4 | 7.1E+14 | 0.97 |
| Temperatura x Solute | 4 | 1.0E+15 | 1.40 |
| Residual | 196 | 3.0E+14 | |

*, significant at $P<0.05$
**, significant at $P<0.01$

Figure legends

Figure 1. Effect of water activity modified with glycerol and NaCl at 25 ºC (a,b), 30 ºC (c, d) and 35 ºC (e, f) on colony diameter of *Aspergillus niger* L11

Figure 2. Comparison of growth rate (mm/day) of three strains of *Aspergillus niger* (L11, B1 and native strain W). Water activity modified with a) glycerol b) NaCl at 35 ºC

Figure 3. Contour surface of $a_w$ vs temperature effects on growth rate (mm $day^{-1}$).

Figure 4. Effect of water activity modified with a) glycerol and b) NaCl on spore production (spores/$cm^2$) at 35$^o$C in three strains of *A. niger*.

Figure 5. *A. niger* L11 spore production (spores/$cm^2$) contour surface of water activity and temperature.

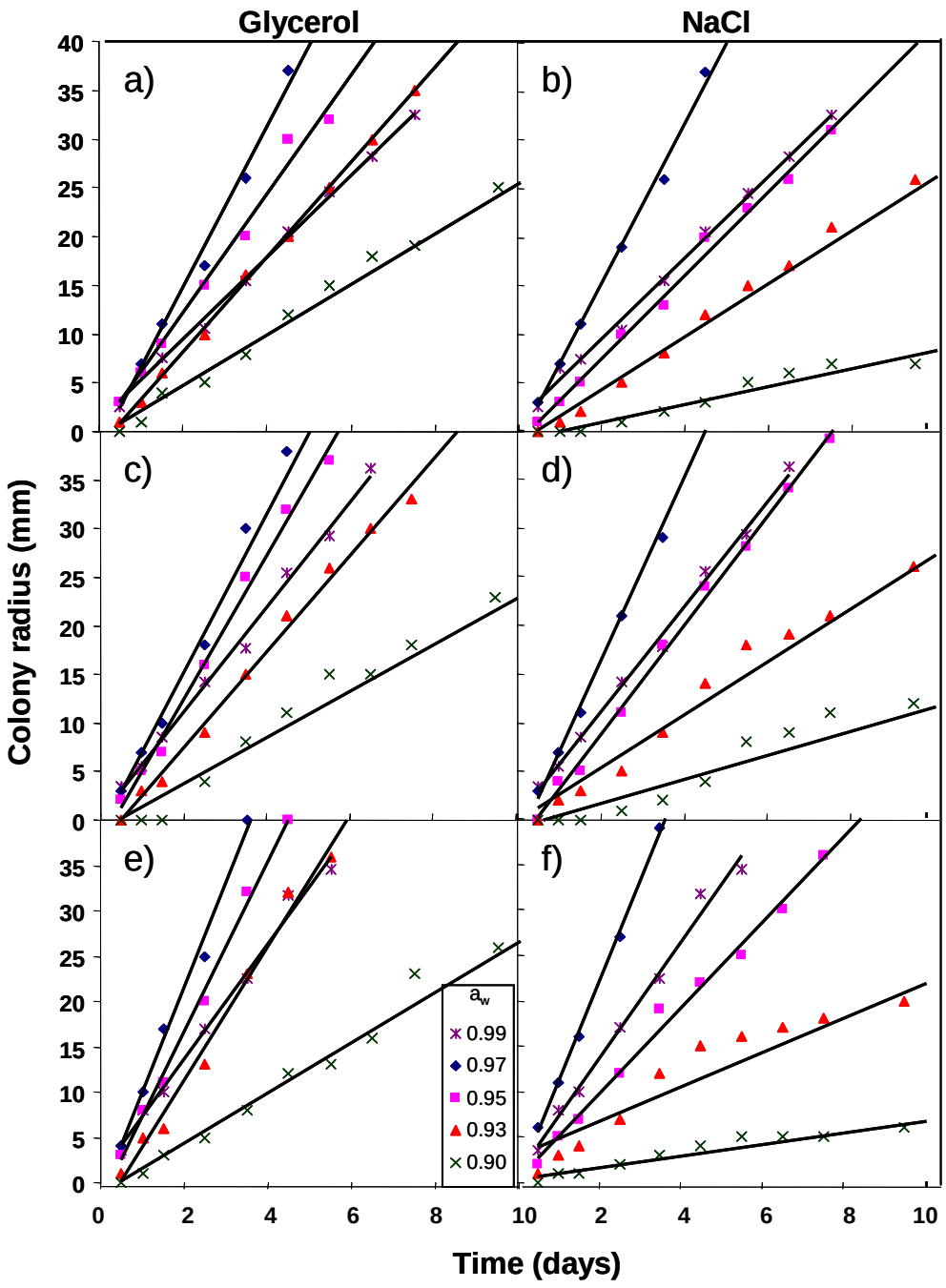
Glycerol
NaCl
a)
b)
c)
d)
e)
f)
Colony radius (mm)
Time (days)
0
5
10
15
20
25
30
35
40
2
4
6
8
aw
0.99
0.97
0.95
0.93
0.90

Figure 1

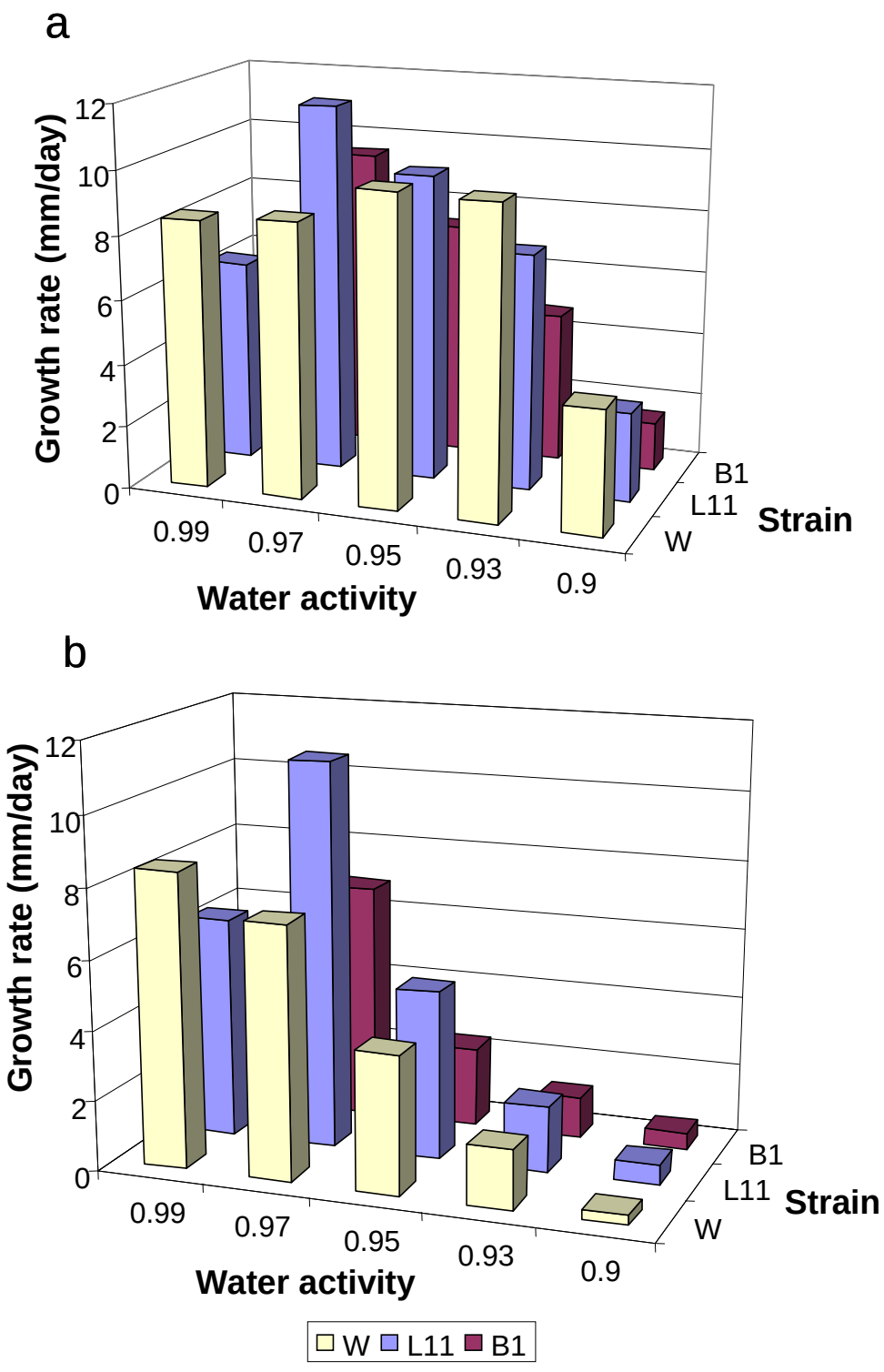
a
Growth rate (mm/day)
12
10
8
6
4
2
0
0.99
0.97
0.95
0.93
0.9
Water activity
B1
L11
W
Strain
b
Growth rate (mm/day)
12
10
8
6
4
2
0
0.99
0.97
0.95
0.93
0.9
Water activity
B1
L11
W
Strain
W
L11
B1

Figure 2

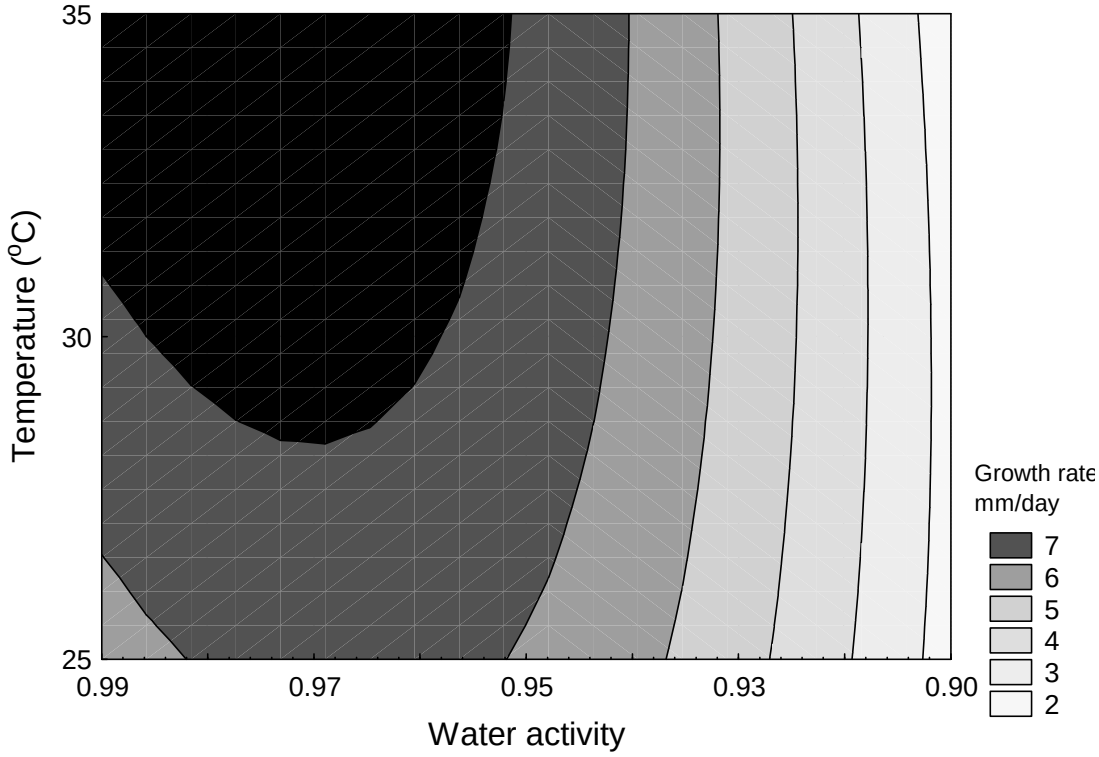
35
30
25
Temperature (°C)
0.99
0.97
0.95
0.93
0.90
Water activity
Growth rate
mm/day
7
6
5
4
3
2

Figure 3

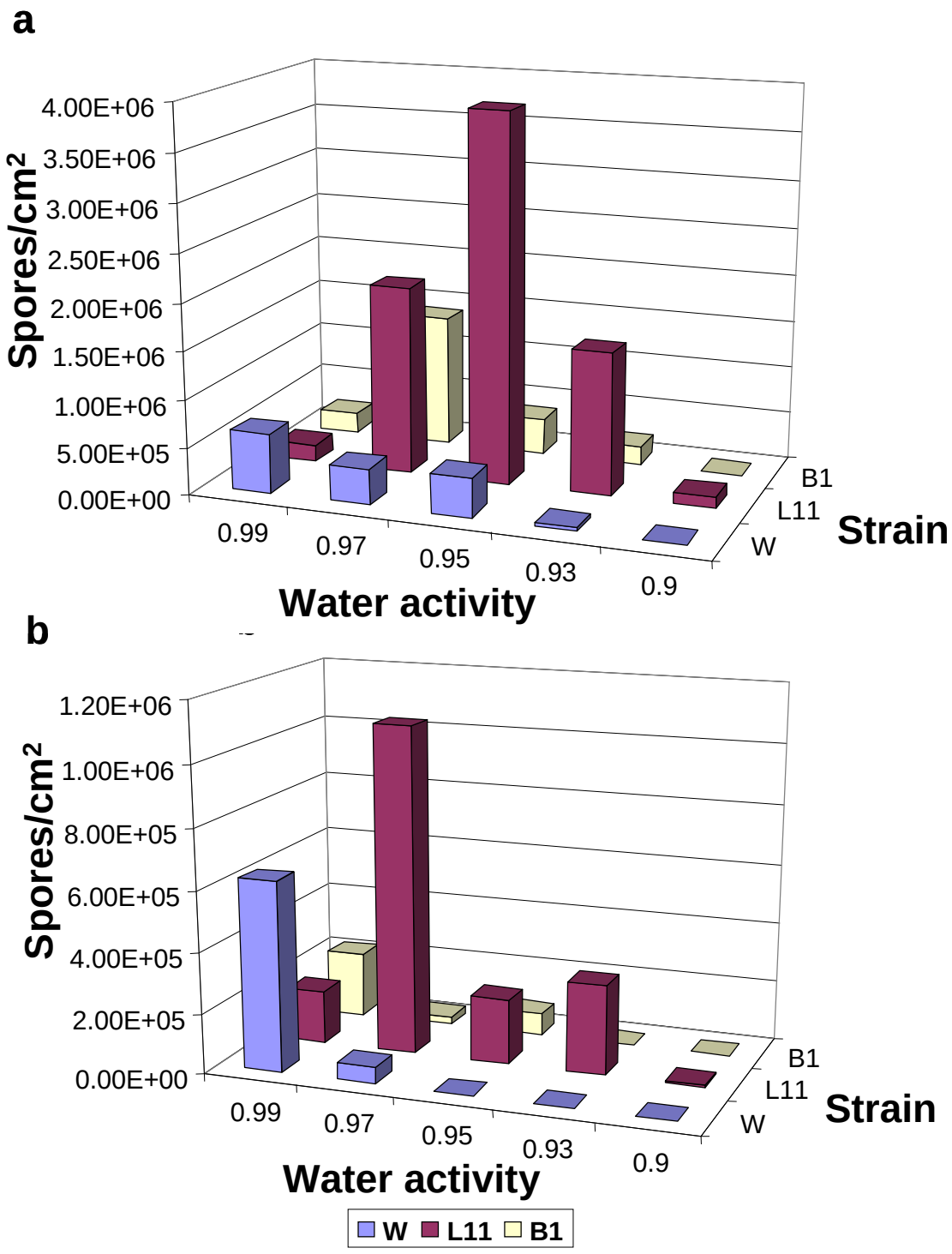
a
4.00E+06
3.50E+06
3.00E+06
2.50E+06
2.00E+06
1.50E+06
1.00E+06
5.00E+05
0.00E+00
Spores/cm²
0.99
0.97
0.95
0.93
0.9
Water activity
B1
L11
W
Strain
b
1.20E+06
1.00E+06
8.00E+05
6.00E+05
4.00E+05
2.00E+05
0.00E+00
Spores/cm²
0.99
0.97
0.95
0.93
0.9
Water activity
B1
L11
W
Strain
W
L11
B1

Figure 4

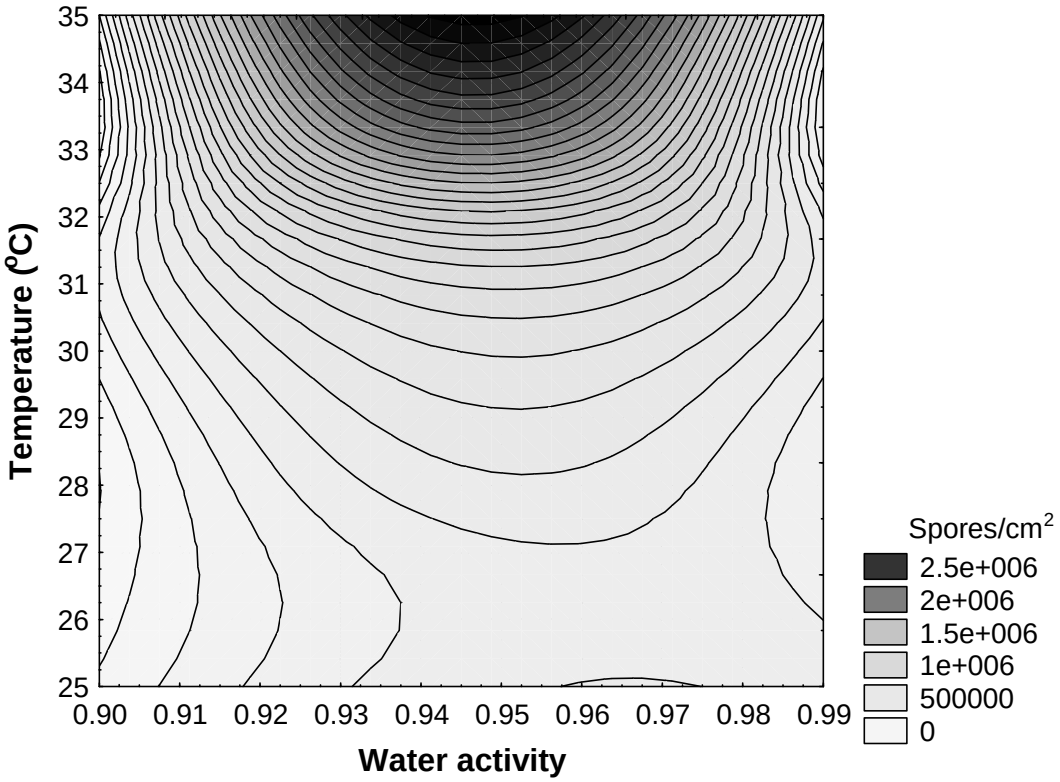
Temperature (°C)
35
34
33
32
31
30
29
28
27
26
25
0.90
0.91
0.92
0.93
0.94
0.95
0.96
0.97
0.98
0.99
Water activity
Spores/cm2
2.5e+006
2e+006
1.5e+006
1e+006
500000
0

Figure 5

# Water activity, solute and temperature modify growth and spore production of wild type and genetically engineered Aspergillus niger strains.

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