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Fungal growth modelling: the effect of water activity on *Penicillium roqueforti*

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SUMMARY. Growth data of *Penicillium roqueforti* were modelled to describe the effect of water activity on colony growth rate and lag-phase. The model coefficients estimated with linear regression and mathematical transformation of Baranyi et al. (Food Microbiol., *10*, 1993, p. 43-59) were used to predict colony growth rate. They showed that the growth of *P. roqueforti* was significantly dependent on the a_w values.

Final model predicting the time of lag-phase was constructed. It comprised the stable "initial lag-phase" at the a_w values higher than 0.92 and the fraction determined with the a_w as the „environmental factor". At the a_w values higher tnan 0.92, the time needed for the growth of a visible colony was not affected by the a_w. A significant prolongation of the lag-phase was determined at a_w values lower than 0.92.

KEYWORDS: lag-phase, growth rate, mathematical modelling, predictive microbiology

Penicillia play an important role in ripening of mould-fermented food. Species of the genus *Penicillium* are exclusively used in the production of mould-fermented food in Europe: *P. roqueforti* for blue cheeses e.g. Roquefort, Gorgonzola, Stilton, Gammelost; *P. camemberti* for white cheeses e.g. Brie and Camembert; and *P. nalgiovense* and *P. chrysogenum* for meat products (salami-type sausages) [1].

Penicillium species cause food spoilage, too. Penicillia were the most frequent in hard, semi-hard and semi-soft cheeses as associated mycoflora. 91 % of the strains isolated from cheese surface by Lund et al. [2] were penicillia. Similarly, Jesenská [3] identified penicillia as the most common species (92 %) in the surface mycoflora of sausages produced in Slovakia. She has

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also reported on the proportion of *Penicillia* spp. isolated from dried milk powder, where they represented 45 % of almost 44 000 isolated strains. Penicillia are able to attack fruits; as much as 30 % of all fruit decay can be attributed to this genus [4].

Although *P. roqueforti* is able to produce several mycotoxins, such as PR-toxin, eremofortin, roquefortin C, mycophenolic acid, patulin, penicillinic acid, and isofumigaclavins, these toxins are unstable or of low concentration. Moreover, only a limited number of strains that could be considered as GRAS ("generally recognized as safe") is available in cheese production [5].

Predictive food microbiology deals with models that describe the behaviour of microorganisms under different physical, physico-chemical or chemical conditions, such as temperature, water activity, pH, or antimicrobial compounds. These models allow prediction of microbial safety or shelf life of food products, identification of critical points of the production and distribution process, and optimisation of production and distribution chains [6,7].

This study was carried out to apply the modelling methods of Baranyi et al. [8] and Gibson et al. [9] to the growth data of *P. roqueforti*. The objective was to provide quantitative information on the effect of NaCl on mould growth. Only a few studies have been published on the effect of solutes like glycerol on the germination and growth of *P. roqueforti* [10, 11]. The water activity of a food product is commonly reduced by the addition of NaCl. Models resulting from this study would allow a food manufacturer to predict the germination and growth of *P. roqueforti* with respect to water activity in roquefort-type cheeses. The results could also be used to prevent the growth of *P. roqueforti* as a spoilage mould in other food products.

Materials and methods

Strain

Penicillium roqueforti PR3 was used throughout the study. The strain, originally from Hans Christians Lab (Denmark), is used to produce roquefort-type cheese in Slovakia.

Media and cultivation

Sabouraud agar (Fluka, Switzerland) was used as a basal medium. Media of different water activity (a_w) were prepared by adding adequate volumes of a sterile saturated NaCl solution. The actual a_w values were determined by Novasina TH 200 (Novasina, Pfäffikon, Switzerland). The thermocon-

stanter was calibrated against all 6 saturated salt solutions in the range of the a_w from 0.98 to 0.11. The pH of the medium was adjusted to the values of 4, 5, 6, 7 and 8 with hydrochloric acid or NaOH solutions.

The spores were inoculated by a touch of bacteriological loop. The cultivation was carried out on Petri dishes (diameter 170 mm) containing agar with the appropriate a_w and pH. Inoculated parallel Petri dishes were incubated at 25±1 °C in plastic boxes. No change of a_w of the media was detected after 1 and 5 days of the experiment. The colony diameters were measured in two parallel Petri dishes using an eye-piece micrometer in two vertical directions. The average value of four diameters was used in modelling.

Mathematical and statistical methods

The diameter (y, expressed in mm) of each colony was measured in vertical and horizontal directions daily at the same time (t, expressed in days). The growth function of Baranyi et al. [8] was applied. It enabled to model both, the limited and the limitless growth, i.e. curves with or without an upper asymptote.

The maximum colony growth rate (g), and the lag-phase (λ), estimated by the above curve-fitting, were modelled as a function of water activity (a_w) and pH values. A useful transformation of water activity for the modelling purposes was applied, as introduced by Gibson et al. [9]:

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

Using this transformation, the natural logarithm of the colony growth rates of the strain was modelled by a two-variate quadratic function:

$$\ln g = C_0 + C_1 b_w + C_2 b_w^2 + C_3 pH + C_4 pH^2 + C_5 b_w pH \qquad (1)$$

The coefficients C_0, C_1, C_2, C_3, C_4, C_5 were estimated by linear regression.

The question whether the growth model based only on b_w is significantly different from model (1) was analysed by an F-test comparing the respective residual mean squares [12].

The modelling of a lag-phase included the similar experimental lag-phases of *P. roqueforti* that were found up to the a_w of 0.92. That is why the final equation predicting the time of the lag-phase of *P. roqueforti* (1) comprised λ_0, the "initial lag-phase" and the fraction - the „environmental a_w factor" that prolonged the time of colony to be visible at the a_w values lower than 0.92:

$$\sqrt{\lambda} = \lambda_0 + \frac{C_0^2}{(a_w - a_w(\min))^2} \quad (2)$$

C_0, λ_0 and $a_w$min are coefficients to be calculated. Using this equation, it was possible to fit our experimental lag-phase data in a novel way that described their hyperbolic shape in relation to a_w values.

Results and discussion

The growth data modelled in this work include growth curves for *Penicillium roqueforti* PR3 at six a_w values and four pH values. The growth curves were typical of microbial growth with a lag-phase, followed by a linear phase and an upper asymptote.

The coefficients and statistical parameters for the quadratic functions (used in the second step of modelling, see the Table 1) show that the model (2), including b_w as the only variate, explains the variance of the growth rate satisfactorily.

As the Table 1 shows, the calculated F-value is close to 1 and less than $F_{0.001}$ which means that reducing the number of parameters, by disregarding the effect of pH in the range of 5-8, does not significantly change the goodness of fit. Therefore, it is sufficient to consider the colony growth rates of *P. roqueforti* as a function of the a_w values. This unified model is demonstrated in the Figure 1.

On the basis of these experimental data, the growth rate model of *P. roqueforti*, as a function of water activity, is demonstrated in the Figure 2. The optimum a_w value 0.998 for growth, and the respective colony growth rate of 13.4 mm.d^{-1}, were calculated using the procedure of Gibson et al. [9].

TABLE 1. Coefficients and statistical parameters for the growth models of *Penicillium roqueforti* PR3.
TABUĽKA 1. Koeficienty a štatistické parametre pre model rastu *Penicillium roqueforti* PR3.

Model	C_0	C_1	C_2	C_3	C_4	C_5	SS (df)	$F = \frac{SS_2/df_2}{SS_1/df_1}$
(1)	1.173	2.498	-30.510	1.121	-0.040	-0.644	0.577 (18)	
(2)	2.534	2.498	-26.324				0.637 (21)	0.946

df – degree of freedom, SS – sum of square, F – F value (F test).
df – stupeň voľnosti, SS - súčet druhých mocnín v štatistickom súbore, F – F hodnota (F test).

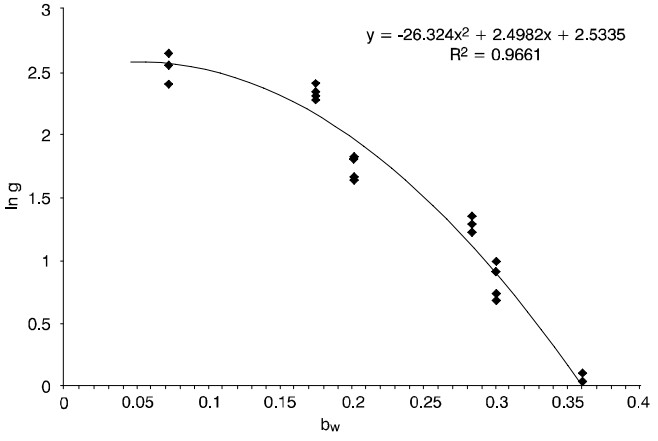

Fig. 1. Plots for the natural logarithm of the colony growth rate (g) against $b_w = \sqrt{1-a_w}$. The continuous line indicates the fitted ln g vs. b_w function where $\ln g = C_0 + C_1 b_w + C_2 b_w^2$. The a_w values were adjusted with NaCl.

Obr. 1. Grafické znázornenie logaritmu rýchlosti rastu kolónií (g) v závislosti od $b_w = \sqrt{1-a_w}$. Súvislá čiara znázorňuje ln g oproti b_w, kde $\ln g = C_0 + C_1 b_w + C_2 b_w^2$. Hodnoty a_v boli upravené prídavkom NaCl.

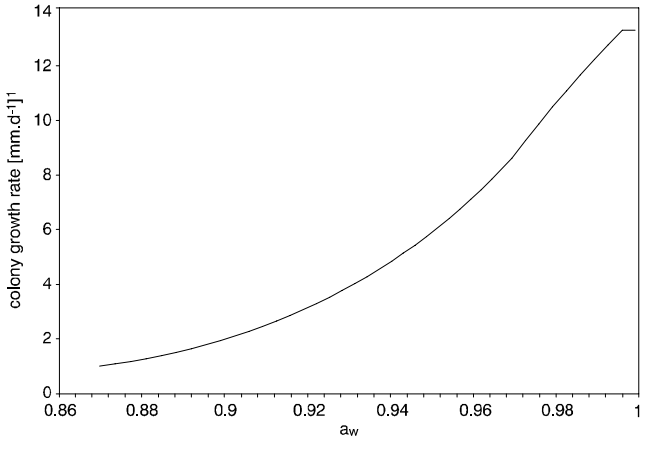

FIG. 2. Plots for the predicted colony growth rate g [mm.d^{-1}] of *Penicillium roqueforti* PR3 against a_w. The a_w values were adjusted with NaCl.

OBR. 2. Grafické znázornenie predpovede rýchlosti rastu g [mm.d^{-1}] *Penicillium roqueforti* PR3 v závislosti od a_v. Hodnoty a_v boli upravené prídavkom NaCl. 1 - rýchlosť rastu kolónií [mm.d^{-1}].

Comparison with published data

The published optimum a_w for *P. roqueforti* at 25 °C, using glycerol and NaCl as the humectants, was 0.97 and 1.0, respectively [13,14]. The predicted value from this model, $a_w$opt 0.998, shows good agreement with the data of Lacey [14] who used the same humectant. No other comparable growth data of *P. roqueforti* on media with NaCl were found in the literature. The growth rates published by Gervais et al. [10], who regulated the a_w value with glycerol or sorbitol were similar to those obtained at a_w 0.99. At lower a_w values, our predicted and experimental rates were different from those observed by Gervais et al. [10] because the growth as well as the osmoregulatory mechanism are influenced by the type of the humectants used [15,16].

Modelling the lag-phase

The modelling of the lag-phase was based on equation (2). The model for lag-phase prolongation (represented here by the a_w fraction in the Eq. 2) describes the growth of mycelium until reaching a measurable colony (Fig. 3). The less is the difference between the a_w of the environment and minimum a_w for mould growth, the more time is needed to fill the surface of medium by visible mycelium.

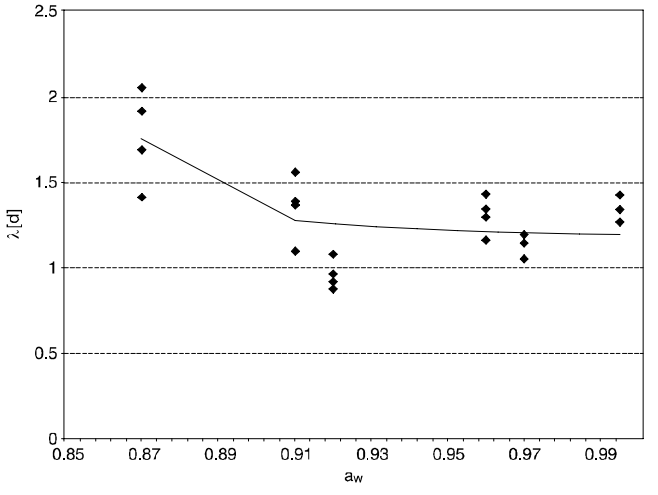

FIG. 3. Lag-phase of *Penicillium roqueforti* as a function of water activity within pH values 5 to 8. The a_w values were adjusted with NaCl.

OBR. 3. Lag fáza *Penicillium roqueforti* PR3 ako funkcia aktivity vody v intervale pH 5-8. Hodnoty a_v boli upravené prídavkom NaCl.

The minimum a_w value for growth ($a_w$min) was calculated by the least square method. Thus the predicted $a_w$min value was 0.86.

The minimum a_w value was also determined in a simple experiment. The experimental $a_w$min = 0.84, which allowed *P. roqueforti* strain PR3 to germinate and grow on bread crusts at 25 °C, was less than 0.86 and more than 0.83 found previously by Gervais et al. [10] and Magnan a Lacey [17], respectively. Using the equation for the lag-phase (2) we fixed the $a_w$min as the experimental $a_w$min value, 0.84. Thus, the lag-phase of *P. roqueforti* can be modelled with this final equation:

$$\sqrt{\lambda} = 1.63 + \frac{0.023^2}{(a_w - 0.84)^2}$$

The Mean Square Error (MSE) of the differences between the observed and predicted data by this model was 0.039 which was very close to the MSE = 0.037 calculated using the mathematically predicted $a_w$min = 0.86. The close similarity of the MSEs indicates reliability of the model when applied to foods. Use of the experimental $a_w$min 0.84 resulted from the independent trials in our model as well as from the conformity of $a_w$min = 0.86 calculated from the model with the data from literature support this claim.

Predictive mathematical models must be validated to demonstrate their applicability to different strains or foods. As we could not find studies on the effect of NaCl-controlled regulated water activity on germination and growth of *P. roqueforti*, we applied the model presented above on the lag-phase of *P. roqueforti* observed by Gervais et al. [10]. The observed lag-phases were independent on the a_w values in the range of 0.99 to 0.95. (The a_w values of the media were adjusted with glycerol.) The comparison of the lag-phase predicted by the Eq. 2 and the lag-phase in the whole range of a_w 0.99-0.88 is demonstrated in the Fig. 4. The Mean Square Error of the differences between the observed and predicted data was 0.212. Excluding the data below a_w 0.92, MSE = 0.150. Both, data in Fig. 4 and calculated MSE, confirmed the fact that our model is useful for fitting of the lag-phase of *P. roqueforti* on media with the a_w values controlled with NaCl as well as with glycerol. On the other hand, the decrease of this MSE value confirmed the fact, that prolongation of the lag-phase does not depend on a_w in the range of its higher values.

The radial colony growth rate was fitted by the function of Baranyi et al. [8], then the procedures of Gibson et al. [9] were used in this work. It was shown that the growth rates of *P. roqueforti* were not dependent

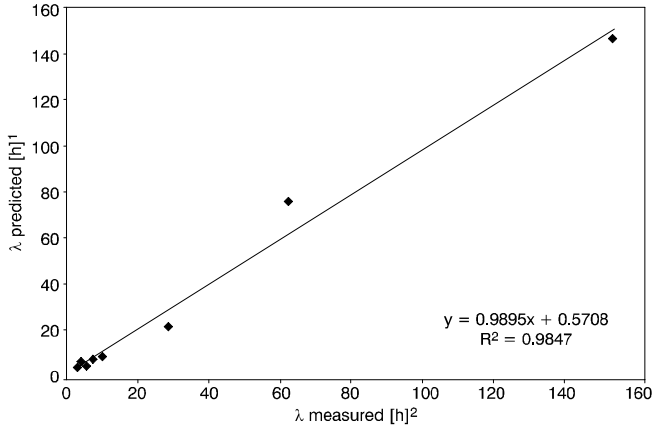

FIG. 4. Plots of the predicted data for the lag-phase of *P. roqueforti* by the model represented with Eq. 2 vs. observed data by Gervais et al. [10]. The a_w values were adjusted with glycerol as a humectant.

OBR. 4. Grafické porovnanie hodnôt získaných na základe predpovede lag fázy *P. roqueforti* naším modelom reprezentovaným v tejto práci rovnicou 2 s hodnotami získanými autormi Gervais a kol. [10].
Hodnoty a_v boli upravené glycerolom. 1 - predpovedaná lag fáza [h], 2 - nameraná lag fáza [h].

on the pH values in the range of 5-8. On the other hand, the effect of the water activity, as one of the most important intrinsic factors of food environment, was significant in the whole range of growth.

From a practical view, the finding that the lag-phase is relatively stable in the range of a_w 0.92-1, can be an advantage for the application of *P. roqueforti* as a starter culture in ripening of blue-veined cheeses. The final a_w values of roquefort-type cheeses that are in the range of 0.91-0.94 [18,19], allow *P. roqueforti* to germinate as quickly as possible and grow during the whole phase of cheese processing including ripening.

From the point of food spoilage prediction, it can be summarized that the time to identify a visible growth of *P. roqueforti* can be significantly prolonged at the a_w values lower than 0.92.

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Modelovanie rastu vláknitých húb: vplyv aktivity vody na *Penicillium roqueforti*

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SÚHRN. V práci sa modelovali parametre rastu *Penicillium roqueforti* vzhľadom na vplyv aktivity vody. Koeficienty použitých modelov sa určili lineárnou regresnou analýzou, v rámci ktorej sa využila matematická transformácia Baranyiho a kol. (Food Microbiol., *10*, 1993, s. 43-59), a ďalej sa použili na predikciu rastovej rýchlosti kolónií. Výsledky ukázali, že rastová rýchlosť *P. roqueforti* bola významne závislá na hodnote a_v.

Prínosom v práci bol model predpovedajúci trvanie lag fázy. Tento model sa skladal z konštantnej lag fázy pri hodnotách a_v vyšších ako 0,92 a frakcie určovanej aktivitou vody ako faktorom prostredia. Pri hodnotách a_v vyšších ako 0,92 sa čas na vytvorenie viditeľnej kolónie nepredlžoval v závislosti od aktivity vody. Významné predĺženie lag fázy *P. roqueforti* sa zistilo v oblasti hodnôt a_v nižších ako 0,92.

KĽÚČOVÉ SLOVÁ: lag fáza, rýchlosť rastu, matematické modelovanie, prediktívna mikrobiológia