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Influence of water activity and temperature on the growth of *Wallemia sebi*: application of a predictive model

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Abstract

Germination and growth of *Wallemia sebi* were examined on media of a_w adjusted with glycerol in the range of 0.96–0.77, at 25°C and 30°C. The effect of temperature on the germination time was significant except between 0.95 and 0.88 a_w. At low a_w levels as well as above 0.95, the increase of temperature produced an increment in the germination time. The minimum a_w for germination was also affected by temperature, being lower at 25°C (0.80 a_w) than at 30°C (0.82 a_w). Radial growth rates at 25°C were higher than at 30°C. The optimum a_w value for growth of *W. sebi* was 0.94 at both temperatures. The minimum a_w for growth was higher than minimum for germination and was also dependent on temperature (0.84 at 25°C and 0.86 at 30°C). An empirical mathematical model was fitted to the measured growth data, providing a good approach to the description of the effect of a_w on the radial growth rate of *W. sebi*.

Keywords: *Wallemia*; Water activity; Temperature; Growth; Predictive microbiology

1. Introduction

Wallemia sebi is a xerophilic mould able to grow in a wide range of water activities (a_w). Pitt and Hocking (1997) consider it ubiquitous, although records of its occurrence in foods are scarce. This is probably due to the use of inadequate techniques of isolation, since *W. sebi* does not grow well on common high a_w laboratory media. Considered as one of the main spoiling fungi of salted and dehydrated fish (Wheeler et al., 1988), it has also been isolated from prunes, syrups, grape raisins, jams, jellies, dry peppers, bread, condensed milk, dates and grains such as rice, corn, wheat, peanut and soybeans (Pitt and Hocking, 1997). Wood et al. (1990) showed that an isolate of *W. sebi* produced toxic effects in several bioassays. A toxic compound (walleminol A) was isolated and characterized as a tricyclic dihydroxisesquiterpene with a minimum inhibitory dose in the bioassays comparable with such mycotoxins as penicillic acid and citrinin.

In our laboratory, diverse strains of *W. sebi* have been isolated from foods such as sweet potato jam, vanilla pudding, peanut, wheat, peach jam, dairy caramel (dulce de leche) and sweet potato syrup. In

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some cases the foods presented remarkable signs of deterioration due to the superficial formation of numerous small brown colonies.

Water activity and temperature are the most important parameters that allow control of the mould growth responsible of the deterioration of foods. The objective of the present work were to study the influence of these two factors on the growth of *W. sebi* in model systems, to determine the optimum and minimum a_w level for germination and growth, as for the formation of a visible colony that can cause rejection of the product by the consumer.

Predictive models used to describe bacterial growth have recently been applied to data related to fungal growth. Some empirical models have been used not only to describe growth, but also to predict optimum growth conditions, growth rates in different conditions, or a certain colony size. In the present work, the model of Gibson et al. (1994) used to predict the effect of a_w on the growth of *Aspergillus flavus* and related species was applied to the obtained data.

2. Materials and methods

2.1. Fungi

Four strains of *W. sebi*, respectively isolated from peach jam, dairy caramel (dulce de leche), sweet potato jam and sweet potato syrup were used for this study. The strains are maintained in the culture collection of Facultad de Ciencias Exactas y Naturales de la Universidad de Buenos Aires, Buenos Aires, Argentina. Storage at 1–4°C in slopes of G25N agar (Pitt and Hocking, 1997) has prevented loss of viability often observed in *W. sebi*.

2.2. Media

The basal medium consisted of 1% malt extract (Merck 5398; Merck, Darmstadt, Germany), 1% yeast extract (Merck 3753), 0.1% K_2HPO_4 and 2% agar. Adding glycerol 87% p.a. (Merck 4094) to this basal medium, media of different a_w levels were prepared in the range 0.96–0.77, falling in intervals of 0.01 a_w between 0.96 and 0.90, and of 0.02 a_w between 0.90 and 0.78. Glycerol concentrations were calculated from the data of Northolt (1979). By adding small amounts of 10% NaOH or 10% HCl (both w/v), the pH of all media was adjusted between 6.5 and 7.5. Media were sterilized by autoclaving (121°C, 15 min).

The final a_w values were verified for each medium after their sterilization using a hygrometer Vaisala Humicap HMI31 (Vaisala, Helsinki, Finland) equipped with a sensor HMP35. The values measured never varied more than 0.005 a_w from the calculated values.

2.3. Inoculum

A cocktail inoculum was prepared with the four strains according to Hocking and Miscamble (1995). Spores of each strain coming from 7 days of cultivation in G25N agar were placed with an inoculation needle in a haemolysis tube containing 0.5 ml of Tween 80 solution (0.05%). After homogenizing, the suspension was counted using a haematocytometer. Under these conditions the inoculum concentration varied between 3.4 and 4.0×10^6 spores ml^{-1}.

2.4. Inoculation and incubation

Petri plates (5.5 cm diameter) containing approximately 7 ml of medium were inoculated centrally with a calibrated loop containing 5 μl of spore suspension. The plates were incubated at 25°C and 30°C for a maximum period of 100 days. These temperatures are representative of ambient temperature at which food susceptible to *Wallemia* deterioration are stored in warm temperate regions. To minimize the transfer of water to or from the media, the plates were placed inside plastic bags containing dishes with glycerol solutions adjusted to the corresponding a_w.

Each set of a_w and temperature conditions was carried out five times.

2.5. Examination

To observe germination, Petri plates were initially examined twice a day under transmitted light microscope ($100\times$), diminishing the periodicity up to

three times a week in the final stages. The criterion for germination was the production of a germination tube of longitude similar to the diameter of the conidia in at least 50% of the inoculum (Hocking and Miscamble, 1995).

Growth was initially assessed by measuring the hyphal extension using an eyepiece micrometer. As the colonies grew larger the diameters were measured by stage verniers. Diameters were measured daily in the initial phase, diminishing the periodicity to twice a week in the final stages. Averages of two perpendicular readings for each colony were used.

Growth rates were calculated as the linear regression from the linear phase of the growth curve. An average radial growth rate (k_r) was calculated from the different independent experiment for each set of conditions.

2.6. Mathematical and statistical methods

A mathematical model proposed by Gibson et al. (1994) was applied. This model introduces the following transformation:

$$b_w = \sqrt{1 - a_w}\,.$$

Applying this transformation, the $\ln(k_r)$ vs. b_w curves are appropriately adjusted by a degree 2 polynomial function:

$$\ln(k_r) = C_0 + C_1 b_w + C_2 b_w^2,$$

where the coefficients C_0, C_1 and C_2 are estimated by lineal regression. Starting from the equation, it is also possible to calculate the optimum a_w value for the maximum radial growth rate:

$$a_w(\text{opt}) = 1 - \left(\frac{C_1}{2C_2}\right)^2,$$

And the radial growth rate at the optimum a_w:

$$k_r(\text{opt}) = \exp\left(C_0 - \frac{C_1}{4C_2}\right)^2.$$

The influence of temperature and a_w on the radial growth rate (k_r), and the existence of an interaction between both factors, was determined by an ANOVA analysis.

3. Results and discussion

3.1. Germination

Fig. 1 shows the influence of a_w on the germination time at 25°C and 30°C. The effect of temperature on the germination time was significant except between 0.95 and 0.88 a_w. At low a_w levels as well as above 0.95, the higher temperature caused an increase in the germination time. The minimum a_w for germination was also affected by temperature, being lower at 25°C (0.80 a_w) than at 30°C (0.82 a_w). The same effect of temperature on the germination of *W. sebi* was observed by Wheeler et al. (1988) working with other solutes (NaCl and a glucose and fructose mixture) and at temperatures of 20°C, 30°C and 34°C.

At 30°C, a considerable increment of germination time was observed when a_w increased from 0.95 to 0.96 (2 and 20 days, respectively), demonstrating the xerophilic nature of *W. sebi* whose growth is delayed at high a_w values. This effect is evident at the higher temperature probably because this temperature is less favourable than 25°C for germination of *W. sebi.*

At 25°C, germination was followed by growth over the whole range of a_w studied. At 30°C, growth was only observed after germination at values above 0.86 a_w.

3.2. Growth

The influence of a_w on the average radial growth rate for *W. sebi* at 25°C and 30°C is shown in Fig. 2. At 25°C, the maximum growth rate was observed in the range of 0.94–0.96 a_w and growth became slower as a_w decreased down to 0.84. At 30°C, the maximum growth rate occurred at 0.94 a_w, with a higher radial growth rate than at 25°C. For all other a_w values, measured k_r at 30°C was lower than at 25°C. The effect of temperature on the radial growth rate was marked, but the optimum a_w was not affected. The optimum a_w value for growth of *W. sebi* found in the present work is in agreement with that reported by Pitt and Hocking (1977) at 30°C.

Slower growth of *W. sebi* above 0.95 a_w at 30°C in culture media of a_w adjusted with glucose/fruc-

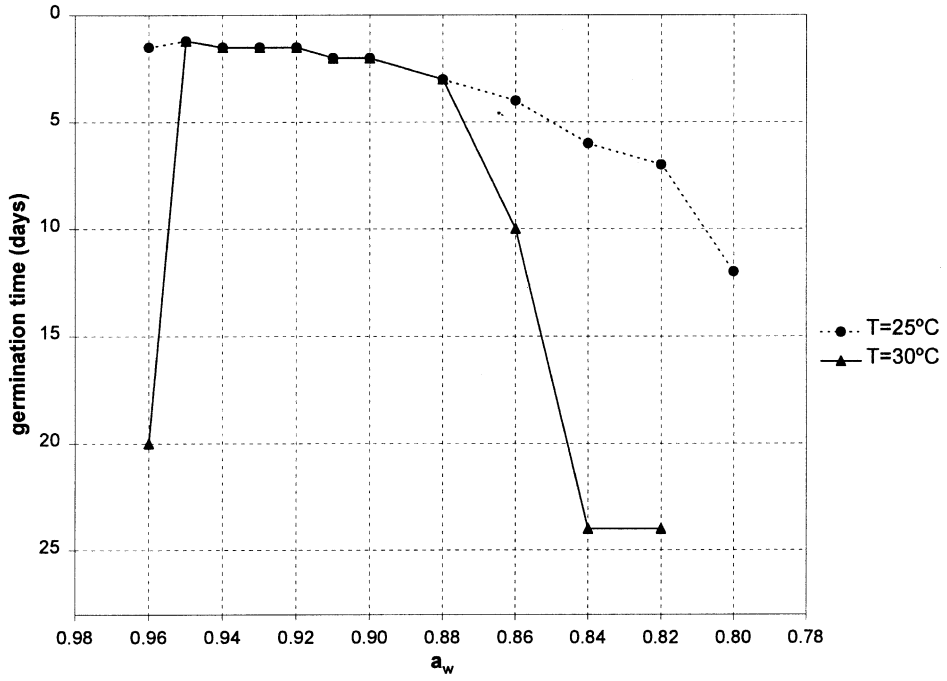

Fig. 1. Effect of a_w and temperature on germination time for *W. sebi*.

tose and NaCl was observed by Wheeler et al. (1988). Beuchat (1992) reported 0.92 a_w as the optimum for growth of heat-stressed *W. sebi* conidia regardless of solute (NaCl, glucose or sorbitol). He

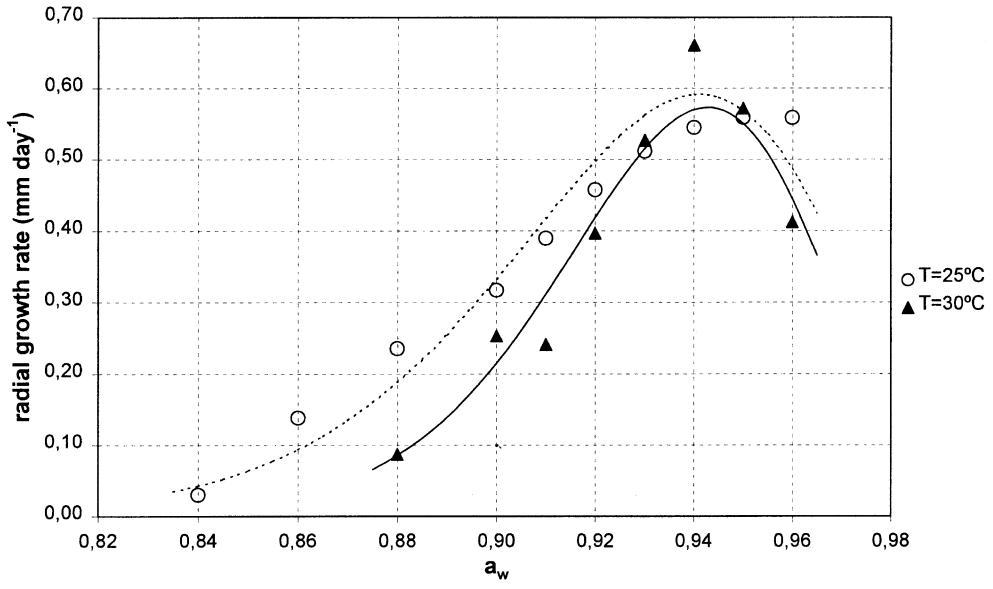

Fig. 2. Effect of a_w and temperature on radial growth rate of *W. sebi*. Symbols represent the growth rates at each a_w. The lines indicate the fitted k_r vs. a_w function, where $k_r = \exp(C_0 + C_1 b_w + C_2 b_w^2)$ and $b_w = \sqrt{1 - a_w}$.

observed, as we did, that the fungus was less tolerant of a_w above the optimum at 30°C.

The minimum a_w for growth was observed to be higher than the minimum for germination at both temperatures examined. At 25°C below 0.84 a_w, growth was very slow and irregular. At 30°C and 0.86 a_w, colonies were not bigger than 0.05 mm after 100 days and at lower a_w values germination was only followed by the production of abortive germinative tubes. Minimum a_w values for germination and growth registered in the present work are slightly higher than those reported by Wheeler et al. (1988). These differences could be due to differences in the strains and solutes used.

The analysis of variance performed confirmed a marked influence of a_w on the growth rate. The effect of temperature, although smaller, was also significant, as well as the interaction that exists between both factors ($p < 0.001$ in all cases).

It was found that 25°C was a more favourable temperature for germination and growth of *W. sebi* than 30°C. Wheeler et al. (1988), working with other solutes, also reported optimal growth of *W. sebi* at 25°C with an upper limit near 30°C and growth inhibition at 34°C.

3.3. Application of the predictive model

Fig. 3 shows the growth rates obtained here for *W. sebi* fitted to the model of Gibson et al. (1994). As can be observed, the model provides a good approach to the description of the effect of water activity on the radial growth rate for *W. sebi*. The coefficients C_i of the fitted equation as well as the a_w(opt) and k_r(opt) values predicted by the model for both temperatures are shown in Table 1.

The results obtained by the application of the model agree with that experimentally observed in relation to the weak influence of temperature on the optimum a_w, i.e. 0.94 a_w for both temperatures. The radial growth rates under these conditions were slightly higher at 25°C (0.59 mm day^{-1} or 25 μm h^{-1}) than at 30°C (0.57 mm day^{-1} or 24 μm h^{-1}). These figures were very similar to those obtained by Wheeler et al. (1988) for *W. sebi* in a medium with a_w adjusted with a mixture of glucose and fructose (18–20 μm h^{-1}).

W. sebi is a very slowly growing mould even at the optimum a_w. For comparison, Pitt and Hocking (1977) reported maximum growth rate between 150 and 180 μm h^{-1} for *A. flavus* and *Eurotium cheva-*

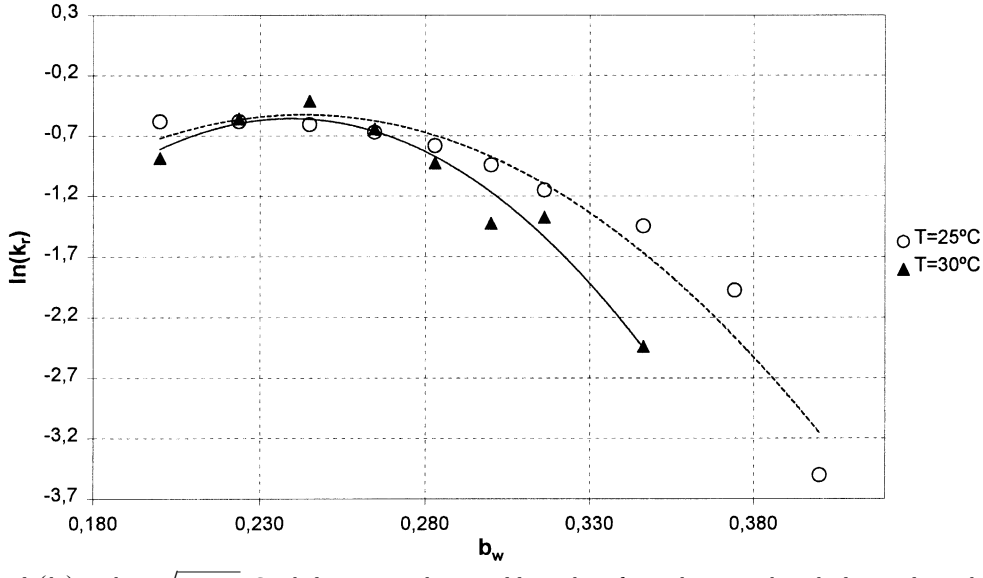

Fig. 3. Plot for $\ln(k_r)$ vs. $b_w = \sqrt{1 - a_w}$. Symbols represent the natural logarithm of growth rates at b_w. The lines indicate the fitted $\ln(k_r)$ vs. b_w function, where $\ln(k_r) = C_0 + C_1 b_w + C_2 b_w^2$.

Table 1
Coefficients and some characteristic predictions of the radial growth rate and t_v models for *W. sebi*

Temperature	Growth rate model					t_v Model				
	Coefficients			Characteristics		Coefficients			Characteristics	
	C_0	C_1	C_2	a_w (opt)[1a]	k_r (opt)[b]	D_0	D_1	D_2	a_w (opt)[2c]	k_r (opt)[d]
25°C	−6.80	51.71	−106.47	0.941	0.591	6.40	−44.97	92.29	0.941	2.521
30°C	−10.01	79.00	−165.12	0.943	0.573	6.30	−47.40	103.24	0.947	2.361

[a] Predicted optimum a_w for the radial growth rate.
[b] Predicted colony growth rate (mm day^{-1}) at optimum a_w.
[c] Predicted a_w for the shortest time to reach a visible colony.
[d] Predicted time (days) to reach a visible colony at a_w(opt)[2].

lieri, while Wheeler et al. (1988) reported optima of about 60 μm h^{-1} for *Xeromyces bisporus* and 40 μm h^{-1} for *Chrysosporum fastidium.*

Another parameter of practical utility is the time required for a fungal inoculum to form a colony of 2 mm of diameter (Horner and Anagnostopoulos, 1973). This is an arbitrary definition and equivalent parameters are commonly found in the predictive microbiology literature, such as the t_3 or time to form colonies of 3 mm of diameter (Gibson et al., 1994). These parameters represent the time necessary for colony formation on the surface of a food large enough to be visible and to cause rejection of the product.

In the present study, we have defined as time of visibility (t_v), the time to form a visible colony starting from the initial inoculum. We chose this parameter that does not imply a fixed size of colony due to the simplicity in its experimental determination. As a consequence of the method used for the

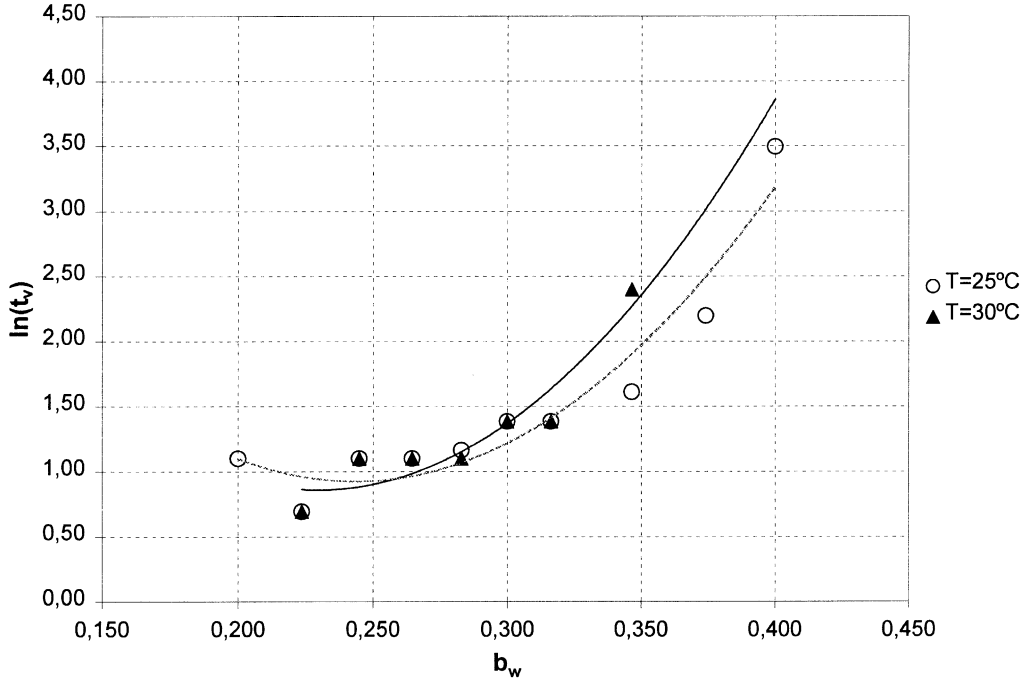

Fig. 4. Plot for $\ln(t_v)$ vs. $b_w = \sqrt{1 - a_w}$. Symbols represent the natural logarithm of t_v at b_w. The lines indicate the fitted $\ln(t_v)$ vs. b_w function where $\ln(t_v) = D_0 + D_1 b_w + D_2 b_w^2$.

Table 2
Predicted time to spoilage (t_v) for foods frequently contaminated by *W. sebi*

Product	a_w	Predicted t_v (days) at 25°C	Predicted t_v (days) at 30°C
Sweet potato jam	0.87[a]	8.9	13.9
Vanilla pudding	0.841 (interior)[b]	23.2	45.4
Peach jam	0.83[a]	34.7	74.2
Dairy caramel	0.84[a]	24.0	47.4
Dairy caramel	0.825[b]	42.0	93.5
Sweet potato syrup	0.888[b]	5.4	7.4

[a] Data obtained from Vigo et al. (1981).
[b] Experimental data.

inoculation, the initial inoculum resulted in a drop of approximately 3–5 mm of diameter, so that when the colony was visible its diameter was usually bigger than 3 mm. By the use of the t_v the arbitrariness introduced by the experimental conditions was minimized.

Gibson et al. (1994) demonstrated the possibility of applying the same model used for the growth rate to the parameter t_3. In the same way, the modelling was repeated with the t_v data, applying again the transformation mentioned above. It was confirmed that such an equation provided a good adjustment of the experimental values (Fig. 4). The coefficients were calculated and the a_w(opt) and t_v(opt) values are shown in Table 1.

The practical utility of t_v modelling is that it provides, through a simple calculation, an estimate of what would happen in a food of a certain a_w when being contaminated by *W. sebi*. Table 2 lists the t_v values predicted at 25°C and 30°C for some of the foods that have been reported as susceptible to spoilage by *W. sebi*. Predicted values could be useful in estimating mould-free life of these foods. Taking into consideration the limitations of the empirical mathematical models, the obtained predictions can be interpreted as a tool to describe the behaviour of *W. sebi* in different foods based on their a_w and the storage temperature. In a later stage the model should be validated in different foods whose a_w is comprehended in the evaluated range.

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