Evaluation of *Rhodotorula* Growth on Solid Substrate *via* a Linear Mixed Effects Model

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Abstract

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The growth of *Rhodotorula glutinis* and *Rhodotorula mucilaginosa* was studied under optimal and stress cultivation conditions at 10°C and 20°C for 14 days. The method of image analysis was used to determine the size of colonies. The linear mixed effects model implemented in the statistical program S-PLUS was applied to analyse the repeated measurements. Two-phase kinetics was confirmed and the mean growth rates in the second linear phase under various stress conditions were estimated. The results indicated a higher growth rate of *R. mucilaginosa* than was that of *R. glutinis* under all cultivation conditions. The highest growth rate of was observed during the cultivation of *R. mucilaginosa* in media with 2% of NaCl at 20°C. The impact of neglecting the fact that repeated data are not independent and using the classical regression model instead of the mixed effects model was demonstrated through the comparison of the confidence intervals for the parameters based on both approaches. While the point estimates of the corresponding parameters were similar, the width of the confidence intervals differed substantially.

Keywords: confidence intervals; giant colony; growth curves; image analysis; solid substrate cultivation

Rhodotorula belongs to a genus of imperfect yeasts, is well known as a producer of carotenoid pigments, and has both positive and negative significance in agriculture and food industry (Yeeh 2000). Its ability to suppress the growth and patulin production of *Penicillium expansum* makes it a potential good biocontrol agent usable for the reduction of postharvest decay of apples (Castoria *et al.* 2005) or pears (Zhang *et al.* 2008). This *Rhodotorula* use could increase the safety of fruit products like juices. At the same time, *Rhodotorula* belongs to food contaminants (cheese, fruit, fruit juices and meat products) (Viljoen & Greyling 1995; Yeeh 2000; Restuccia *et al.* 2006). *Rhodotorula* was chosen for our experiments for its advantageous features: pseudomycelium is formed occasionally; colonies are smooth with clearly defined boundaries (Yeeh 2000). Until now, the main interest in the literature has been concentrated on *Saccharomyces cerevisiae*; there is still a lack of information about the behaviour of *Rhodotorula*.

Solid-substrate cultivation (SSC) is defined as any fermentation process performed on a non-soluble material in the absence of free-flowing water. In comparison with submerged liquid cultivation, it has several advantages, e.g.: higher yields, environmental conditions similar to natural ones, a simple design of reactors. The main disadvantage of the process is the lack of information about the overall growth kinetics (Mitchel & Lonsane 1992; Peréz-Guerra *et al.* 2003)

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The growth of giant colonies of yeast on agar plates may be regarded as the simplest example of solid substrate cultivation (Mitchel 1992b).

Several models of the growth of yeasts or fungi on solid substrates have been presented in the literature (Pirt 1967). These models are based on the measurement of biomass or colony diameter (Valik & Pieckova 2001; Hamidi-Esfahani *et al.* 2004; Marín *et al.* 2007). Direct biomass determination in most SSC is difficult due to the problems connected with the separation of the organism from the substrate. Even indirect methods of biomass estimation (e.g. monitoring of metabolic activities) cannot be always applied (Mitchel 1992a; Araya *et al.* 2007). The colony diameter is usually measured several times in horizontal and vertical directions which is very inaccurate and time consuming. The modern method of image analysis can be used instead (Vecht-Lifshitz & Ison 1992; Thomas & Paul 1996; Couri *et al.* 2006).

A common way of analysis consists in modelling averages over different specimens exposed to the same experimental conditions. Because the true covariance structure of the data due to repeated observations of the specimens made over time is not taken into account, the inference is not quite correct. The mixed effects model can be used for the data where the observations of the same item are correlated and a higher correlation can be expected between adjacent observations than between those more distant in time. A detailed description of the mixed effects models (both linear and nonlinear) can be found in Pinheiro and Bates (2000). The mixed effects model was used e.g. by Miguez *et al.* (2008) for the analysis of the growth curves of biomass crops or by Shorten *et al.* (2004) as a tool for variance component analysis in a microbial problem.

The aim of our study was to evaluate the growth of *Rhodotorula* during SSC under various cultivation conditions. The data were obtained by the image analysis and the linear mixed effects model was used for the analysis. To the best of our knowledge, *Rhodotorula* growth in SSC process has never been studied.

MATERIAL AND METHODS

Yeasts. *Rhodotorula mucilaginosa* (DBM 19) was obtained from DBM-Culture Collection of the Department of Biochemistry and Microbiology, Institute of Chemical Technology in Prague, Czech Republic. *Rhodotorula glutinis* (CCY 20-2-20) was obtained from CCY-Culture Collection of Yeasts, Slovak Academy of Sciences, Bratislava.

Inoculum preparation. The following liquid medium was used for the yeast growth: glucose 25 g/l, yeast extract 10 g/l, K_2HPO_4 2 g/l, KH_2PO_4 2 g/l, $MgSO_4 \cdot 7H_2O$ 0.1 g/l; pH was adjusted to 6.0. The liquid media in Erlenmeyer flasks were inoculated with the yeast grown on agar slant. Shaken flasks (300 rpm) were incubated at 28°C for two days.

Giant colony cultivation. A drop of the cell suspension was laid on Sabouraud's agar (14 ml) in the middle of a Petri dish (Ø = 60 mm). Agar plates were inoculated with suspensions of young cells, which contained about 10^7–10^9 cells/ml. The plates were cultivated at 10°C or 20°C for approximately 14 days. NaCl (0%, 10%, and 2%) was used as exogenous osmotic stress factor. Low concentrations of the stress factor (NaCl, 1% or 2%) were chosen to ensure agar solidification. Two temperatures were chosen according to Bhosale and Gadre (2002) who described significant differences in total carotene pigment concentration during cultivation at 10°C and 20°C. Six specimens under the same treatment conditions were observed at one-day intervals.

Measurement of giant colony size. The colony growth was measured by the method of image analysis. The photos of the colonies were taken by the digital camera FinePix S7000 (Fujifilm, Japan). The camera was fixed on a Kaiser RS1 (support with Kaiser RB 5000 DL (Germany) illuminating system. Image processing procedures were done in software Lucia (Laboratory Imaging Ltd., Prague, Czech Republic). A simple macro was created for photo evaluation.

An appropriate threshold was used for the object separation from the background. Afterwards, mathematical operations such as clean, close and fill-holes were applied to produce the final binary image. The area of the colony and other parameters were measured. In one of the treatments, the consistency of the automatically determined results (macro) was checked by manual measurement. A high degree of correlation (0.999, sample size 56, $P < 0.0001$) was confirmed.

Software. Sigma Stat software (version 3.1) and S-PLUS (version 6.2) were used for statistical analysis. All graphs were created using Sigma Plot software (version 10.0).

Growth model. The equivalent colony diameter, i.e. the diameter of a circle having the same area as the colony was chosen as a response variable. In matrix notation, the linear mixed effects model is written as

$$\mathbf{y} = \mathbf{X\beta} + \mathbf{Zb} + \mathbf{e} \quad (1)$$

where:
y – vector of responses
X – known design matrix linking **β** to **y**
β – vector of unknown parameters (fixed effects)
Z – design matrix linking **b** to **y**
b – vector of unknown random effects
e – vector of random errors.

Assuming $\mathbf{b} \sim N(\mathbf{0},\mathbf{D})$, $\mathbf{e} \sim N(\mathbf{0},\mathbf{R})$, and **b** and **e** being independent, the mean profile is given by $E(\mathbf{y}) = \mathbf{X\beta}$ and the covariance structure depends on the matrices **D** and **R**, namely $\text{var}(\mathbf{y}) = \mathbf{ZDZ}^T + \mathbf{R}$.

With time taken as continuous and on the assumption that our measurements record the period of the linear growth (Pirt 1967) where the starting time point is t_0, the colony diameter of the i-th specimen at the j-th level of temperature, the k-th level of NaCl concentration, and time t_l is expressed in the form:

$$y_{ijkl} = \beta_{0,jk} + b_{0,i}) + (\beta_{1,jk} + b_{1,i})(t_l - t_0) + e_{ijkl} \quad (2)$$

(i = 1, 2, ..., 36; j = 1, 2; k = 1, 2, 3; l = 1, 2, ..., 288 in our study)

where:
$\beta_{0,jk}$ – corresponds to the mean diameter at $t = t_0$
$\beta_{1,jk}$ – mean growth rate (in cm per day) in the "linear" period
$b_{0,i}$ – amount by which the "intercept" of the i-th straight line differs from $\beta_{0,jk}$
$b_{1,i}$ – difference between the slope of the i-th straight line and $\beta_{1,jk}$

The random variables b_0 and b_1 are called random effects and their variations across different specimens are described by parameters σ_0^2 and σ_1^2, respectively. These parameters lie on the main diagonal of **D** (2 × 2). In a special case of uncorrelated b_0 and b_1, **D** is a diagonal matrix. A more general covariance model with heterogeneous variation of random effects across different experimental conditions may be considered; in that case, **D** will have a larger dimension. Random errors e_{ijkl} denote the departures of observations from the model. In the case of independence and homoscedasticity, their covariance matrix would be $\mathbf{R} = \sigma^2\mathbf{I}$. When the growth curves are analysed, often the autoregressive scheme AR(1) is considered (the model $e_t = \Phi\, e_{t-1} + \nu_t$, where Φ is an autocorrelation coefficient and random component ν_t has the properties of the white noise, see e.g. Pinheiro & Bates 2000). This special form of **R** is described by two parameters; σ denotes standard deviation of the error component ν_t, Φ is the autocorrelation coefficient.

It follows that the linear mixed effects model has both the mean structure and the covariance structure. The mean profile representing the growth under the specific treatment conditions has the form:

$$E(y) = \beta_{0,jk} + \beta_{1,jk}\,(t - t_0) \quad (3)$$

The indices j and k stand for the j-th level of temperature and the k-th level of NaCl concentration. Both parameters are supposed to be affected by the treatment (note that $\beta_{0,jk}$ does not represent the intercept at $t = 0$ but $t_0 = 5$). In fact, $\beta_{0,jk}$ and $\beta_{1,jk}$ are the sums of several fixed-effects parameters depending on which fixed effects are included in the model. The covariance structure is described by matrices **D** and **R**.

The fixed-effects parameters and covariance parameters of the selected structures are usually estimated by the maximum likelihood method or by the restricted maximum likelihood method, see e.g. Pinheiro and Bates (2000). Here the latter was used. During the model building, conditional t-tests are applied to test the significance of the fixed-effects parameters, and the information criteria AIC and BIC (see e.g. Pinheiro & Bates 2000) are used to find appropriate forms of **D** and **R**. Besides, the checking of confidence intervals for covariance parameters in **D** and **R** is recommended by Pinheiro and Bates (20006).

Approximate confidence intervals for $\beta_{0,jk}$ are given by

$$\hat{\beta}_{0,jk} \pm t_{1-\alpha/2,\nu}\, \hat{\sigma}\,(\hat{\beta}_{0,jk}) \quad (4)$$

where:
$\hat{\beta}_{0,jk}$ – denotes the estimates of $\beta_{0,jk}$
$t_{1-\alpha/2,\nu}$ – 1–α/2 quantile of the i-distribution with ν degrees of freedom
$\hat{\sigma}(\hat{\beta}_{0,jk})$ – denotes the estimate of the standard error of $\hat{\beta}_{0,jk}$

Similarly for the confidence intervals for $\beta_{0,jk}$.

Remark: The terms *conditional t*-tests and *approximate* confidence intervals are used in Pinheiro and Bates (2000) to suggest that the tests and intervals are based on estimates of unknown covariance parameters instead of on their true values.

Table 1. Estimated covariance parameters for *R.* glutinis (procedure lme, S-PLUS)

Matrix **D**	Matrix **R**	
$\hat{\sigma}_0$	$\hat{\sigma}$	$\hat{\Phi}$
0.0477	0.0182	0.6986

$\hat{\sigma}_0$ – standard deviation of b_0; $\hat{\sigma}$ – error standard deviation; $\hat{\Phi}$ – autocorrelation coefficient

RESULTS

The camera and illuminating systems used in the experiment did not enable us to monitor the initial stages of the colony growth due to a low contrast between the colony and the background (agar plates). The first results were obtained after five days. From then on, most growth curves exhibited linear dependence of equivalent diameter on time (Figure 1).

The data for the two *Rhodotorula* species were analysed separately. The observations on specimens No. 19 (Figure 1e) and No. 31 (Figure 1f) of *R. glutinis*, the growth curves of which differed strikingly from those obtained under the same treatment, were excluded from the analysis.

Rhodotorula glutinis

Parallel lines under the same treatment conditions suggested that only the random effects b_0, representing the variation around $\beta_{0,jk}$, were needed in the model and D was reduced to scalar σ_0^2. The matrix **R** corresponded to the autoregressive scheme AR(1). The estimates of the corresponding parameters are shown in Table 1.

The variation of random effects b_0 described by σ_0^2 might have been caused either by different initial conditions of the individual specimens or by random variation of the growth rates in the initial period. Owing to the fact that the growth rates of all specimens under the same treatment conditions in the following period were practically the same, the former cause seemed to be more probable.

The estimates of the fixed-effects parameters are displayed in Table 2. Both the main effects and interaction effects were included in the model. The parameters were estimated by the procedure *lme* in S-PLUS.

Using these estimates and their approximate covariance matrix provided by *lme* in S-PLUS (not displayed), the estimates of $\beta_{0,jk}$ and $\beta_{1,jk}$ including

Table 2. Estimated fixed-effects parameters for *R. glutinis* (the output of lme, S-PLUS)

	Value	Std. Error	DF	*t*-value	*P*-value
(Intercept)	0.7620732	0.02057925	232	37.03115	< 0.0001
c2	–0.1011466	0.02910345	28	–3.47542	0.0017
c3	0.0086074	0.02910345	28	0.29575	0.7696
T2	0.1052754	0.02910345	28	3.61728	0.0012
c2:T2	0.1856616	0.04217490	28	4.40218	0.0001
c3:T2	0.1327114	0.04217490	28	3.14669	0.0039
time	0.0280388	0.00103676	232	27.04448	< 0.0001
c2:time	0.0114039	0.00146621	232	7.77785	< 0.0001
c3:time	0.0222477	0.00146621	232	15.17368	< 0.0001
T2:time	0.0273091	0.00146621	232	18.62570	< 0.0001
c2:T2:time	–0.0070224	0.00212473	232	–3.30506	0.0011
c3:T2:time	–0.0201103	0.00212473	232	–9.46485	< 0.0001

c2 (c3) – 2nd (3rd) level of NaCl concentration; T2 – 2nd level of temperature, time is a continuous variable
The estimated fixed-effects parameters in the 2nd column are used to compute $\hat{\beta}_{0,jk}$ and $\hat{\beta}_{1,jk}$ in Table 3. The value at Intercept corresponds to $\hat{\beta}_{0,11}$ at 10°C and 0% NaCl, the value at c2 (c3, T2) is added to obtain $\hat{\beta}_{0,12}$ ($\hat{\beta}_{0,13}$, $\hat{\beta}_{0,21}$), the value at c2:T2 (c3 T2) is added together with those at c2 (c3) and T2 to obtain $\beta_{0,22}$ ($\beta_{0,23}$). The value at time corresponds to $\hat{\beta}_{1,11}$, i.e. to the growth rate at 10°C and 0% NaCl, the other values of $\hat{\beta}_{1,jk}$ are obtained in a similar way as $\hat{\beta}_{0,jk}$

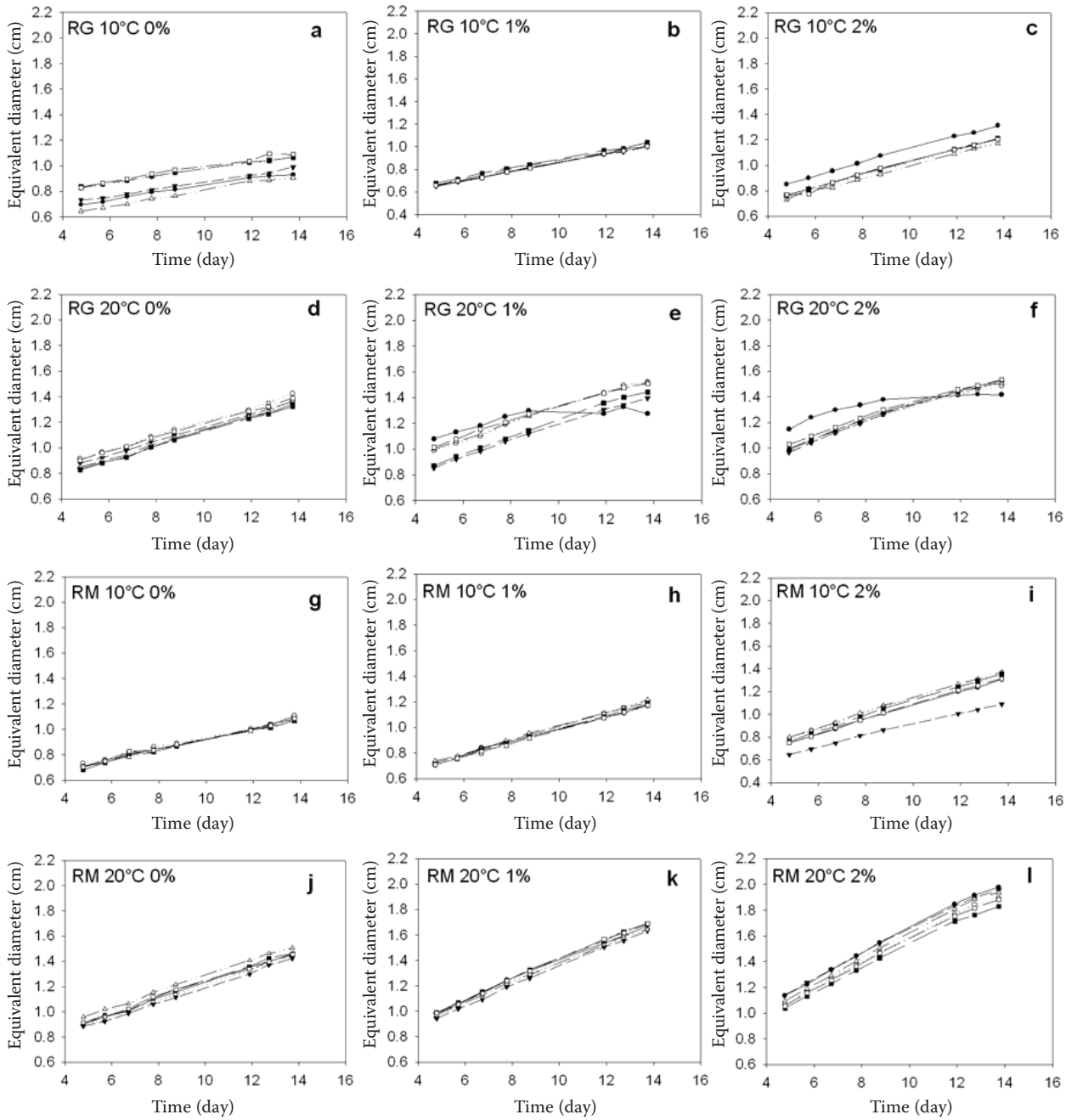

Figure 1. Growth curves of *Rhodotorula* giant colonies. *Rhodotorula glutinis* (RG) and *Rhodotorula mucilaginosa* (RM) were cultivated on Sabouraud agar plates at 10°C or 20°C for 14 days. Different NaCl concentrations (0%, 1%, and 2%) were added to the media as a stress factor. First results were observed after 5 days

conventional confidence limits were computed (Table 3) and displayed (Figure 2).

In general, the temperature and concentration affect both the mean diameter $\beta_{0,jk}$ at t_0 and the mean growth rate $\beta_{1,jk}$. The significance of the individual terms can be checked through *P*-values. It can be said that the temperature had a positive effect on $\beta_{0,jk}$ and $\beta_{1,jk}$. Due to the fact that there were three levels of concentration in the experiment, we could observe a nonlinear effect of concentration on $\hat{\beta}_{0,jk}$ in the range 0–2%. The impact of the interaction of the temperature and concentration can be seen best in Figure 2 where the interval midpoints correspond to $\hat{\beta}_{0,jk}$ or $\hat{\beta}_{1,jk}$.

Table 3. Estimated parameters of the straight lines with 95% confidence limits for *R. glutinis*

Experimental conditions	T (°C)	c (%)	$\hat{\beta}_1$	95% lcl	95% ucl	$\hat{\beta}_1$	95% lcl	95% ucl
I	10	0	0.7621	0.7215	0.8026	0.0280	0.0260	0.0301
II	10	1	0.6609	0.6204	0.7015	0.0394	0.0374	0.0415
III	10	2	0.7707	0.7301	0.8112	0.0503	0.0482	0.0523
IV	20	0	0.8673	0.8268	0.9079	0.0553	0.0533	0.0574
V	20	1	1.0530	1.0086	1.0974	0.0483	0.0461	0.0506
VI	20	2	1.0001	0.9556	1.0445	0.0352	0.0330	0.0375

$\hat{\beta}_0$ – mean diameter after 5 days; $\hat{\beta}_1$ – mean growth rate during the linear growth period

Considering the same initial mean diameter regardless of the treatment as a reasonable assumption, the differences between the estimates $\hat{\beta}_{0,jk}$ reflected different mean rates under various treatment conditions in the previous growth period.

Rhodotorula mucilaginosa

Although the growth lines at 20°C and 2% NaCl indicated a slight variation of slopes (Figure 1), only the random effects b_0 were included in the model based on the information criteria (see Model validation). The differences between σ_0 (standard deviations of random effects b_0) under different treatment conditions were apparent. The effects of both the temperature and concentration are distinguishable in Figure 1, however, based on the information criteria, the form $\mathbf{D} = \text{diag}\{\sigma^2_{0,1}, \sigma^2_{0,2}, \sigma^2_{0,3}\}$ was chosen, where the three parameters corresponded to the three levels of NaCl concentration. The estimates of the standard deviations supplemented by the estimates of the matrix **R** parameters are given in Table 4. The estimates of the fixed-effects parameters are given in Table 5, the estimates of $\beta_{0,jk}$ and $\beta_{1,jk}$ including conventional confidence limits are displayed in Table 6.

Due to the positive signs of all estimated effects, the results were more straightforward than with *R. glutinis*. Both the temperature and concentration had a positive effect on $\beta_{0,jk}$ and $\beta_{1,jk}$ (though insignificant in the case of concentration and $\beta_{0,jk}$) and the nonlinear effect of concentration almost did not exhibit. Again, the interaction of temperature and concentration affected both $\beta_{0,jk}$ and $\beta_{1,jk}$ significantly. The impact of the interaction can be detected in Figure 3.

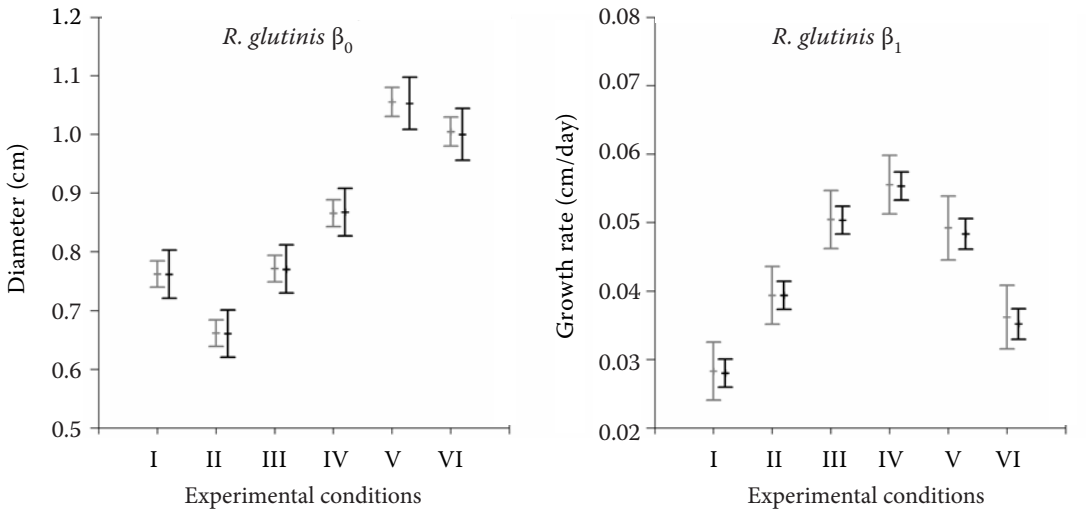

Figure 2. Confidence intervals for β_0 and β_1 based on LME (black bar) and LM (grey bar) under different experimental conditions, *Rhodotorula glutinis*

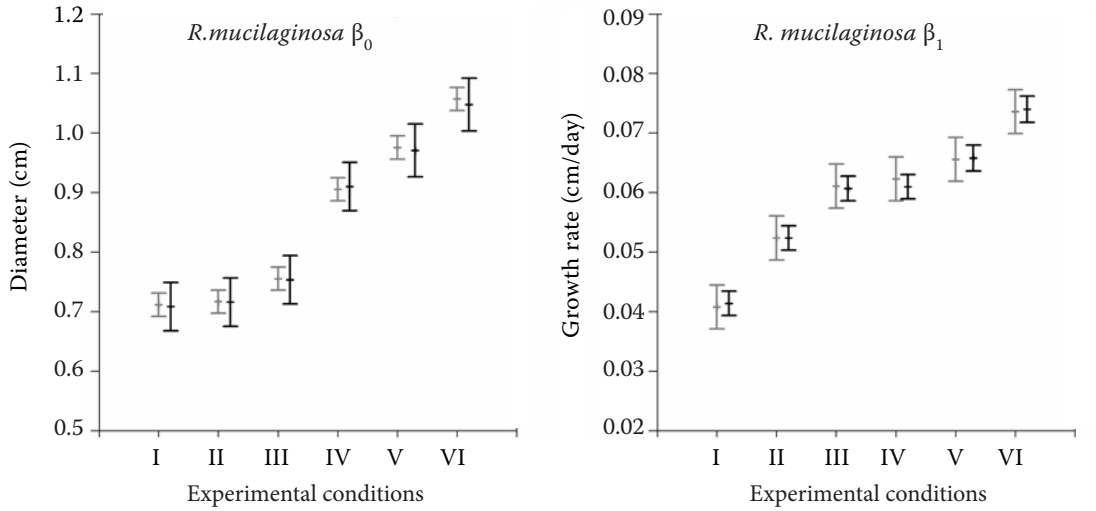

Figure 3. Confidence intervals for β_0 and β_1 based on LME (black bar) and LM (grey bar) under different experimental conditions, *Rhodotorula mucilaginosa*

Model validation

The lack-of-fit test was performed to check the mean structure model as a linear function of time. Although *P*-values were only approximate in this case of the dependent data, the values of 0.999978 and 0.996675 for *Rhodotorula glutinis* and *Rhodotorula mucilaginosa*, respectively, clearly indicated the fitness of the linear relationship.

Various forms of **D** (2 × 2 diagonal, 2 × 2 general, and various kinds of structures with heterogeneous variances) and two forms of **R**, i.e. AR(1) scheme or independent errors were examined. Only covariance models with diagonal **D** are involved in Tables 7 and 8 because either the restricted maximum likelihood method failed due to a large number of parameters or an infinitive confidence interval for off-diagonal parameters indicated a redundancy of the parameters. As for *R. glutinis* (Table 7), AIC indicated RG1 and BIC indicated RG4 as the best model (the lower the values of AIC and BIC, the better). The criterion BIC penalising models with a greater number of parameters was preferred. Another reason for the choice of RG4, the third best by AIC, was the fact that σ_0 did not appear to be dependent either on the temperature or on NaCl concentration. In the case of *R. mucilaginosa*, both criteria indicated RM3 as the best (Table 8)

Table 4. Estimated covariance parameters for *R. mucilaginosa* (procedure lme, S-PLUS

Matrix D				Matrix R	
c (%)	0	1	2	$\hat{\sigma}$	$\hat{\Phi}$
$\hat{\sigma}$	0.0126	0.0013	0.0629	0.0234	0.7998

$\hat{\sigma}_0$ – standard deviation of $b_0$dependent on NaCl concentration; $\hat{\sigma}$ – error standard deviation; $\hat{\Phi}$ – autocorrelation coefficient

Q-Q plots (Figure 4) are nearly straight, indicating no serious evidence against the assumption of normality. Plots in Figure 5 show a very good agreement between the observed data and the fitted model including random effects and may serve as a further confirmation of the adequacy of both the mean and the covariance structure models.

Besides the mixed effects model, the classical model was applied so that we could demonstrate the differences between the lengths of the confidence intervals obtained by the two approaches. All intervals are displayed in Figures 2 and 3. It is obvious that the corresponding estimates obtained by the two approaches did not differ essentially. But the differences in the lengths are striking, though expected. The length of the interval for $\beta_{0,jk}$ based on the classical model is roughly half the length of the interval based on the mixed effects model, the opposite is true in the case of $\beta_{1,jk}$.

DISCUSSION

Although it was impossible to measure the area at the beginning of the process in our experiment, the data analysis indicated a two-phase kinetic profile. This was based on the fact that the intercepts of

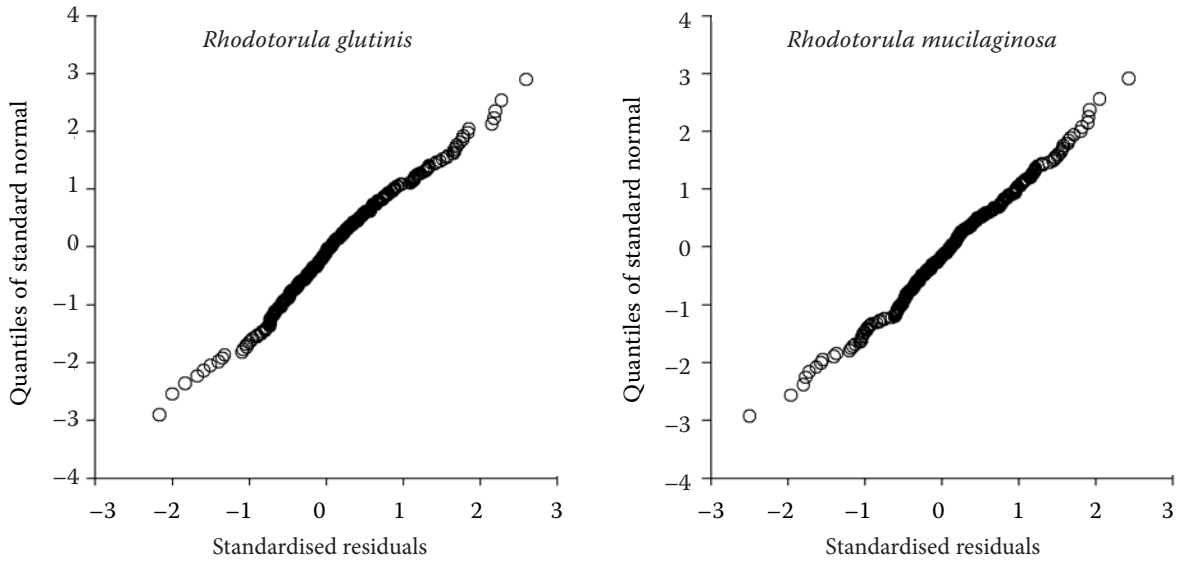

Figure 4. Q-Q plot to check on the assumption of normality (Standardised residuals correspond to the model including random effects)

the growth lines obtained by extrapolation for $t = 0$ differed across the experimental treatments which contradicted the reasonable assumption that only random variation of the specimen sizes was possible at $t = 0$ because different experimental conditions could not yet manifest themselves. It followed that the growth rate in the first phase before t_0 must have differed from the constant rate observed past $t = t_0$. This finding was not unexpected (Mitchel 1992b; Valik & Pieckova 2001).

Based on the estimates $\hat{\beta}_{0,jk}$ reflecting the mean growth rate in the unobserved initial phase and

Table 5. Estimated fixed-effects parameters for *R. mucilaginosa* (*lme*, S-PLUS)

	Value	Std. Error	DF	*t*-value	*P*-value
(Intercept)	0.7087315	0.01033568	246	68.57136	< 0.0001
c2	0.0074252	0.01369474	30	0.54219	0.5917
c3	0.0449595	0.02910547	30	1.54471	0.1329
T2	0.2015940	0.01461686	30	13.79189	< 0.0001
c2:T2	0.0604287	0.01936729	30	3.12014	0.0040
c3:T2	0.1372389	0.04116136	30	3.33417	0.0023
time	0.0414132	0.00123243	246	33.60285	< 0.0001
c2:time	0.0109370	0.00174292	246	6.27512	< 0.0001
c3:time	0.0192538	0.00174292	246	11.04683	< 0.0001
T2:time	0.0196192	0.00174292	246	11.25652	< 0.0001
c2:T2:time	0.0047745	0.00246486	246	1.93703	0.0539
c3:T2:time	0.0129767	0.00246486	246	5.26467	< 0.0001

c2 (c3) – 2nd (3rd) level of NaCl concentration; T2 – 2nd level of temperature, time is a continuous variable, time is a continuous variable

The estimated fixed-effects parameters in the 2nd column are used to compute $\hat{\beta}_{0,jk}$ and $\hat{\beta}_{1,jk}$ in Table 6. The value at Intercept corresponds to $\hat{\beta}_{0,11}$ at 10°C and 0% NaCl, the value at c2 (c3, T2) is added to obtain $\hat{\beta}_{0,12}$ ($\hat{\beta}_{0,13}$, $\hat{\beta}_{0,21}$), the value at c2:T2 (c3:T2) is a1dded together with those at c2 (c3) and T2 to obtain $\beta_{0,22}$ ($\beta_{0,23}$). The value at time corresponds to $\hat{\beta}_{0,11}$, i.e. to the growth rate at 10°C and 0% NaCl, the other values of $\hat{\beta}_{1,jk}$ are obtained in a similar way as $\hat{\beta}_{0,jk}$

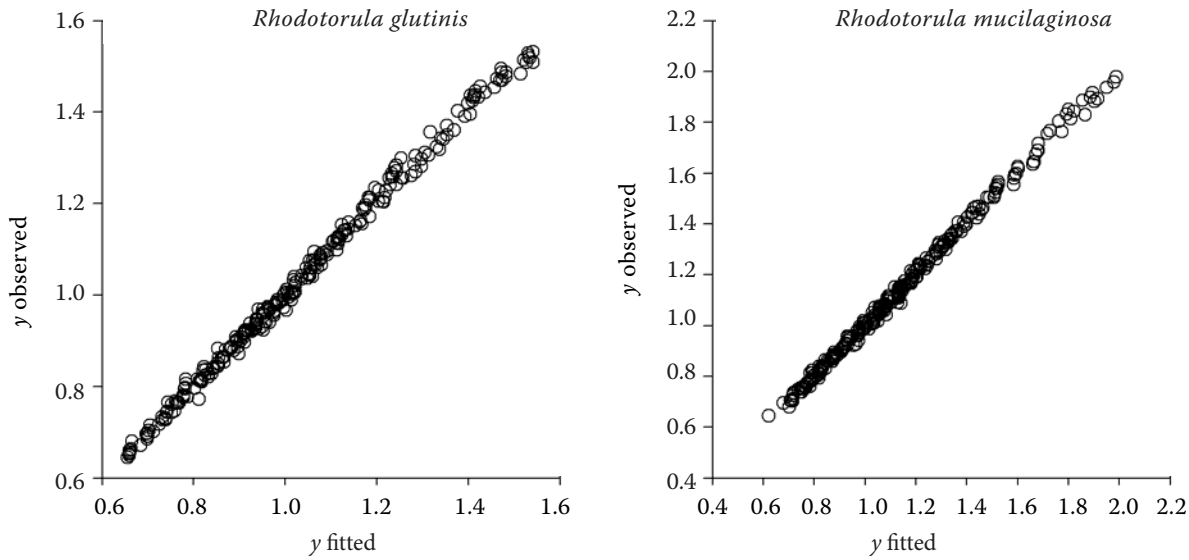

Figure 5. Comparison of fitted and observed values of colony diameter. Fitted values correspond to the model including random effects, observed values are the response values

$\hat{\beta}_{1,jk}$ representing the mean growth rates in the observed linear phase, it can be concluded that in the case of *R. mucilaginosa* both rates were positively affected by the temperature and NaCl concentration. As for *R. glutinis*, the results were not so straightforward. The positive effect of temperature existed in both phases of the growth with the exception of the highest NaCl concentration in the observed phase. The dependence on NaCl concentration was different both at the two temperatures and in the two phases. The reason can hardly be found due to the observations missing in the initial phase. The results clearly indicate that the growth rate of *R. mucilaginosa* was higher than that of *R. glutinis* under all cultivation conditions. The highest growth rate was observed during the cultivation of *R. mucilaginosa* in the media with 2% of NaCl at 20°C. The covariance structures of the models describing SSC growth of the two yeasts were slightly different but this can be explained by the fact that the greater variation of b_0 comes about at the higher growth rate. This supposition is also supported by the larger variation around $\hat{\beta}_{0,jk}$ at 20°C and 2% NaCl for *R. glutinis* (not considered in the model) and obviously a larger variation around $\hat{\beta}_{1,jk}$ at 20°C and 2% NaCl for *R. mucilaginosa* (not considered in the model).

The use of the linear mixed effects model offers a more precise analysis than does the classical linear model. As shown in our paper, the impacts of neglecting assumptions of the classical linear model on the length of confidence intervals for the parameters of the model may be quite essential. The use of the model is not restricted to

Table 6. Estimated parameters of the of the straight lines with 95% confidence limits for *R. mucilaginosa*

Experimental conditions	T (°C)	c (%)	$\hat{\beta}_0$	95% lcl	95% ucl	$\hat{\beta}_1$	95% lcl	95% ucl
I	10	0	0.7087	0.6682	0.7493	0.0414	0.0394	0.0435
II	10	1	0.7162	0.6756	0.7567	0.0524	0.0503	0.0544
III	10	2	0.7537	0.7132	0.7942	0.0607	0.0586	0.0627
IV	20	0	0.9103	0.8698	0.9509	0.0610	0.0590	0.0631
V	20	1	0.9708	0.9264	1.0152	0.0658	0.0636	0.0680
VI	20	2	1.0477	1.0033	1.0921	0.0740	0.0718	0.0762

$\hat{\beta}_0$ – mean diameter after 5 days; $\hat{\beta}_1$ – growth rate during the linear growth period

Table 7. Covariance structure models specified by matrices **D** and **R** and corresponding information criteria AIC and BIC for *R. glutinis* (*lme*, S-PLUS)

Model	**D**	**R**	*h*	*AIC*	*BIC*
RG1	$diag\{\sigma^2_{0,jk}\}$, $j = 1,2$; $k = 1,2,3$	AR(1)	8	–1356.121	–1284.908
RG2	$diag\{\sigma^2_{0,1}, \sigma^2_{0,2}\}$	AR(1)	4	–1346.230	–1289.259
RG3	$diag\{\sigma^2_{0,1}, \sigma^2_{0,2}, \sigma^2_{0,3}\}$	AR(1)	5	–1346.520	–1285.988
RG4	σ^2_0	AR(1)	3	–1347.683	–1294.273
RG5	σ^2_0	σ^2**I**	2	–1276.245	–1226.395

The covariance matrix of random effects **D** of model RG1 has six different variances $\sigma^2_{0,jk}$ corresponding to the different experimental conditions on its diagonal. In RG2 $\sigma^2_{0,1}$, $\sigma^2_{0,2}$, correspond to different temperatures, in RG3 $\sigma^2_{0,1}$, $\sigma^2_{0,2}$, $\sigma^2_{0,3}$, correspond to different concentrations. In RG4 and RG5 the same variation under all experimental conditions is considered. The covariance matrix of random errors **R** corresponds either to the autoregressive scheme AR(1) or to the independent random errors with equal variances (**I** is the identity matrix). By *h* the overall number of covariance parameters in **D** and **R** is denoted

Table 8. Covariance structure models specified by matrices **D** and **R** and corresponding information criteria AIC and BIC for *R. mucilaginosa* (*lme*, S-PLUS)

Model	**D**	**R**	*h*	*AIC*	*BIC*
RM1	$diag\{\sigma^2_{0,jk}\}$, $j = 1,2$; $k = 1,2,3$	AR(1)	8	–1430.699	–1358.291
RM2	$diag\{\sigma^2_{0,1}, \sigma^2_{0,2}\}$	AR(1)	4	–1415.369	–1357.443
RM3	$diag\{\sigma^2_{0,1}, \sigma^2_{0,2}, \sigma^2_{0,3}\}$	AR(1)	5	–1432.362	–1370.815
RM4	$diag\{\sigma^2_0, \sigma^2_1\}$	AR(1)	4	–1416.333	–1358.406
RM5	σ^2_0	AR(1)	3	–1415.867	–1361.561
RM6	σ^2_0	σ^2**I**	2	–1276.245	–1226.395

The covariance matrix of random effects **D** of model RM1 has six different variances $\sigma^2_{0,jk}$ corresponding to the different experimental conditions on its diagonal. In RM2 $\sigma^2_{0,1}$, $\sigma^2_{0,2}$, correspond to different temperatures, in RM3 $\sigma^2_{0,1}$, $\sigma^2_{0,2}$, $\sigma^2_{0,3}$, correspond to different concentrations. In RM4 σ^2_0 and σ^2_1 and in RM5 and RM6 only σ^2_0 independent on experimental conditions is considered. The covariance matrix of random errors **R** corresponds either to the autoregressive scheme AR(1) or to the independent random errors with equal variances (**I** is the identity matrix). By *h* the overall number of covariance parameters in **D** and **R** is denoted

the functions linear in parameters; non-linear mixed effects models are applied in a similar way. Both linear and non-linear mixed effects models are implemented in the best known commercial software products such as S-PLUS or SAS.

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