

**Impact of environmental factors on growth and satratoxin G production by strains of *Stachybotrys chartarum***

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Running title: Environment and satratoxin production by *Stachybotrys*

**ABSTRACT**

The black mould *Stachybotrys chartarum* and its mycotoxins have been linked to damp building-associated illnesses. The objective of this study was to determine the effects of water availability (water activity,  $a_w$ ) and temperature on growth and production of the satratoxin G (SG) by a macrocyclic trichothecene-producing strain (IBT 7711) and non-producing strain (IBT 1495) of *S. chartarum*. Growth studies were carried out on potato dextrose agar modified with glycerol to 0.995-0.92 water activity ( $a_w$ ) at 10-37°C. Growth extension was measured and the cultures were extracted after 10 days and a competitive enzyme-linked immunosorbent assay (ELISA) method used to quantify the SG content. Growth was optimal at 25 to 30°C at 0.995  $a_w$ , but this was modified to 0.98  $a_w$  at 30°C for

both strains (1.4-1.6 mm day<sup>-1</sup> respectively). The ELISA method revealed that, in contrast to growth, SG production was maximal at 20°C with highest production at 0.98 a<sub>w</sub> (approx. 250 µg/g mycelia). When water was freely available (0.995 a<sub>w</sub>), SG was maximally produced at 15°C and decreased as temperature was increased. Interestingly, the strain classified as a non-toxigenic produced very low amounts of SG (<1.6 µg g<sup>-1</sup> mycelia) that were maximal at 25°C and 0.98 a<sub>w</sub>. Contour maps for growth and SG production were developed from these data sets. These data have shown, for the first time, that growth and SG production profiles are very different in relation to key environmental conditions in the indoor environment. This will be very useful in practically determining the risk from exposure to *S. chartarum* and its toxins in the built environment.

## 1. Introduction

*Stachybotrys chartarum* (Ehrenb.) Hughes is a frequent contaminant of water-damaged buildings that has been suspected of adversely affecting human health (Peat *et al.* 1998; Sahakian *et al.* 2008; Lu *et al.* 2009; Pestka *et al.* 2008). Two different chemotypes of *S. chartarum* have been described (Andersen *et al.* 2002, 2003). Chemotype S produces the macrocyclic trichothecenes, including satratoxins and roridins whereas Chemotype A produces the non-macrocyclic trichothecenes, atranone and dolabellanes. Metabolite profiling suggested that production of satratoxins occurred in many *Stachybotrys* strains isolated from water-damaged buildings in Finland (Nielsen *et al.*, 1999; 2003; Peltola *et al.*, 2001). Using immunocytochemistry, Gregory *et al.* (2004) showed that *S. chartarum* spores contained high satratoxin concentrations whereas mycelium contained lesser amounts.

Fungal growth and toxigenesis are influenced by key abiotic factors such as water activity ( $a_w$ ), temperature and surface pH (Nielsen *et al.* 2004; Magan, 2007). While there have been detailed studies on the ecology of a range of xerotolerant fungi including *Penicillium*, *Aspergillus* and *Eurotium* species (Ayerst 1969; Magan 2007), much less information is available on non-xerophilic indoor air moulds like *S. chartarum*. Ayerst (1969) reported the minimal  $a_w$  for growth at 0.94 and an optimal temperature of 23°C for a *Stachybotrys atra* Corda strain isolated in the U.S.A. Previously, Nielsen *et al.* (1998a,b) showed that the trichothecene mycotoxins satratoxin G and H were both present on water damaged gypsum boards in Denmark and that growth occurred only at  $>0.95 a_w$  on gypsum board building materials (Nielsen *et al.* 2004). There is thus a clear need here for improved understanding of the role of the environment in growth and toxigenesis of *S. chartarum*.

The objective of this study was to determine the effects of  $a_w$  and temperature on growth and satratoxin G (SG) production by two strains of *S. chartarum* considered to be a chemotype S (IBT 7711), a macrocyclic trichothecene producer, and chemotype A (IBT 14915), a non-producer. The results were used to produce contour maps that enable the prediction of optimum and marginal conditions for growth and SG production by this important environmental mould.

## 2. Materials and methods

### Fungal strains used in this study

Two representative strains of *S. chartarum* isolated from damp buildings were used based on previous studies of a number of strains (Nielsen et al., 1998a, b; 2004). *S. chartarum* IBT (Institut for BioTeknologi) 7711 was chosen as a representative macrocyclic trichothecene producer (Chemotype S) and *S. chartarum* IBT 14915 was selected to represent non-macrocyclic producing isolate (Chemotype A). Both were kindly provided by The Mycology Group, Systems Biology Department, Technical University of Denmark. This Culture Collection is a member of the World Fungal Culture Collections. Two additional strains of *S. chartarum* (Portsmouth 4, Denbeigh) isolated from damp buildings in the UK were also used in some growth studies for comparison. These were characterised as Chemotype S strains and were found to be morphologically similar to IBT 7711 and in toxicity assays. These strains are in the Applied Mycology Culture Collection and the names identify the source of the strains. It should be noted that this study has used only four different strains of *S. chartarum* in these ecophysiological studies. There may be wider strain differences which may occur, especially on a regional basis.

### Growth studies

Basal growth media was prepared by boiling 39 g L<sup>-1</sup> of potato dextrose agar (PDA) and then autoclaving at 121°C for 15 minutes. This basic medium (0.995 water activity;  $a_w$ ) was modified with the addition of the non-ionic solute glycerol to obtain  $a_w$  levels of 0.98, 0.96 and 0.92  $a_w$ . Autoclaved media were poured into 9 cm diameter sterile Petri plates (15 ml/plate). The  $a_w$  of all media were verified with a Thermconstanter (NovaSina Sprint).

Fungi were grown on PDA for 7-10 days at 25°C to obtain heavily sporulating cultures. The spores were dislodged using a sterile loop and then suspended in 10 ml sterile distilled water containing one drop of a wetting agent (Tween 80). The spore concentration was determined using a haemocytometer slide and adjusted to 10<sup>6</sup> spores ml<sup>-1</sup> with sterile

water. A stock spore suspension (1 ml) was added to 9 ml glycerol/water solutions at the same water activity ( $a_w$ ) levels as the treatment and replicate agar media in 9 cm Petri plates. The final water activities of the treatments were 0.995, 0.98, 0.95 and 0.92  $a_w$ .

A 3  $\mu$ l aliquot of the spore suspension was point inoculated in the centre of each of three replicate PDA plates for each treatment. Inoculated plates of the same  $a_w$  treatment were kept in closed polyethylene bags and incubated at 10, 15, 20, 25, 30 and 37 °C for 14 days. Every two days, two colony diameters at right angles were measured. Temporal mycelial extension data were used to determine the growth rates ( $\text{mm day}^{-1}$ ) by linear regression. All experiments were carried out twice.

### **Satratoxin G (SG), extraction and quantification**

SG production in cultures was measured after 10 days. Using a sterile cork borer (5 mm diam) 5 discs were taken across the colony, frozen at -20 °C, freeze dried overnight and weighed. This lyophilized material was then used for extraction and quantification of SG with three replicates of each treatment for each of the representative strains.

Freeze dried samples were soaked in phosphate buffered saline (PBS) (pH 7.2, 10 mM) overnight at 4 °C. Because of the amounts of material available 1.5 ml of PBS was added to Chemotype S (satratoxin G producer, IBT 7711) samples and 1 ml to chemotype A (non-producer, IBT 14915). Samples were centrifuged for 15 min ( $13,000 \times g$ ) at 4 °C and the resulting supernatant analyzed for SG.

SG was quantified by a competitive ELISA method (Chung *et al.*, 2003). This method has a detection limit of 100 pg/ml of SG. This method was compared with the HPLC method and found to give a good correlation (Chung *et al.*, 2003). Briefly, SG-specific polyclonal antibodies (100  $\mu$ l) diluted (0.5  $\mu\text{g ml}^{-1}$ ) in phosphate buffered saline (PBS) (pH 7.2, 10 mM) were incubated in 96-well ELISA plates (NUNC) overnight at 4 °C. Plates were washed three times with PBS containing 0.05% (v/v) Tween 20 (PBS-T) and blocked with 300  $\mu$ l of 1% (w/v) bovine serum albumin in distilled water (BSA), and then incubated 60 min at 37 °C. After washing four times with PBS-T, 50  $\mu$ l of standard or samples with 50  $\mu$ l SG-horseradish peroxidase conjugate was diluted (0.5  $\mu\text{g ml}^{-1}$ ) in BSA at room temperature (25 °C) for 60 min. Plates were washed seven times with PBS-T and bound peroxidase was determined

after incubation for 30 min at 25 °C with 100 µl/well of K-Blue Substrate (Neogen, Lansing, USA). The reaction was terminated with 100 µl/well of 2 N sulphuric acid stopping reagent and the plate read at 450 nm using a Vmax Kinetic Microplate Reader (Molecular Devices, Menlo Park, CA, USA). Three replicates of each treatment were analysed. The results are presented as µg g<sup>-1</sup> culture.

### **Statistical analyses**

Analyses were done using the JMP 8 Statistical Software package (SAS Institute Cary NC, USA). Generally, the independent variable distribution was assessed for each strain using the Shapiro-Wilk W Test. Both datasets failed the test so logarithm and square root transformations were tested in order to increase the normality and homoscedasticity (Levene's test). Taking into account the dataset characteristics, non-parametric Kruskal-Wallis rank sum test was used to study the effect of  $a_w$  and temperature on SG toxin data from *S. chartarum* 7711 and parametric ANOVA test was used to test the same factors on the log SG toxin for *S. chartarum* IBT 14915. Afterwards *post-hoc* Tukey-Kramer Honestly Significant Difference (HSD) test was applied to investigate the relationship between the different treatment means.

For developing the contour maps two different models were considered initially and growth curves were fitted using the Baranyi biphasic model and a linear model obtained by plotting the results against time (Medina and Magan, 2011). Results obtained by both approaches were very similar and based on the studies of Marin *et al.* (2008), the Baranyi biphasic model was used for all data to minimise subjectivity in the calculation of the growth rate (mm day<sup>-1</sup>). The conditions under which equivalent growth rates occurred under different environmental conditions were joined to generate contour lines that mapped the relative optimum and marginal conditions for growth of the *S. chartarum* strains. Profiling graphs were similarly obtained for the data on SG production using Sigma Plot v.10.0 (Systat Software Inc. Hounslow, London, UK).

## **3. Results**

### **Effects of water activity and temperature on growth**

The effects of temperature (15-37°C) at a steady state  $a_w$  (0.98) and at a steady state temperature (25°C) on mycelial extension rates of the toxigenic strain IBT 7711 and non-toxigenic strain 14915 of *S. chartarum* strains, respectively are shown in Figures 1 and 2. *S. chartarum* IBT 7711 extension was similar at 25 and 30°C. However, *S. chartarum* IBT 14915 extension rate was faster at 30°C. Growth of both strains was most rapid at 0.995  $a_w$  and decreased as the water availability was reduced. Strain IBT 7711 was able to initiate growth at 0.92  $a_w$ , while the non-toxigenic strain IBT14915 was unable to grow at this water stress level. Comparisons were also made between these type strains and two UK ones isolated from damp buildings in the UK in relation to  $a_w$  tolerance (Figure 3). This shows that the behaviour of the strains was relatively similar in terms of growth rates and the effect of changing  $a_w$  with little growth at 0.91-0.92  $a_w$  and with optima at 0.995  $a_w$ . The Denbeigh strain grew at a slower rate than the other three strains examined. For subsequent studies we concentrated on the IBT 7711 and IBT 14951 strains because of their overall similarity to the other strains examined.

Contour maps of relative growth rates in relation to the whole  $a_w$  x temperature range revealed that both strains grew over a broad temperature range, with an optimum at 25°C, (Figure 4). At marginal  $a_w$  conditions, IBT 7711 was able to grow best at 20°C (0.2 mm day<sup>-1</sup>) and 0.91-0.92  $a_w$ . This contrasted with strain IBT 14915 which was limited at about 0.93-0.94  $a_w$ .

### **Effects of water activity and temperature on SG production**

The interactive effects of  $a_w$  x temperature on SG production was assessed for the two type strains (Figure 5). SG production by IBT 7711 was optimum at 0.98  $a_w$  and 20°C, followed by that at 20°C and 0.995  $a_w$ . There was very little production at 0.96  $a_w$  in all treatments. Notably, SG was not produced at >30°C or <15°C. Although IBT 14915 is classified as a non-producer of SG, it appeared to produce very low levels of the toxin with an optimum at 25°C and 0.98  $a_w$  and at 20°C and 0.96  $a_w$ . At 0.995  $a_w$  the highest SG concentrations were produced at 15°C and this decreased with increasing temperature. Again, SG was not produced at >30°C. Table 1 shows the statistical analyses of the effect of  $a_w$  and temperature on the two strains. This shows that  $a_w$  and temperature had a significant effect on SG production by the producing strain (IBT 7711) while for the non-producing strain,

where SG concentrations produced were very low, there was no effect of  $a_w$ , but some effect of temperature.

SG data were used to produce contour maps by joining mean concentrations produced in relation to  $a_w$  x temperature conditions (Figure 6). Optimum SG production for IBT 7711 occurred over a wide temperature range and  $>0.98 a_w$  (Figure 6a). SG production is most pronounced at 20 to 25°C regardless of water availability. For IBT 14915, patterns were less consistent because of the very small amounts of SG produced (Figure 6b). Nevertheless, high  $a_w$  and temperature of 25°C appeared to be optimal for this modest output of SG.

#### 4. Discussion

The results presented here are the first to systematically relate growth and macrocyclic trichothecene production to temperature and water availability. Notably, growth occurs at a lower minimum  $a_w$  than that for SG production. This is consistent with other studies of mycotoxigenic fungi demonstrating that  $a_w$  x temperature conditions for toxin production are usually narrower than those for germination or growth (Sanchis and Magan 2004).

For the SG-producing strain, growth was limited to  $>0.92 a_w$  under optimum temperature conditions (25-30°C). For the other strain, 0.93-0.94  $a_w$  appeared to be the minimum for growth, also at 30°C. It was interesting to note that as water stress was imposed (e.g. 0.98  $a_w$ ) the optimum temperature for growth changed. The temperature range was very wide for both strains of *S. chartarum*, being from  $<10^\circ\text{C}$  to 37°C. This is different from the results of Grant *et al.* (1989) who found no germination or growth at 37°C. Ayerst (1969) suggested a minimal  $a_w$  for growth of 0.94 and an optimum temperature of 23°C for a strain of *S. atra* (= *S. chartarum*). The absolute minimum may be influenced by nutritional considerations and time scales of experiments. The present experiments were limited to 14 days. Recently Frazer *et al.* (2011) showed that germination was very rapid over a range of temperature x  $a_w$  conditions (15-37°C, 0.995-0.95  $a_w$ ). Interestingly, sporulation of *S. chartarum* occurred over a wide temperature range (15-30°C) and 0.995-0.95  $a_w$ . However, no sporulation was observed at 37°C regardless of environmental conditions.



Studies by Nielsen *et al.* (2004) on colonisation of a wide range of building material under a matrix of temperature and  $a_w$  conditions showed *Stachybotrys* growth only occurred in gypsum board at  $>0.95 a_w$ . At lower  $a_w$  levels ( $<0.95 a_w$ ) only *Penicillium*, *Aspergillus* and *Eurotium* species were able to grow. The contour diagrams in the present study join conditions of similar growth rates by isopleths lines and shows the growth/no growth boundaries which may also be applicable to sporulation. These may be very useful as a guideline of threshold conditions above and below which this species may cause problems in damp building environments, especially after flooding and during drying out.

There has been surprisingly little information on the ecophysiology of SG production by strains of *S. chartarum*. With the exception of the studies of Andersen *et al.* (2002), which showed the metabolite profiling of strains of *S. chartarum*, there has been practically no information on the effect of ecological conditions on SG production. The available literature on this mould in relation to human health problems was reviewed by Miller *et al.* (2003). While the presence of *S. chartarum* has not been directly related to putative neurological damage it has been associated with pulmonary haemorrhage. Thus, it is still important to have information on the production of satratoxin G and other macrocyclic trichothecenes by strains of this species. Recent work suggests that high concentrations of the toxin can accumulate in spores which can be deposited in the respiratory tract (Pestka *et al.*, 2008).

The present study has shown that the conditions for SG production are much narrower than those for growth in terms of  $a_w$  x temperature, especially for the toxigenic strain. The most interesting findings here were that optimum conditions for SG production were different from those for growth (20 vs 25-30°C, respectively). Production was also limited to about 0.96  $a_w$  in the SG producing strain. In the chemotype A strain production of SG was very low and varied much more with environmental conditions. The contour plots show clearly that no SG toxin is produced at  $\geq 30^\circ\text{C}$ . This is important and suggests that in damp building environments the risk of exposure to the SG toxin may be much higher at  $<25^\circ\text{C}$  and  $>0.95 a_w$ . This suggests that in water damaged buildings which are still occupied, drying regimes could result in *S. chartarum* spores being released into the air which can then cause problems to those occupants who may be immuno-compromised. The observation that the

non-producer strain (IBT 14915) produced low amounts of SG could reflect the high sensitivity of the ELISA method employed in this study. The data for SG production was however more variable and the contour plots reflect this. Previous studies by Nielsen *et al.* (1998a, b) demonstrated that this strain produced low amounts of verrucarol when it was grown on building materials.

## 5. Conclusions

This study has shown that environmental factors have a profound effect on growth and SG production by strains of *S. chartarum*. The optimum  $a_w$  and temperature conditions for growth and SG production were shown to be different for the first time. The contour maps developed in this study will be beneficial for determining practically, the conditions which represent (i) risk from growth of *S. chartarum* and (ii) risk from SG production and exposure in damp buildings by this environmentally important fungus.

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Table 1. Statistical analyses of the mean satratoxin G ( $\mu\text{g g}^{-1}$ ) production by two strains of *S. chartarum* under different water activity ( $a_w$ ) and temperature regimes using the Tukey-Kramer HSD test. Treatments followed by different letters are significantly different ( $P=0.05$ ).

<i>S. chartarum</i> IBT 7711			<i>S. chartarum</i> IBT 14915		
Treatment		Satratoxin ( $\mu\text{g g}^{-1}$ )	Treatment		Satratoxin ( $\mu\text{g g}^{-1}$ )
<b>Aw</b>			<b>Aw</b>		
0.98	a	74598.64	0.98	a	4.76
0.995	a b	55623.12	0.995	a	4.71
0.96	b	850.33	0.96	a	3.47
<b>Temp (<math>^{\circ}\text{C}</math>)</b>			<b>Temp (<math>^{\circ}\text{C}</math>)</b>		
20	a	117140.13	20	a	5.71
25	a b	71024.39	25	a	5.50
15	a b	38068.72	15	a	5.12
30	b	1035.47	30	b	3.07
37	b	46.98	37	b	1.77

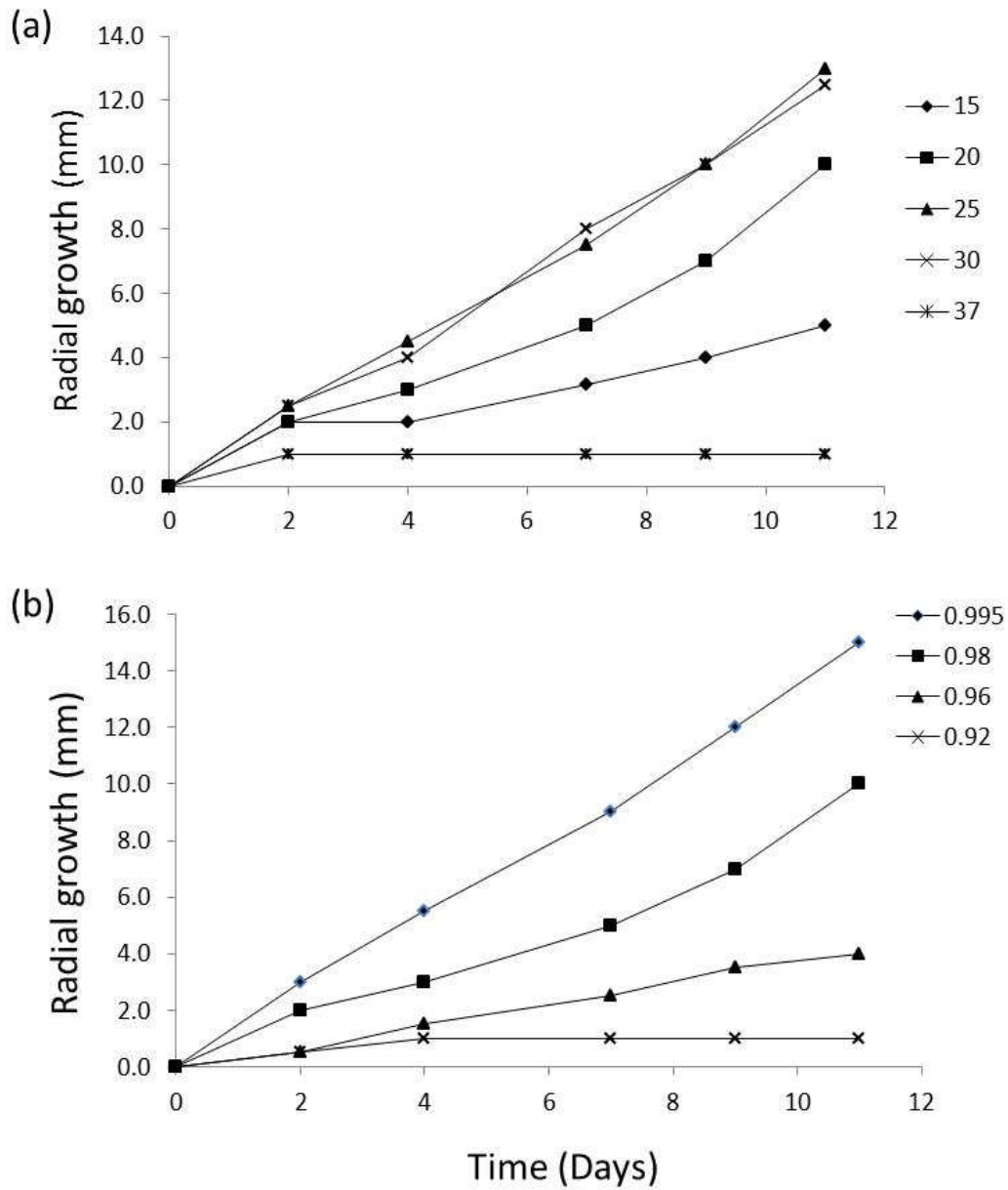


Figure 1. The growth effects of (a) temperature at 0.98 water activity ( $a_w$ ) (b) effect of  $a_w$  at 25°C radial extension by *S. chartarum* strain IBT 7711. Data are mean of three replicates per treatment.

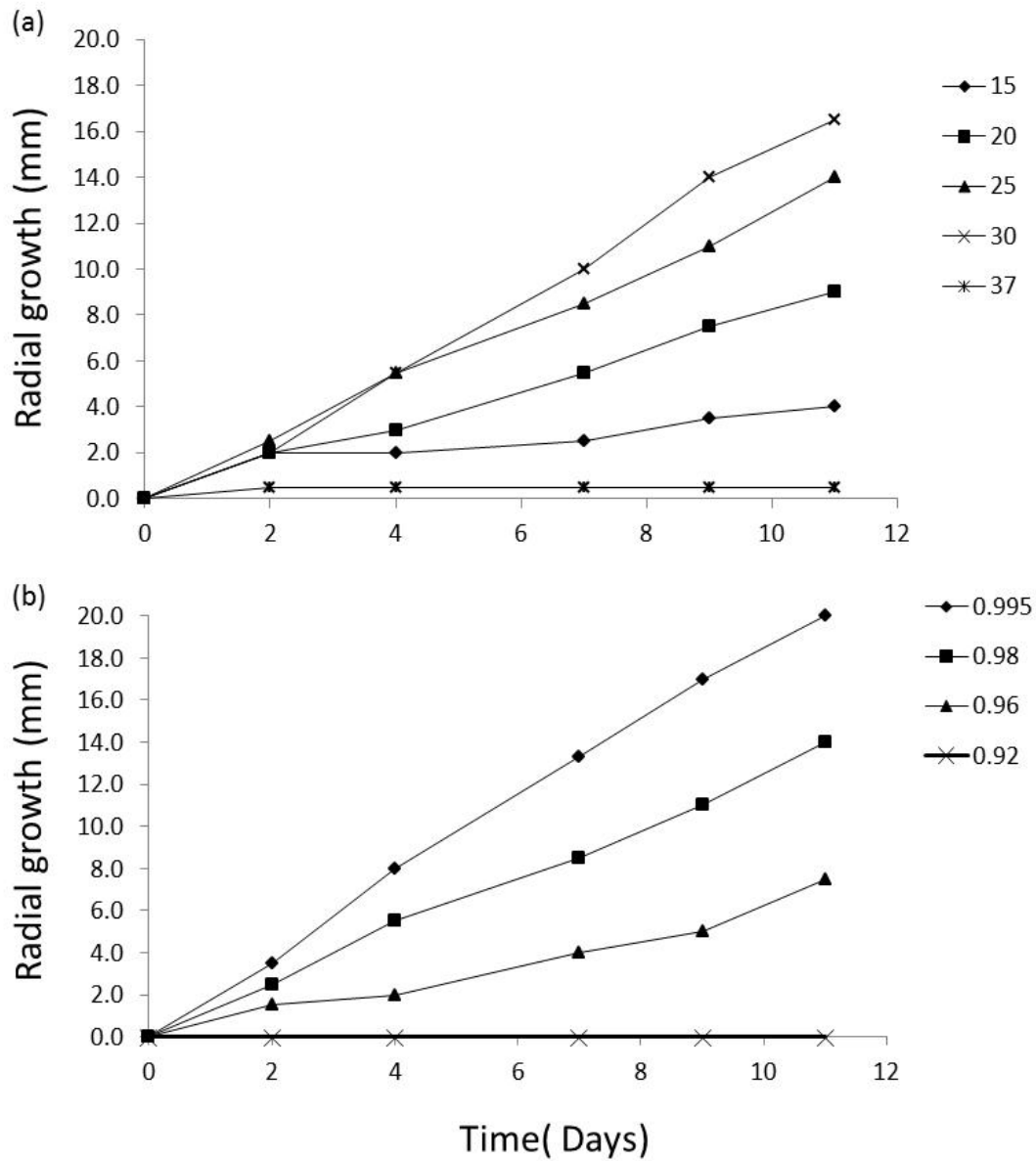


Figure 2. The growth effects of (a) temperature at 0.98 water activity ( $a_w$ ) (b) effect of  $a_w$  at 25°C radial extension by *S. chartarum* strain IBT 14915. Data are mean of three replicates per treatment.

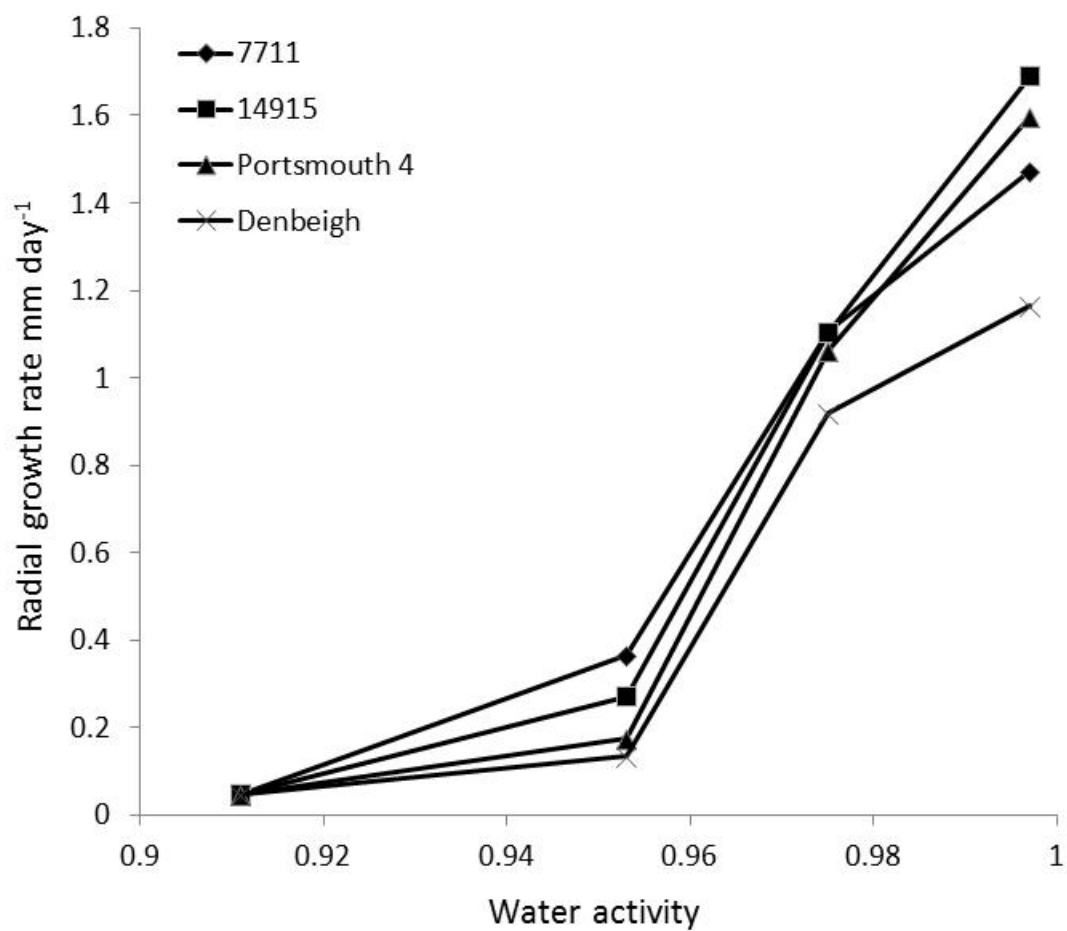


Figure 3. Comparison of the growth rate (mm day<sup>-1</sup>) of four strains of *Stachybotrys chartarum* in relation to water activity at 25°C. Data are means of three replicates of each strain.



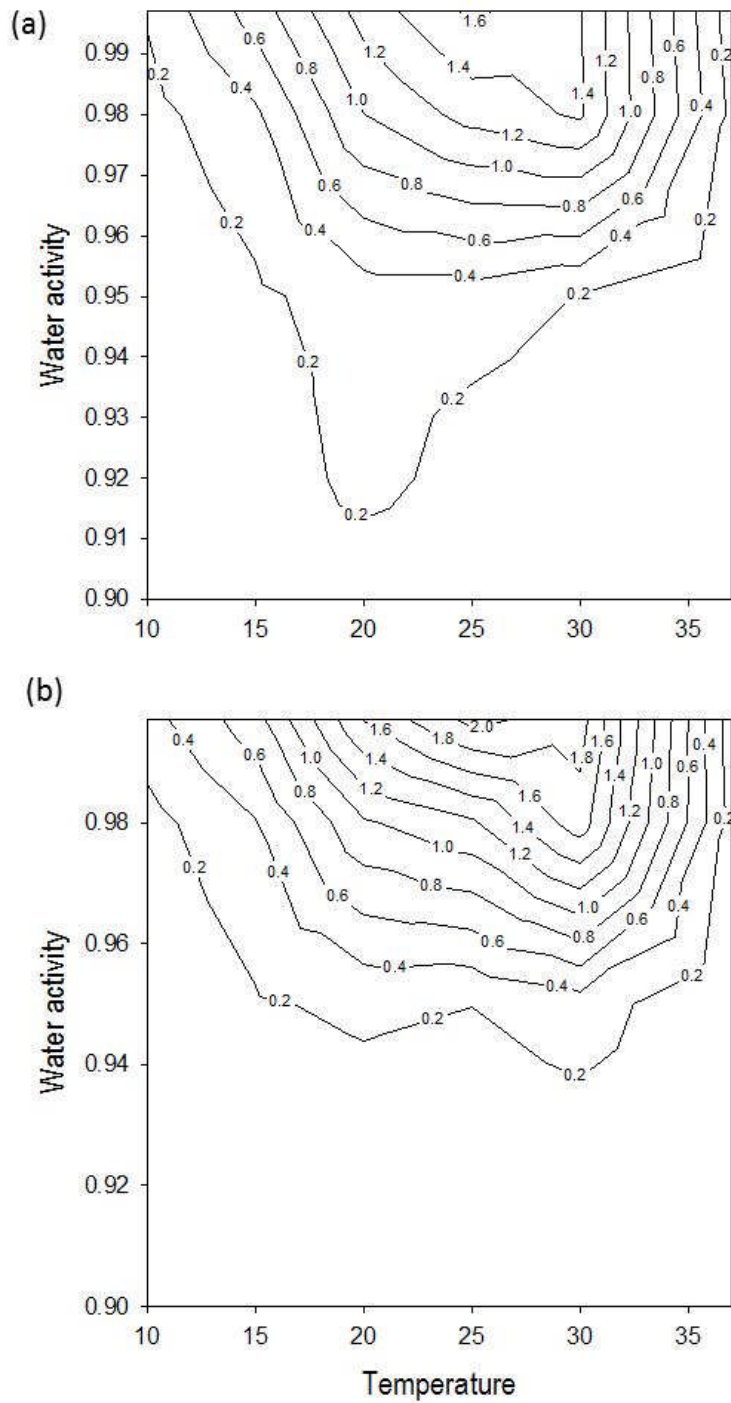


Figure 4. Contour plots of growth rates of (a) *S. chartarum* strains IBT 7711 and (b) IBT 14195. Numbers on the isopleths represent similar growth rates (mm day<sup>-1</sup>) under different conditions of water activity x temperature conditions.

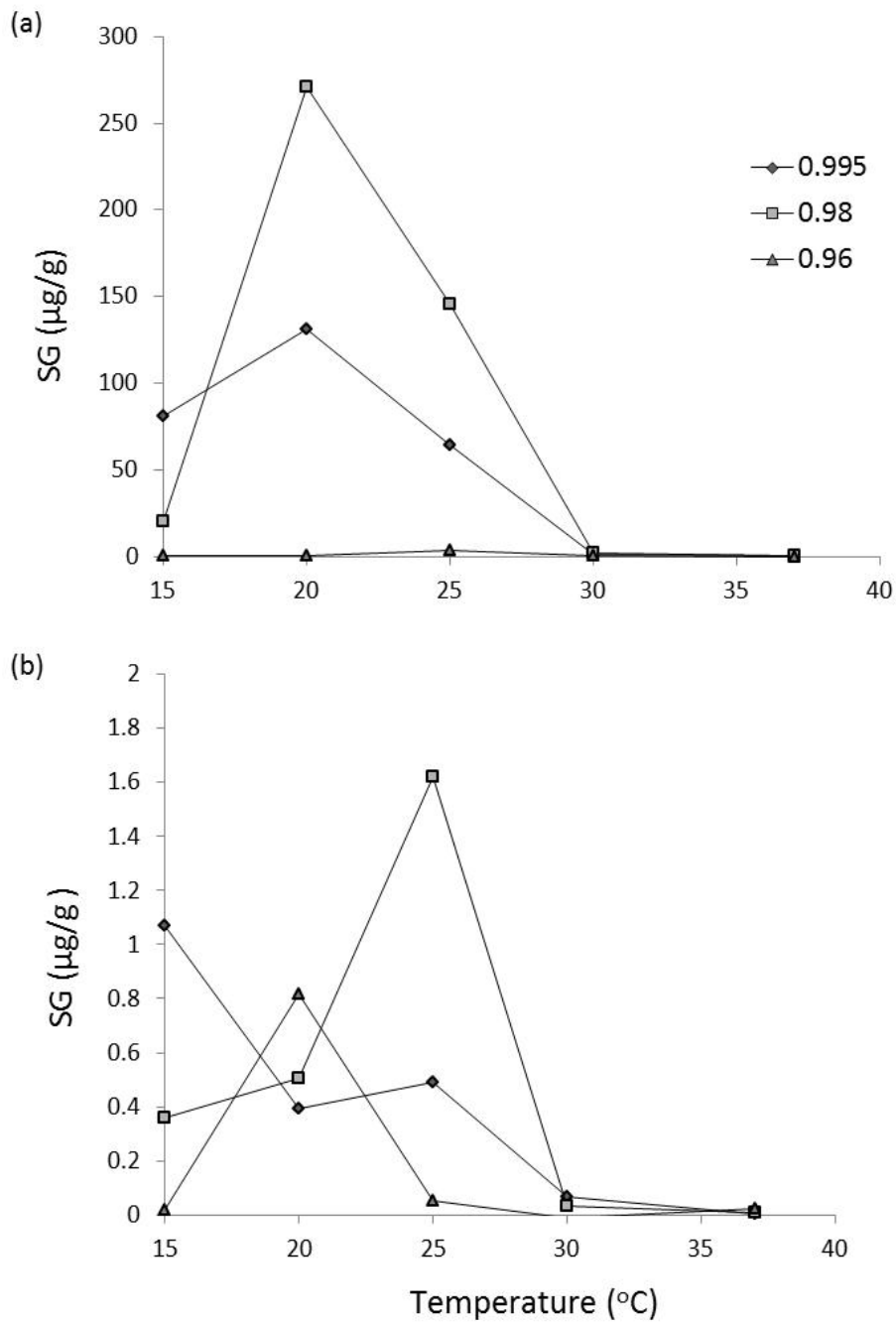


Figure 5. Satratoxin G production ( $\mu\text{g g}^{-1}$ ) by *S. chartarum* strains (a) IBT 7711 and (b) IBT 14195 in relation to water activity and temperature. These are means of three replicates per treatment.

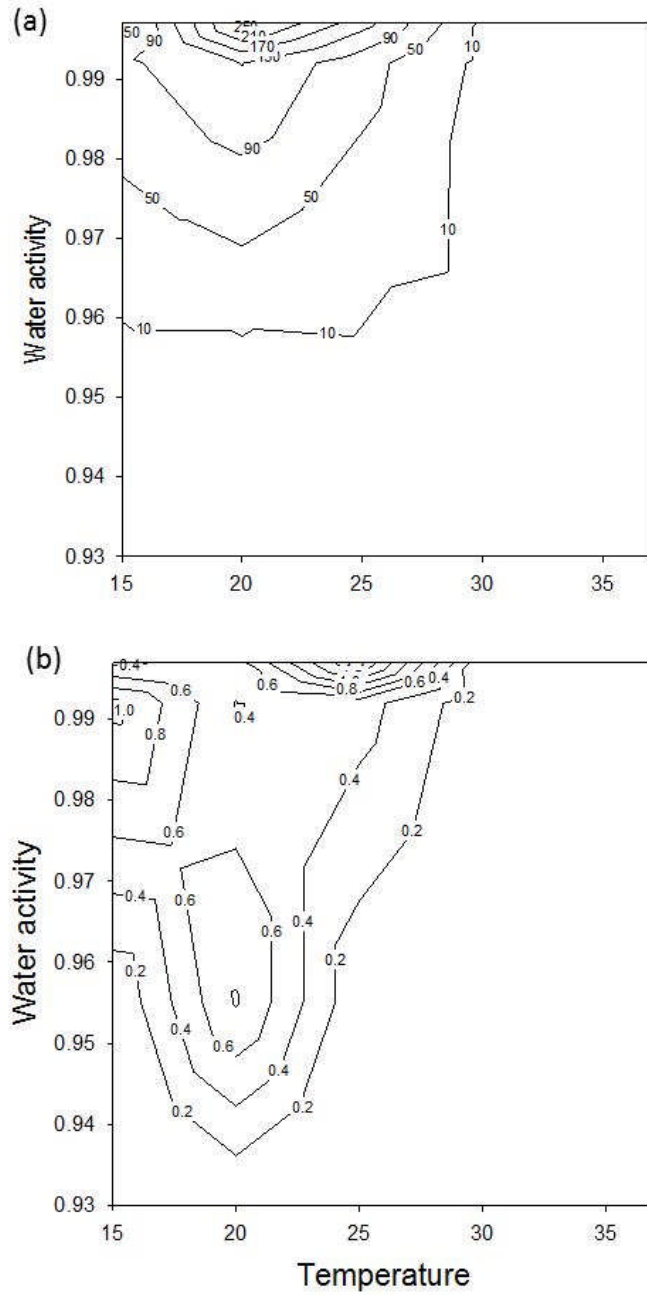


Figure 6. Contour plots of SG ( $\mu\text{g g}^{-1}$  freeze dried sample) production by *S. chartarum* strains (a) IBT 7711 and (b) IBT 14195. Numbers on the isopleths represent similar amounts of toxin produced under different conditions of water activity x temperature conditions.