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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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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.

References

[1] Jeenes DJ, MacKenzie DA, Roberts IN, Archer DB. Heterologous protein production by filamentous fungi. Biotechnol Gen Eng Rev 1991;9:327-67.

[2] Archer DB. Filamentous fungi as microbial cell factories for food use. Current Opinion in Biotechnology 2000;11:478-83.

[3] Cullen D, Gray GL, Wilson LJ, Hayenga KJ, Lamsa MH, Rey MW, Norton S, Berka RM. Controlled expression and secretion of bovine chymosin in *Aspergillus nidulans*. Bio/Technology 1987;5:369-76.

[4] Gwynne DI, Buxton FP, Williams SA, Garven S, Davies RW. Genetically engineered secretion of active human interferon and a bacterial endoglucanase from *Aspergillus nidulans*. Bio/Technology 1987;5:713-19.

[5] Upshill A, Kumar AA, Bailey MC, Parker MD, Favreau MA, Lewison KP, Joseph ML, Maraganore JM, McKnight GL. Secretion of active human tissue plasminogen from the filamentous fungus *Aspergillus nidulans*. Bio/Technology 1987;5:1301-4.

[6] Turnbull IF, Rand K, Willets NS, Hynes MJ. Expression of the *Escherichia coli* enterotoxin subunit B gene in *Aspergillus nidulans*. Bio/Technology 1989;7: 169-74.

[7] Christensen T, Woeldike H, Boel E, Mortinsen SB, Hjortshoej K, Thim L, Hansen MT. High level expression of recombinant gene in *Aspergillus oryzae*. Bio/Technology 1988;6:1419-22.

[8] Ward M, Wilson LJ, Kodama KH, Rey MW, Berka RM. Improved production of chymosin in *Aspergillus* by expression as a glucoamylase-chymosin fusion. Bio/Technology 1990;8:435-40.

[9] Conesa A, Punt PJ, Van Luijk N, Van den Hondel CAMJJ. The secretion pathway in filamentous fungi: A biotechnological view. Fungal Genetics & Biology 2001 33:155-7.

[10] Punt PJ, Van Biezen N, Conesa A, Alberts A, Mangnus J, Van den Hondel C. Filamentous fungi as cell factories for heterologous protein production. Trends Biotechnol 22:200-6.

[11] Magan, N. Fungi in extreme environments. The Mycota IV. Environmental and Microbial Relationships. Wicklow/Soderstrom (Eds.). Springer-Verlag Berlin Heidelberg 1997;7:99-114.

[12] Marin S, Sanchis V, Magan N. Water activity, temperature, and pH effects on growth of *Fusarium moniliforme* and *Fusarium proliferatum* isolates from maize. Can J Microbiol 1995;41:1063-70.

[13] Baxter CJ, Magan N, Lane B, Wildman HG. Influence of water activity and temperature on in vitro growth of surface cultures of *Phoma sp*. and production of pharmaceutical metabolites, squalestatins S1 and S2. Appl Microbiol Biotechl 1998;49: 328-32.

[14] Scott WJ. Water relations of food spoilage microorganisms. Adv Food Res 1957;7:83-127.

[15] Gervais P, Molin P, Bensoussan M.. Influence of water activity of a solid substrate on the growth rate and sporogenesis of filamentous fungi. Biotechnol Bioeng 1988;31:457-463.

[16] Aldred D, Magan N, Lane BS. Influence of water activity and nutrient on growth and production of squalestatin S1 by a *Phoma sp*. J Appl Microbiol 1999;87:842-48

[17] van Hartingsveldt W, Marttern IE, van Zeijl, CMJ., Pouwels PH, van den Hondel, CAMJJ. Development of a homologous transformation system for *Aspergillus niger* based on the pyrG gene. J Mol Gen Genet 1987;206:71-75.

[18] Archer DB, Jeenes DJ, MacKenzie DA, Brightwell G, Lambert N, Lowe G, Radford SE, Dobson CM. Hen egg white lysozyme expressed in, and secreted from, *Aspergillus niger* is correctly processed and folded. Bio/Technology 1990; 8:741-45.

[19] Baxter CJ. Influences of some environmental factors on fungal growth and production of aflatoxins in solid state fermentation. J Scientific Inter Res 1997;55: 365-72.

[20] Trinci APJ. A kinetic study of the growth of *Aspergillus nidulans* and other fungi. J. Gen Microbiol 1969;57:11-24.

[21] Trinci APJ, Collinge A. Influence of $_{L}$-sorbose on the growth and morphology of *Neurospora crassa*. J Gen Microbiol 1973;78:179-192.

[22] Wu Y, Hobbs MW. In: Taguchi GM, editor. Quality Engineering: Product & Process Design Optimization. American Supplier Institute Inc, Derbon, Michigan USA 1987. Pp 469.

[23] Michell D, Aldred D, Magan N. Impact of ecological factors on growth and ochratoxin A production by *Aspergillus carbonarius* from different regions of Europe. Asp. Appl Biol 2003;68:109-16

[24] Ayerst, G. The effects of moisture and temperature on growth and spore germination in some fungi. J Stored Prod Res 1969;5:127-41.

[25] Magan N, Lacey J. Effect of temperature and pH on water relations of field and storage fungi. Trans Br Mycol Soc 1984;82:71-81.

[26] Gervais P, Belin JM, Grajek W, Sarrette M. Infuluence of water activity on aroma production by *Trichoderma viride* TS growing on a solid substrate. J Ferment Technol 1988;4:403-7.

[27] Gervais P, Molin P. The role of water in solid-state fermentation. Biochem Eng J 2003;13:85-101.

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.

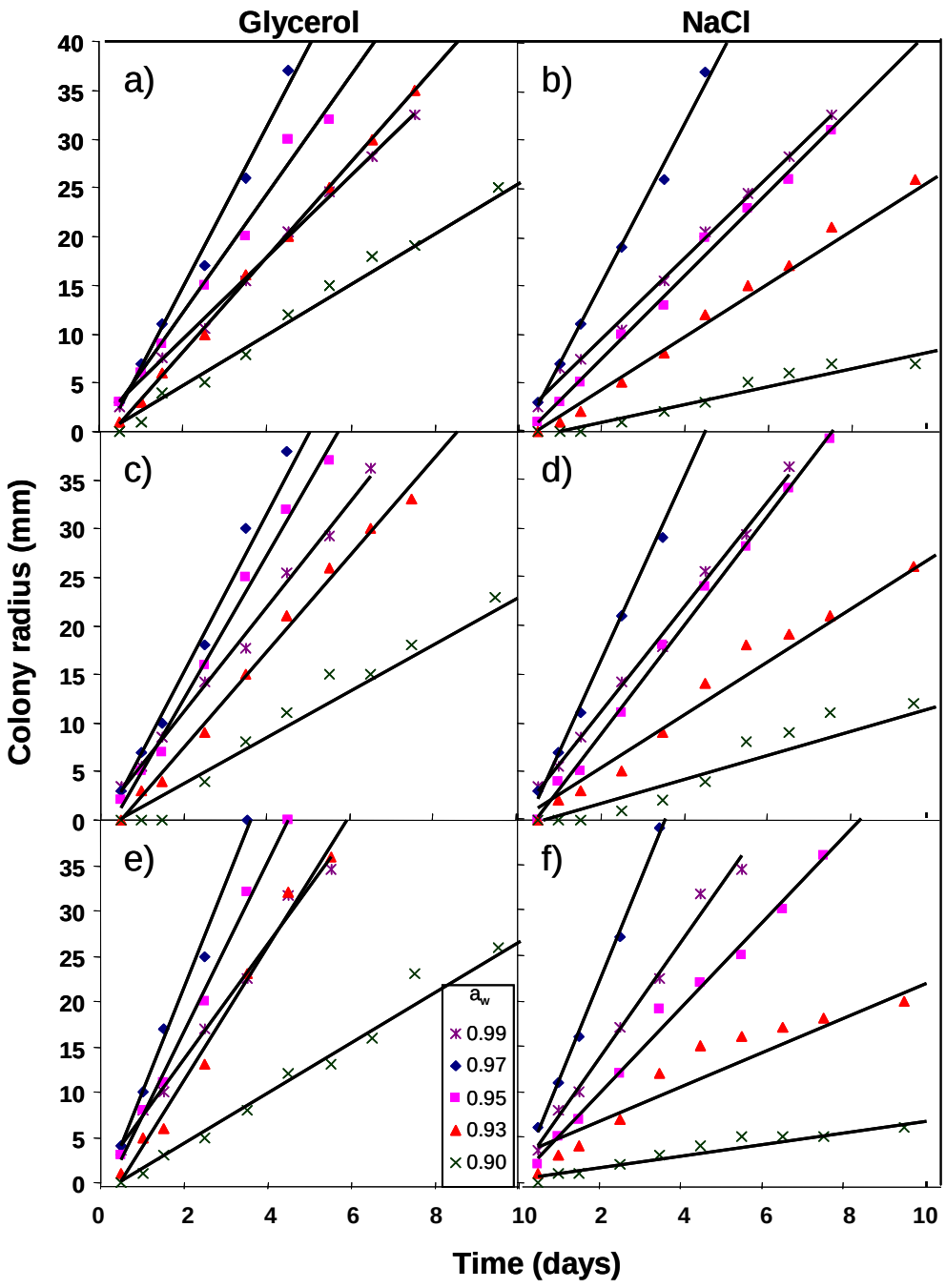

Glycerol
NaCl
a)
b)
c)
d)
e)
f)
Colony radius (mm)
Time (days)
0
5
10
15
20
25
30
35
40
2
4
6
8
aw
0.99
0.97
0.95
0.93
0.90

Figure 1

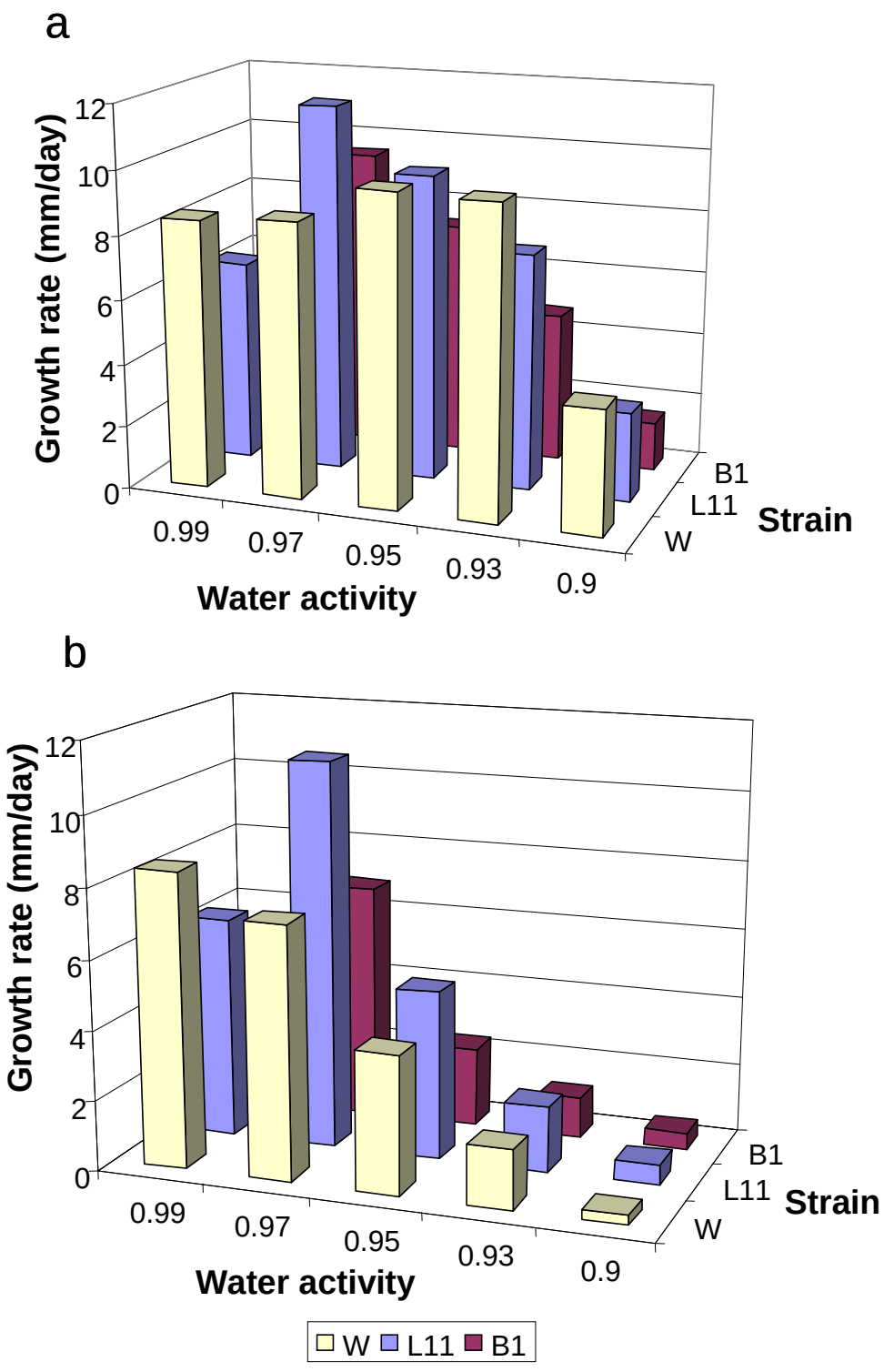

a
Growth rate (mm/day)
12
10
8
6
4
2
0
0.99
0.97
0.95
0.93
0.9
Water activity
B1
L11
W
Strain
b
Growth rate (mm/day)
12
10
8
6
4
2
0
0.99
0.97
0.95
0.93
0.9
Water activity
B1
L11
W
Strain
W
L11
B1

Figure 2

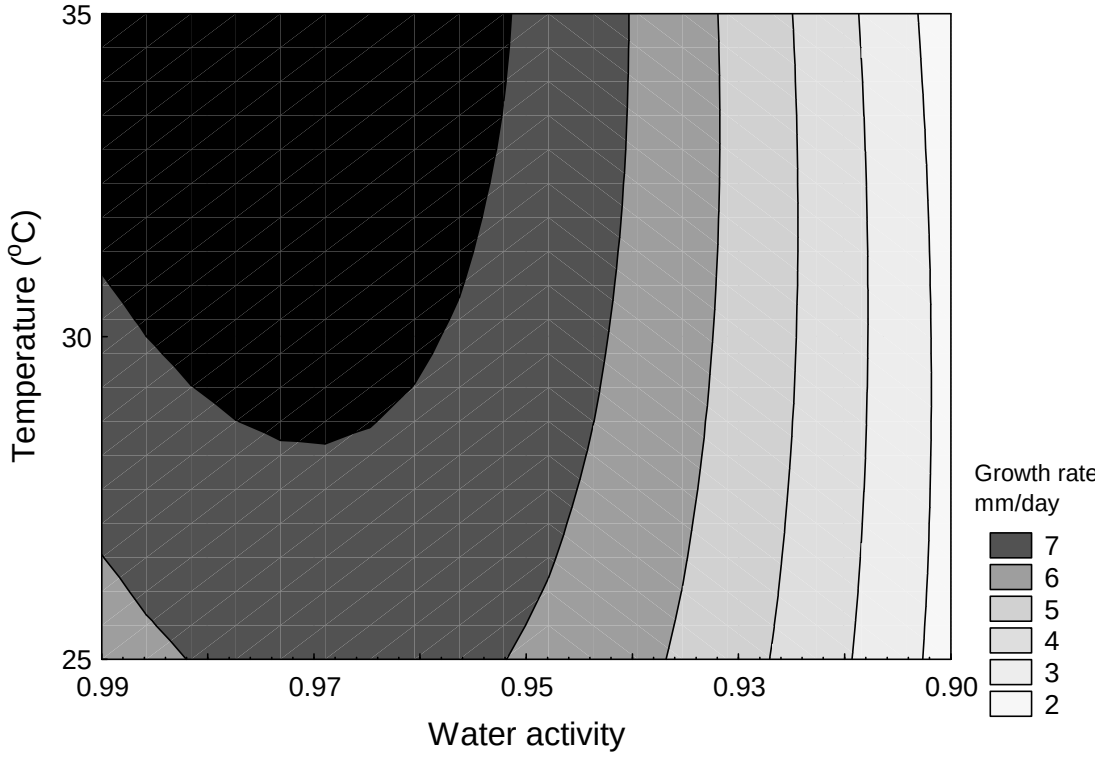

35
30
25
Temperature (°C)
0.99
0.97
0.95
0.93
0.90
Water activity
Growth rate
mm/day
7
6
5
4
3
2

Figure 3

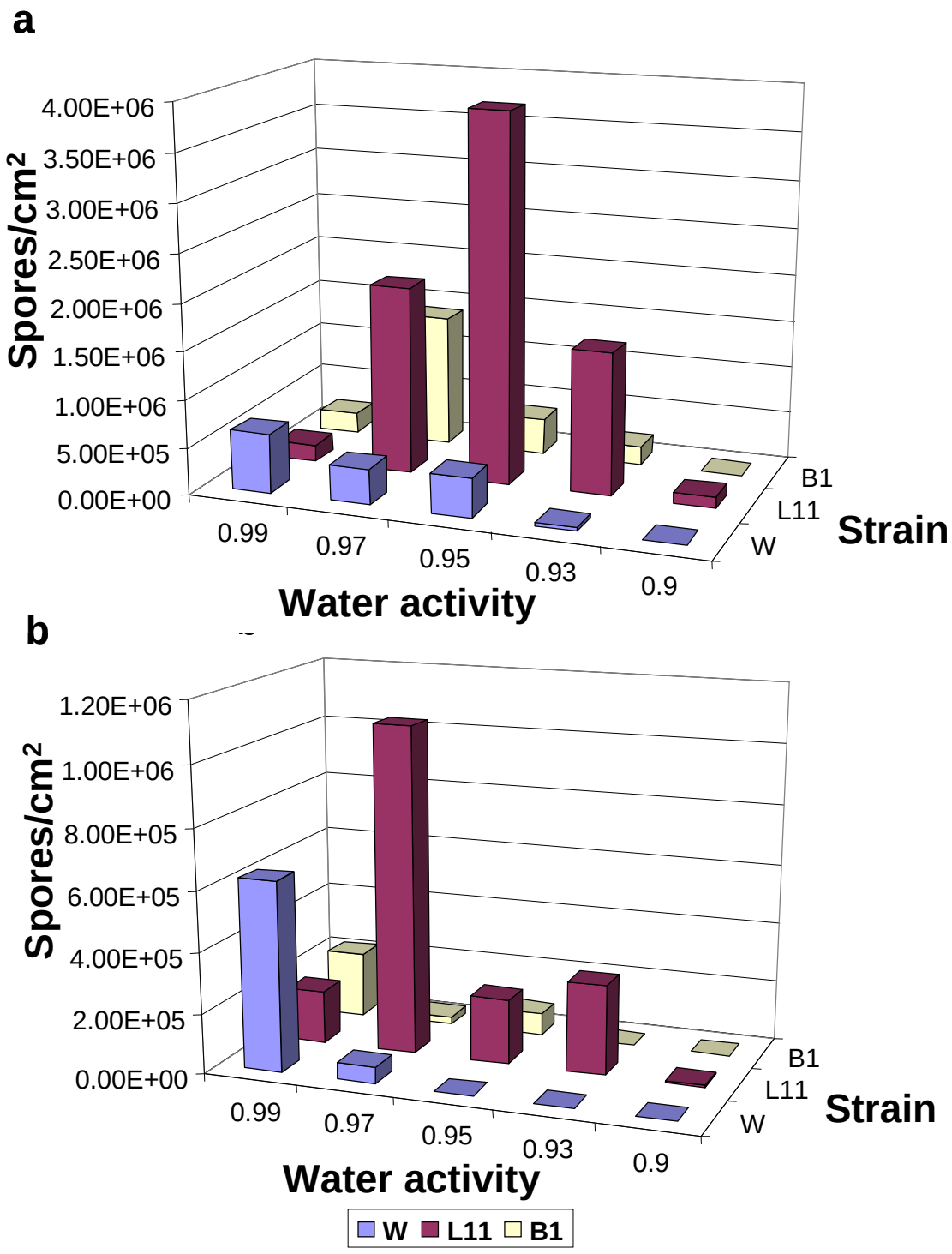

a
4.00E+06
3.50E+06
3.00E+06
2.50E+06
2.00E+06
1.50E+06
1.00E+06
5.00E+05
0.00E+00
Spores/cm²
0.99
0.97
0.95
0.93
0.9
Water activity
B1
L11
W
Strain
b
1.20E+06
1.00E+06
8.00E+05
6.00E+05
4.00E+05
2.00E+05
0.00E+00
Spores/cm²
0.99
0.97
0.95
0.93
0.9
Water activity
B1
L11
W
Strain
W
L11
B1

Figure 4

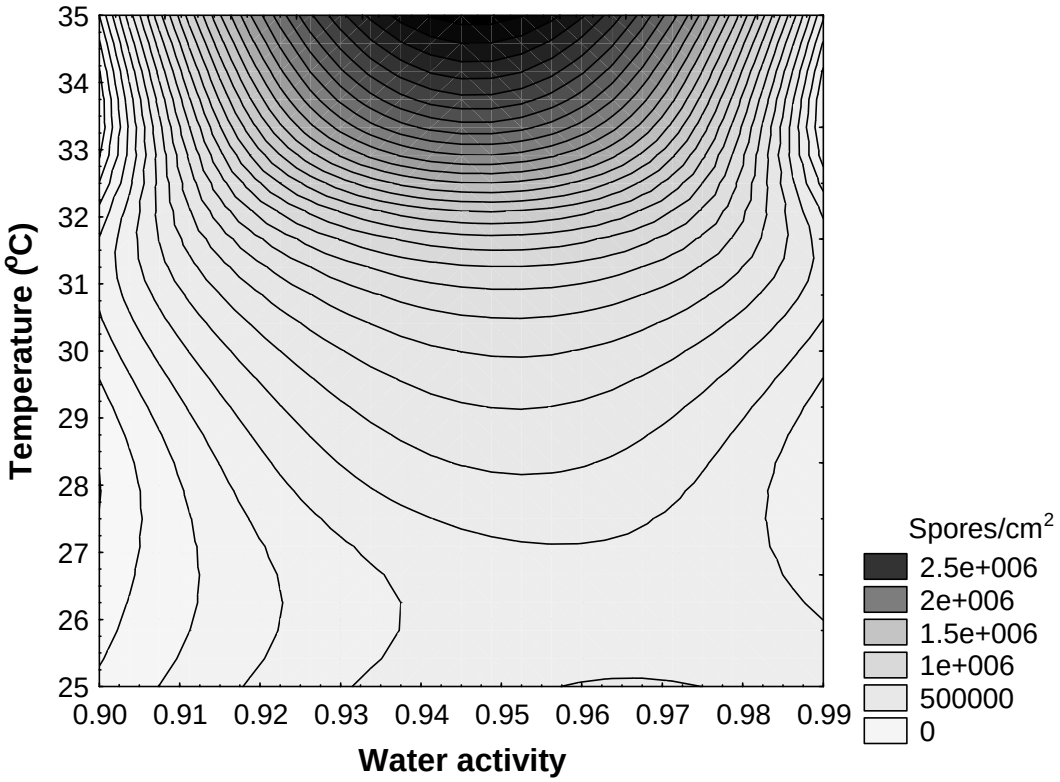

Temperature (°C)
35
34
33
32
31
30
29
28
27
26
25
0.90
0.91
0.92
0.93
0.94
0.95
0.96
0.97
0.98
0.99
Water activity
Spores/cm2
2.5e+006
2e+006
1.5e+006
1e+006
500000
0

Figure 5



https://dspace.org/

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