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World Journal of Microbiology and Biotechnology

Han, B.Z.; Nout, M.J.R.

<https://doi.org/10.1023/A:1008974621698>

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Effects of temperature, water activity and gas atmosphere on mycelial growth of tempe fungi *Rhizopus microsporus* var. *microsporus* and *R. microsporus* var. *oligosporus*

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Received 12 October 1999; accepted 7 November 2000

Keywords: Carbondioxide, fungi, oxygen, *Rhizopus*, solid-substrate fermentation SSF, tempe modelling, temperature, water activity

Summary

Rhizopus microsporus var. *microsporus* and var. *oligosporus* are used in the manufacture of various Asian fermented foods (tempe, black oncom, sufu). In view of solid-substrate fermentation (SSF) control, mycelial growth of strains of both varieties was tested for sensitivity to fluctuations of temperature, water activity and interstitial gas composition. This was achieved by measuring radial growth as well as biomass dry weight of pre-germinated microcolonies on defined media. The optimum conditions were temperature 40 °C, a_w 0.995 and a gas composition of air for the growth of both strains on a model medium. Whereas radial growth rates of var. *microsporus* and var. *oligosporus* were similar, biomass growth rates of var. *oligosporus* were higher than those of var. *microsporus* under optimum conditions. The temperature-dependent growth of *Rhizopus* spp. at $a_w > 0.98$ could be described by the Ratkowsky Equation. Carbon dioxide (5–10% v/v) inhibited the growth of *Rhizopus* spp. at non-limiting levels of oxygen. The two strains were able to grow at low (0.5% v/v) oxygen levels, but the mycelial density was rather low. No interrelation of water activity and gas composition was observed, but at high water activity the fungi were more sensitive to changes of temperature. The implications for process control are discussed.

Introduction

Rhizopus spp. are used in solid substrate fermentations (SSF) for the production of several Asian fermented foods such as tempe, made of cooked soybeans (Nout & Rombouts 1990) and certain kinds of sufu made of soybean curd (Su 1986). Most of these SSF are static, i.e. no mixing of the substrate takes place during incubation. Whereas fungi and their metabolites can be produced in liquid culture, SSF is of specific interest in the manufacture of solid foods, enzyme and flavour substances (Smits *et al.* 1998). Optimization and control of fermentation (incubation) conditions is of essential importance for maximum and sustained productivity of biomass and/or metabolites.

Previous studies were conducted on the importance of singular environmental parameters such as temperature. Ratkowsky *et al.* (1982, 1983) proposed mathematical models to describe the effect of incubation temperature on bacterial growth rate. Much work has been done on the development of models for bacterial growth as a function of temperature (Zwietering *et al.* 1994a,b). To

our knowledge, only a few papers have described mould growth on a solid surface (Mitchell *et al.* 1990; Peñaloza *et al.* 1991; Cuppers *et al.* 1997). Water activity (a_w) is defined as the relative humidity of the gaseous atmosphere in equilibrium with the substrate. The importance of water activity has been studied by Oriol *et al.* (1988) in SSF conditions. However, little information is available concerning the effect of a_w on the mycelial growth of *Rhizopus* spp.

De Reu *et al.* (1995) studied the effect of oxygen and carbon dioxide in the incubation atmosphere on the germination and mycelial growth of *Rhizopus oligosporus* and observed that at oxygen levels as low as 0.2% (v/v) mycelial growth still occurred but was insufficient for tempe making. Carbon dioxide had a stimulatory effect at 5–10% (v/v) but became inhibitory at levels exceeding 20% (v/v).

Due to limited dissipation of heat and mass in SSF, increases of temperature and carbon dioxide occur simultaneously with decreases of oxygen and water activity, the latter caused by water migration resulting from temperature gradients. It is therefore important to

know the sensitivity of the fungi to these simultaneous and combined shifts of environmental conditions. Our interest in tempe fermentations prompted us to select strains of two fungal strains used for tempe making, i.e. *Rhizopus microsporus* var. *microsporus* and *Rhizopus microsporus* var. *oligosporus*.

Materials and Methods

Micro-organism

Rhizopus microsporus var. *microsporus* (LU573) and *R. microsporus* var. *oligosporus* (LU575; NRRL5905), both isolated from commercial tempe, were grown for 7 days at 30 °C and maintained at 4 °C on malt extract agar (MEA, Oxoid CM 59) slants. Sporangiospore suspensions were obtained by scraping the sporangia off the agar and suspending them into sterile distilled water with 0.85% salt, 0.1% peptone and 0.05% Tween 80. The viable count varied between 5×10^5 and 2×10^6 colony-forming-units (c.f.u.) ml⁻¹. For each series a fresh spore suspension was prepared.

Adjustment of a_w in the medium

Before sterilization, an amount of glycerol calculated using Norrish's equation (Norrish 1966; Gervais *et al.* 1988a) was added to the medium in order to produce a_w values ranging from 0.96 to 0.995.

Incubator

A purpose-built gas-flushed incubator (De Reu *et al.* 1995) (70 × 40 × 40 cm, length × depth × height) was connected to gas cylinders with different gas compositions. Gases were filtered and humidified before entering the incubator. The flow rate was set at 0.5 l/min. Non-inoculated Petri dishes were placed in the incubator to equilibrate with the gas atmosphere for 4 h before the inoculation took place. Incubation temperatures were set and controlled at 30, 35, 40, 45, 50 ± 0.5 °C respectively.

Micro-colony growth

A 1-ml sample of a diluted spore suspension ($\sim 10^2$ c.f.u. ml⁻¹) was used in pour plates with malt extract broth (120 g/l, Oxoid CM57), neutralized bacteriological peptone (30 g/l, Oxoid L34) and agar bacteriological (1% w/v, Oxoid L11) (MEB NBP A1). After 20 h of incubation at 30 °C, colonies were cut out with a sterile cork borer having an internal diameter of 7 mm, and transferred to the centre of a ready-poured MEB NBP A1.5 plate (22 ml; containing 1.5% w/v agar) for the growth experiment.

Biomass monitoring

At regular intervals triplicate petri dishes were removed. After measurement of the diameter of the colony,

mycelium was peeled and then heated with 300 ml demineralized water in a microwave oven until the suspension was boiling. The mixture was filtered through a preweighed filter (No. 595, Schleicher & Schüll, Dassel, Germany). Subsequently the filters were dried for 2 days at 80 °C, and the mycelial dry weight was determined gravimetrically.

Radial growth rate [dR/dt] and biomass growth rate [$d(x^{1/3})/dt$]

The growth curves based on the colony diameters and the cubic root of mycelial dry weights were fitted using linear regression (Microsoft Excel, version 5.0) to determine the radial growth rate dR/dt and the biomass growth rate $d(x^{1/3})/dt$.

Growth rate models for temperature dependence

The temperature dependence of the growth rate was modelled as follows by means of the Ratkowsky Square Root Model (Ratkowsky *et al.* 1982, 1983) with the parameters minimum growth temperature ($T_{\min}$), maximum growth temperature ($T_{\max}$), b , and c : $\sqrt{\mu} = b(T - T_{\min})\{1 - \exp[c(T - T_{\max})]\}$

The computer program of TC (Table Curve 2D v 2.03) was used to fit the Ratkowsky model.

Results

Growth curves with different a_w

Figure 1 shows the colony diameter of microcolonies and biomass dry weight at controlled a_w and 40 °C. Owing to adaptation towards the media, the diameters of the colonies and the biomass dry weight did not increase during the first 4 h. After this period the shape of the increasing colonies was perfectly circular and the colony diameters increased linearly with time. When the cubic root of biomass dry weight was plotted against time, a linear relation was observed. Although at a_w 0.995, the colony of LU573 reached the edge of its Petri dish earliest, LU575 had produced more biomass than LU573 at the same time. The colony diameter and the biomass of both LU573 and LU575 decreased rapidly when a_w level was lowered. At $a_w \leq 0.96$ both strains hardly produced biomass. Thus, the effect of water activity on growth of *Rhizopus* spp. is significant.

Effect of different temperature and a_w on growth rate on MEB NBP A1.5

In Figure 2 the radial growth rate [dR/dt] and the biomass growth rate [$d(x^{1/3})/dt$] are shown for the experiments with different temperature and a_w . The levels of a_w varied from 0.995 to 0.96 at various temperatures from 30 to 50 °C. The optimum growth temperatures and a_w for both LU573 and LU575 were

Figure 1. Colony diameter (mm) and biomass dry weight (g) during growth of LU573 and LU575 on MEB NBP A1.5 at 40 °C.

40 °C and 0.995, respectively. The growth rates at a given temperature decreased sharply with decreasing a_w . Both LU573 and LU575 were less sensitive to the changes of temperature at lower level of a_w . The differences in dR/dt between LU573 and LU575 at the same a_w are small, while the differences in $d(x^{1/3})/dt$ are significant. This implies that the biomass density of LU575 is higher than that of LU573, except for one point of a_w 0.995 at 50 °C.

Growth rate models for the effects of temperature at different a_w

Tables 1 and 2 show that good fits of the Ratkowsky model could be obtained for both LU573 and LU575 at a_w 0.995. The goodness of fit of the model as judged by the correlation coefficient (r^2) and standard error of fit (FtStdErr) became worse with decreasing a_w . The model can be accepted at $a_w > 0.98$ on the basis of statistical comparison. Within limits of the model produced, T_{min} and T_{max} of LU573 are higher than that of LU575. It means that the growth of these *Rhizopus* spp. can be predicted in accordance with the model produced.

Effect of gas composition on MEB NBP A1.5

Figure 3 shows three-dimensional representations of the effect of different gas mixtures and a_w levels on dR/dt and $d(x^{1/3})/dt$ at the optimum temperature 40 °C. Four

different gas compositions were tested (1) Air, (2) Air + 5% CO₂, (3) Air + 10% CO₂ and (4) 99.5% N₂ + 0.5% O₂. The growth rates of LU573 and LU575 decrease when O₂ content lowers and CO₂ content rises. Both strains are more sensitive to the change of gas composition at higher a_w . The biomass growth rates of LU573 and LU575 with air are much higher than that with 99.5% N₂ + 0.5% O₂ at a_w 0.995. But, relatively speaking, there are no great changes in the radial growth rates with those conditions. It means that effect of gas composition on the production of biomass is more significant than that on the radial growth of colonies.

Discussion

In this study, microcolonies were used to standardize the initial conditions for the growth experiments under various conditions. With this technique, the effects of the different factors on the germination of *R. microsporus* var. *oligosporus* and *R. microsporus* var. *microsporus* sporangiospores were excluded.

From Figure 1, the linear increases of colony diameters were observed and the linear increase can also be observed when the cubic root of biomass was plotted against time. De Reu *et al.* (1995) reported that a linear increase was observed when the natural logarithm of biomass was plotted against time, to estimate the specific growth rate (μ_x). In the present paper, we developed a

Figure 2. Radial growth rate dR/dt ($mm h^{-1}$) and Biomass growth rate $[d(x^{1/3})/dt]$ ($g^{1/3} h^{-1}$) of LU573 and LU575 as a function of temperature and water activity.

different approach. In the case of the relationship between biomass and diameter of colony on the solid medium,

$$B = V \times \rho = (1/4) \times \pi \times D^2 \times H \times \rho, \quad (1)$$

where B = biomass, V = mycelium volume, ρ = mycelium specific density, D = colony diameter, and H = mycelium height.

Suppose $H \times \rho = \alpha_1 \times D$, then $B = \alpha_2 \times D^3$.

By definition of radial growth rate: $D = (dR/dt) \times t + D_0$,

$$B = \alpha_2 \times [(dR/dt) \times t + D_0]^3 \text{ or}$$

$$B^{1/3} = \alpha \times [(dR/dt) \times t + D_0]. \quad (2)$$

So the cubic root of biomass is a reasonable way to estimate biomass growth rate, rather than the natural logarithm of biomass for the specific growth rate (μ_x).

When: $t = 0$, $B_0^{1/3} = \alpha \times D_0$

Then:

$$B^{1/3} = \alpha \times (dR/dt) \times t + B_0^{1/3}. \quad (3)$$

Table 1. Parameters for the Ratkowsky model of radial growth rate. Ratkowsky $\sqrt[3]{(dR/dt)} = b(T - T_{min})\{1 - \exp[c(T - T_{max})]\}$.

Strain	a_w	Parameter				Statistical comparison	
		b	c	T_{min}	T_{max}	r^2	FtStdErr
LU573	0.995	0.057	0.085	6.12	59.05	0.979	0.047
LU573	0.982	0.072	0.045	9.91	62.04	0.943	0.071
LU573	0.97	0.109	0.029	14.85	60.66	0.855	0.126
LU573	0.96	0.045	0.047	10.86	63.46	0.918	0.096
LU575	0.995	0.038	0.252	-1.13	52.80	0.993	0.031
LU575	0.982	0.041	0.103	2.29	58.97	0.979	0.037
LU575	0.97	0.095	0.030	13.46	60.90	0.883	0.096
LU575	0.96	0.019	0.398	-6.59	52.51	0.935	0.062

FtStdErr = standard error of fit.

Table 2. Parameters for the Ratkowsky model of biomass growth rate. Ratkowsky $\sqrt[3]{[d(x^{1/3})/dt]} = b(T - T_{min})\{1 - \exp[c(T - T_{max})]\}$.

Strain	a_w	Parameter				Statistical comparison	
		b	c	T_{min}	T_{max}	r^2	FtStdErr
LU573	0.995	0.008	0.031	6.85	63.64	0.989	0.002
LU573	0.982	0.004	0.052	2.12	64.19	0.906	0.004
LU573	0.97	0.208	0.0007	13.67	68.33	0.525	0.014
LU573	0.96	0.215	0.0004	8.56	76.69	0.933	0.005
LU575	0.995	0.004	0.211	1.69	54.11	0.993	0.003
LU575	0.982	0.040	0.005	15.56	68.60	0.990	0.002
LU575	0.97	0.064	0.003	15.13	64.50	0.720	0.011
LU575	0.96	0.003	0.100	-1.47	57.73	0.854	0.006

FtStdErr = standard error of fit.

Figure 3. Radial growth rate dR/dt (mm h^{-1}) and Biomass growth rate $[d(x^{1/3})/dt]$ of LU573 and LU575 at different gas compositions at optimum temperature 40°C .

According to our definition of biomass growth rate $[d(x^{1/3})/dt]$:

$$B^{1/3} = [d(x^{1/3})/dt] \times t + B_0^{1/3} \quad (4)$$

Combining Equations (3) and (4) leads to: $\alpha = [d(x^{1/3})/dt]/[dR/dt]$ and the following relationship between B , $[dR/dt]$ and $[d(x^{1/3})/dt]$:

$$B = [d(x^{1/3})/dt]^3 \times [t + \{1/(dR/dt)\} \times D_0]^3 \quad (5)$$

Thus the biomass of colony could be easily estimated on model medium.

Concerning the effect of a_w , we found an optimum a_w for growth of 0.995 which corresponds with the findings of Gervais *et al.* (1988b) and Sarrette *et al.* (1992). So water activity appeared to be an important and acute factor and it should be controlled as well as possible during the solid-substrate fermentation.

The models developed by Ratkowsky *et al.* (1982, 1983) described the effect of temperature on bacterial growth rate under conditions where temperature was the only variable parameter. We found that the Ratkowsky model can be applied as well to the effect of temperature on fungal (*Rhizopus* spp.) growth rate. Smits *et al.* (1998) used the radial growth rate of *Trichoderma reesei* to fit the model of Ratkowsky. Our data allow a good fit of the effect of temperature on both dR/dt and

$[d(x^{1/3})/dt]$ of *Rhizopus* spp. with the Ratkowsky model under condition of $a_w > 0.98$. Owing to changes of the behaviour of *Rhizopus* spp. at $a_w \leq 0.97$, the model produced could be hardly accepted in terms of statistical significance.

Rosso *et al.* (1993) also proposed another model of temperature dependence that uses three cardinal temperatures [$T_{\min}$, $T_{\max}$ and optimum growth temperature (T_{opt})] and the specific growth rate at the optimum temperature (μ_{opt}) as parameters:

$$\begin{aligned} \mu = \mu_{\text{opt}} & (T - T_{\max})(T - T_{\min})^2 / (T_{\text{opt}} - T_{\min}) \\ & \times [(T_{\text{opt}} - T_{\min})(T - T_{\text{opt}}) - (T_{\text{opt}} - T_{\max}) \\ & \times (T_{\text{opt}} + T_{\min} - 2T)] \end{aligned}$$

Although this model has the advantage that all four parameters have a physiological significance, it did not fit as well as the Ratkowsky model with the same data. Probably, the reason is that the specific growth rate (μ_x) was not found and used to fit Rosso's model in our study.

Hesseltine *et al.* (1985) and Soccol *et al.* (1994) reported that 16 and 19 different strains, respectively, of *Rhizopus* spp. were able to grow under anaerobic conditions. In our experiments, the two strains tested also were able to grow under gas composition of 99.5% $\text{N}_2 + 0.5\% \text{O}_2$. However, the biomass of a colony under anaerobic conditions is much less than that under

aerobic conditions for the same diameter of the colony. It means that the hyphae of two strains spread to scavenge oxygen from the medium while the mycelial density remained rather low.

Concerning the interrelation of the parameters temperature, water activity and gas composition, our data indicate that there is no relation between the effects of water activity and gas composition and that both fungi are more sensitive to the range of water activity tested than to the tested gas compositions. Considering the combination of temperature and water activity, an interrelation appears to be present at high water activity. In principle, the higher the a_w the more sensitive the fungi towards changes of temperature. For both strains optimum conditions for biomass productivity were similar, i.e. $T = 40\text{ }^{\circ}\text{C}$, $a_w = 0.995$, in air. At this optimum, both fungi show the highest sensitivity towards environmental fluctuations. In view of fermentation control, it is therefore essential to seek methods of temperature control that do not affect the water activity of the substrate.

Acknowledgements

The authors thank B. Hasenack for technical assistance. One of the authors (B.H.) was supported by the Sino – Dutch Exchange programme.

References

- Cuppers, H.G.A.M., Oomes, S. & Brul, S. 1997 A model for the combined effects of temperature and salt concentration on growth rate of food spoilage molds. *Applied and Environmental Microbiology* **63**, 3764–3769.
- De Reu, J.C., Griffiths, A.M., Rombouts, F.M. & Nout, M.J.R. 1995 Effect of oxygen and carbon dioxide on germination and growth of *Rhizopus oligosporus* on model media and soya beans. *Applied Microbiology and Biotechnology* **43**, 908–913.
- Gervais, P., Bensoussan, M. & Grajek, W. 1988a Water activity and water content: comparative effects on the growth of *Penicillium roqueforti* on solid substrate. *Applied Microbiology and Biotechnology* **27**, 389–392.
- Gervais, P., Molin, P., Grajek, W. & Bensoussan, M. 1988b Influence of the water activity of a solid substrate on the growth rate and sporogenesis of filamentous fungi. *Biotechnology and Bioengineering* **31**, 457–463.
- Hesseltine, C.W., Featherston, C.L., Lombard, G.L. & Dowell Jr, V.R. 1985 Anaerobic growth of molds isolated from fermentation starters used for foods in Asian countries. *Mycologia* **77**, 390–400.
- Mitchell, D.A., Greenfield, P.F. & Doelle, H.W. 1990 Mode of growth of *Rhizopus oligosporus* on a model substrate in solid state fermentation. *World Journal of Microbiology and Biotechnology* **6**, 201–208.
- Norrish, R.S. 1966 An equation for the activity coefficient and equilibrium relative humidities of water in confectionery syrups. *Journal of Food Technology* **1**, 25–39.
- Nout, M.J.R. & Rombouts, F.M. 1990 Recent developments in temperature research. *Journal of Applied Bacteriology* **69**, 609–633.
- Oriol, E., Raimbault, M., Roussos, S. & Viniegra-Gonzales, G. 1988 Water and water activity in the solid state fermentation of cassava starch by *Aspergillus niger*. *Applied Microbiology and Biotechnology* **27**, 498–503.
- Peñaloza, W., Davey, C. L., Hedger, J. N. & Kell, D. B. 1991 Stimulation by potassium ions of the growth of *Rhizopus oligosporus* during liquid- and solid-substrate fermentations. *World Journal of Microbiology and Biotechnology* **7**, 260–268.
- Ratkowsky, D.A., Olley, J., McMeekin, T.A. & Ball, A. 1982 Relationship between temperature and growth rate of bacterial cultures. *Journal of Bacteriology* **149**, 1–5.
- Ratkowsky, D.A., Lowry, R.K., McMeekin, T.A., Stokes, A.N. & Chandler, R.E. 1983 Model for bacterial culture growth rate throughout the entire biokinetic temperature range. *Journal of Bacteriology* **154**, 1222–1226.
- Rosso, L., Lobry, J.R. & Flandrois, J.P. 1993 An unexpected correlation between cardinal temperatures of microbial growth highlighted by a new model. *Journal of Theoretical Biology* **162**, 447–463.
- Sarrette, M., Nout, M.J.R., Gervais, P. & Rombouts, F.M. 1992 Effect of water activity on production and activity of *Rhizopus oligosporus* polysaccharidases. *Applied Microbiology and Biotechnology* **37**, 420–425.
- Smits, J.P., Rinzema, A., Tramper, J., Van Sonsbeek, H.M., Hage, J.C., Kaynak, A. & Knol, W. 1998 The influence of temperature on kinetics in solid-state fermentation. *Enzyme and Microbial Technology* **22**, 50–57.
- Soccol, C.R., Raimbault, M. & Pinheiro, L.I. 1994 Effect of CO₂ concentration on the mycelium growth of *Rhizopus* spp. *Agricultural Biology and Technology* **37**, 203–210.
- Su, Y-C. 1986 Sufu. In *Legume-based Fermented Foods*. eds. Reddy, N.R., Pierson, M.D. & Salunkhe, D.K. pp. 69–83. Boca Raton, FL, USA: CRC Press. ISBN 0-8493-6286-5.
- Zwietering, M.H., Cuppers, H.G.A.M., De Wit, J.C. & Van 't Riet, K. 1994a Evaluation of data transformations and validation of a model for the effect of temperature on bacterial growth. *Applied and Environmental Microbiology* **60**, 195–203.
- Zwietering, M.H., De Wit, J.C., Cuppers, H.G.A.M. & Van 't Riet, K. 1994b Modeling of bacterial growth with shifts in temperature. *Applied and Environmental Microbiology* **60**, 204–213.