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Study of physicochemical factors limiting the growth of *Kluyveromyces marxianus*

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SUMMARY

The effects of various physicochemical parameters on the growth of two *Kluyveromyces marxianus* strains were investigated, including: pH values, sodium chloride, water activity in the medium and temperature. Both yeast strains were unaffected by pH changes. Optimal pH for growth was found to be 4 with both strains, but they were able to develop within the pH 3–8 range. Suitable growth was obtained at temperatures of 4–44 °C and the optimal temperature for growth was 36 °C for both strains. Modelling of this latter parameter is described. Growth of both microorganisms was considerably modified by increased NaCl or decreased water activity in the medium.

INTRODUCTION

Kluyveromyces lactis (Dombrowsky) van der Walt and *Kluyveromyces marxianus* (Hansen) van der Walt are well-known yeasts in the dairy industry. The former often exhibits a weak fermentative metabolism but in contrast rapidly metabolizes lactic acid [17].

In contrast, some *Kluyveromyces marxianus* strains are able to ferment lactose and its derivative hexoses. They can therefore be used to produce alcohol from whey [14,20–23].

K. marxianus may also play an important role in cavity formation in cheese. Co-culture of *K. marxianus* and *K. lactis* is used for single cell protein production from whey in the 'Fromagerie Bel' process [17,18,24].

Many authors have previously used mathematical models to study the behavior of various microorganisms in well-defined experimental conditions. Their studies concerned with the influence of pH, temperature, NaCl content and water activity on growth [5,7,8,15].

In order to determine the potential of *K. marxianus* strains in the milk industry, we studied the influence of physicochemical parameters on the growth of yeast and modeled the influence of temperature.

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MATERIALS AND METHODS

Biological material

The two strains studied were *Kluyveromyces fragilis* CBS 397 and *Candida pseudotropicalis* IP 513. These two yeasts belong to the *K. marxianus* species according to the classification of Barnett [2].

Culture conditions

Culture. All cultures were carried out in Erlenmeyer flasks filled to one-tenth of their volume and shaken (amplitude, 7 cm; oscillations, 80/min). They were incubated at 28 °C except where otherwise indicated. The G medium [12] contained 5 g glucose, 1 g KH_2PO_4, 2 g $(NH_4)_2SO_4$, 6 g $NH_4H_2PO_4$, 0.1 g NaCl, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2$, 0.5 mg H_3BO_3, 40 mg $CuSO_4 \cdot 5H_2O$, 100 mg KI, 400 mg $MnSO_4$, 200 mg $Na_2Mo_4 \cdot 2H_2O$, 400 mg $ZnSO_4 \cdot 7H_2O$, 200 mg $FeCl_3$, 2 mg calcium pantothenate, 2 mg thyamine, 2 mg inositol, 2 mg pyridoxine, 0.5 mg niacin, 0.02 mg biotin in 1 liter of tartrate/tartaric acid (0.1 M) buffer, excepted where otherwise indicated.

Inoculum was taken from a 12-h-old culture on G medium. The absorbance after inoculation was about 0.5.

Physicochemical parameters control. The following buffers were used: HCl/KCl (0.1 M) at pH 1–2.5; Tartrate/Tartaric acid (0.1 M) at pH 3–5; Sodium hydrogenophosphate/potassium dihydrogenophosphate (0.1 M) at pH 6–8. Tris-HCl (0.1 M) at pH 9–10.

All cultures were carried out in a shaken bain-marie (Clifton) with thermostatic control.

In order to change water activity (a_w), NaCl and poly-(ethylene glycol) (PEG) concentrations were calculated from values given by Tome and Bizot [31] and then used by Guilbert [13]. PEG 400 was used as a water activity depression factor because it was not metabolized by the yeast. The medium water activity without NaCl or PEG was about 0.9975.

Napierian growth rate measure

Growth was monitored using a UVIKON 930 spectrophotometer. Growth curves (napierian logarithm value of cell density over the time) were established for each microorganism and for each studied parameter. From these curves, the method described by Broughall et al. [3] allowed calculation of the napierian growth rate by linear regression.

Growth modelling

Two types of mathematical models of the growth rate as a function of temperature are well described in the literature.

(i) A non-linear model derived from the Arrhenius equation was proposed by Schoolfield et al. [30] and was successfully applied to bacterial growth by Broughall et al. [3,4] and Adair et al. [1]:

$$\mu = \frac{\mu(25) \times T/298 \times \exp[Ha/R \times (1/298 - 1/T)]}{\{1 + \exp[Hl/R \times (1/Tl - 1/T) + \exp[Hh/R \times (1/Th - 1/T)]\}}$$

where μ = growth rate (h^{-1}); $\mu(25)$ = growth rate at 25 °C; T = temperature in Kelvin degrees; Ha = constant describing the enthalpy activation for microbial growth; R = universal gas constant (1.987 cal mol^{-1} K^{-1}); Hl = constant describing the enthalpy for low temperature inactivation of growth; Tl = constant describing the temperature for 50% low temperature inactivation of growth; Hh = constant describing the enthalpy for high temperature inactivation of growth; Th = constant describing the temperature for 50% high temperature inactivation of growth.

(ii) A Belehradek-type model described by Ratkowsky et al. [26,27], in which the square root of the growth rate is related to temperature. Many others have previously used this model [1,6,15,28].

$$\mu = b \times (T - T_{min}) \times \{1 - \exp[c \times (T - T_{max})]\}$$

where μ = growth rate constant (h^{-1}); b = regression coefficient; T = temperature in Kelvin degrees; T_{min} and T_{max} = conceptual temperatures of no metabolic signification (minimum and maximum temperatures, respectively, where the growth rate is zero); c = additional parameters to enable use of the model at temperatures above the optimal temperature.

An algorithmic search of the constants used in the above-cited equations (Ha, Hl, Hh, Tl, Th, b and c) was performed with the Marquardt and Gauss-Newton iterative methods with SAS (Statistical Analysis System) software. Experimental data for μ and T were thus used.

RESULTS

To determine the influence of given parameters on the growth rate of yeasts, we arbitrarily fixed the other parameters at close to optimal values.

Effect of pH

The effect of pH on yeast growth rates is illustrated in Fig. 1.

For pH values ranging from 2.5 to 8 for the IP 513 strain and from pH 3 to 9 for the CBS 397 strain, the growth rates were not significantly affected by pH variations. Interestingly strain IP 513 was able to grow at pH values between 1 and 2. At pH 2, CBS 397 did not grow. On the contrary, CBS 397 grew better at a high pH; the upper limits for growth were pH 9 for IP 513 and pH 10 for CBS 397.

Effect of sodium chloride content

Fig. 2 shows that the growth rate decreased linearly when NaCl content was lower than 7% and then dropped to 0 at 8% for IP 513. In the case of CBS 397, the slope change only occurred at 8% NaCl and growth stopped at 10% NaCl.

Effect of water activity

In some cheeses, a_w may be the limiting growth factor for microorganisms. Therefore, we monitored growth rate

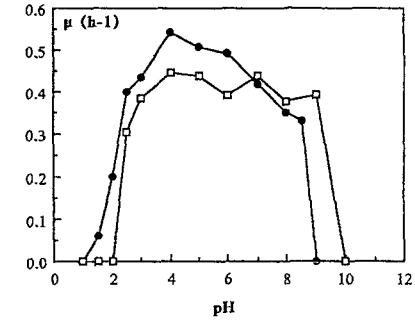

Fig. 1. Variations in the growth rate of IP 513 (●) and CBS 397 (□) strains as a function of pH.

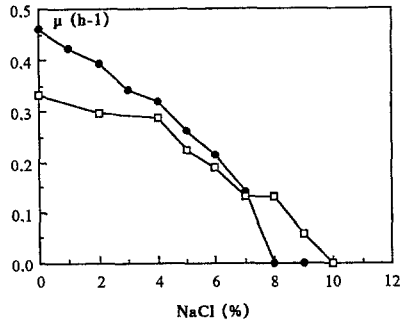

Fig. 2. Variations in the growth rate of IP 513 (●) and CBS 397 (□) strains as a function of NaCl content.

changes for various a_w values using PEG, which lowered the availability of the water molecules.

In Fig. 3, the growth rate variations are given as a function of NaCl or PEG content. On the abscissa, for each water activity value, we gave the corresponding NaCl and PEG content (% w/w), according to the equation obtained from Tome and Bizot [31].

From these results, two remarks may be made: (i) The growth rate decreases almost linearly when water activity declines. This is in agreement with the observations of Troller and Christian [32] who noted that below the optimum a_w growth often declines linearly. (ii) These curves confirm the better resistance of the CBS 397 strain to the a_w decrease. When a_w was around 0.950, IP 513 was unable to grow, whereas the growth of CBS 397 strain was completely inhibited at a_w of 0.934. Clearly, in this case, the bacteriostatic effect of sodium chloride was essentially due to its a_w depression action.

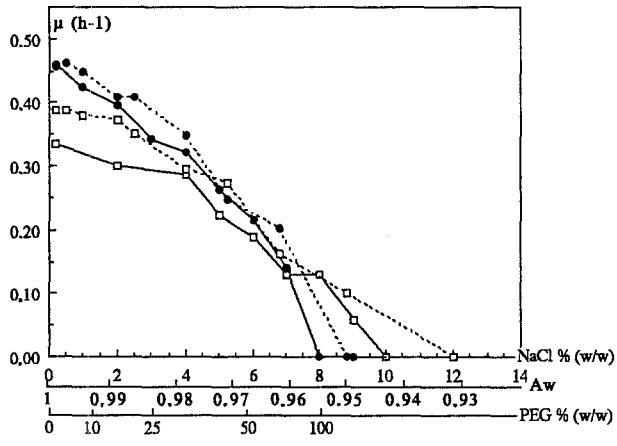

Fig. 3. Variations in the growth rate as a function of water activity with NaCl IP 513 (●) and CBS 397 (□) or PEG 400 (IP 513 (- - -● - - -) and CBS 397 (- - -□ - - -)).

Effect of temperature

Fig. 4a, b, c, and d show the variations in growth rate as a function of temperature for each strain, along with the corresponding curves.

From the experimental values, the optimal and minimal growth temperatures for both microorganisms were calculated using the equation of Ratkowsky. The optimum (T_{opt}) and minimum (T_{min}) temperatures were identical for both strains: the optimum temperature was 36 °C, the minimum was −1 °C.

The minimum temperature (T_{min}) is an intrinsic property of the organism when growth conditions other than temperature are non-limiting [26]. This indicates that these two strains are mesophilic. Indeed, T_{min} values for mesophiles ranges from about −3 to 27 °C.

However, the two microorganisms can grow at high temperatures: growth was only inhibited at 48 °C for IP 513 and at 44 °C for CBS 397.

Comparison of growth modelling

One of the most effective means of comparing two modelling systems is to compare predicted values from the two models with actual observed data points, using the mean square error (MSE) [1]:

$$MSE = \{(obs - pred)^2\}/n$$

where obs = observed growth rate value;

Pred = predicted growth rate value; n = number of observations.

The MSE value of the Schoolfield model (8.4×10^{-4} for IP513 and 2.3×10^{-4} for CBS 397) always remained lower than the MSE value of the Ratkowsky model (22.4×10^{-4} for IP 513 and 18.4×10^{-4} for CBS 397). The observed values did not completely fit to the Ratkowsky model around optimal growth.

The better fit with the Arrhenius model was probably due to the higher number of constants that took into consideration inactivation and activation enthalpy of the growth-regulating enzyme. Moreover, these constants have a biological meaning. The enthalpies of thermal activation (Ha) and thermal inactivation (Hl), calculated according to the Schoolfield model were comparable; Ha was 5096 cal mol^{-1} for IP 513 and 5258 cal mol^{-1} for CBS 397, Hl was −26000 cal mol^{-1} for IP 513 and −27000 cal mol^{-1} for CBS 397.

In contrast, the values of Hh, enthalpy of high temperature inactivation of growth, were quite different: 261000 cal mol^{-1} for CBS 397 and 80000 cal mol^{-1} for IP 513.

The Schoolfield model is very precise and has biological significant constants (Ha, Hl and Hh), but it is difficult to use. The Ratkowsky model seems less exact, but is more accessible to non-mathematician biologists.

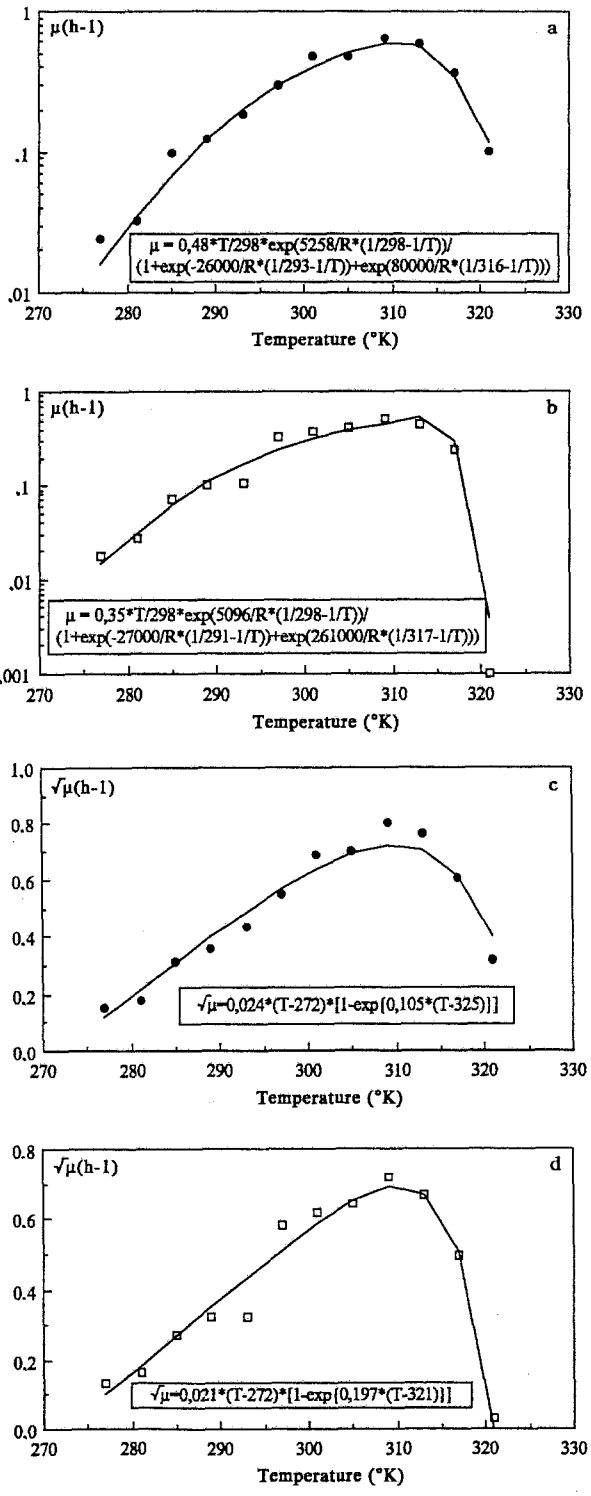

Fig. 4. Variations in the growth rate as a function of temperature with the corresponding curves: (a) IP 513 strains, Schoolfield model; (b) CBS 397 strain, Schoolfield model; (c) IP 513 strain, Ratkowsky model; (d) CBS 397 strain, Ratkowsky model. Experimental IP 513 (●); Modelised IP 513 (–); Experimental CBS 397 (□); Modelised CBS 397 (–).

These models cannot be extrapolated and have to be used within the temperature ranges defined for the calculation.

DISCUSSION

The IP 513 strain grew weakly at pH 2 and there was no growth at pH 1.5. This suggests that the cells might be resistant to human stomach acidity if taken during meals. IP 513 could be used as a 'probiotic' element to induce β-galactosidasic activity in people who do not possess this enzyme. There are many people worldwide who are unable to assimilate lactose; this characteristic occurs in Middle or Far East populations and in many African ethnic groups [10]. It is otherwise generally accepted that yeasts offer a good biotic contribution (vitamins) and participate in the intestinal flora balance [25]. Further experiments will be undertaken with nutritionists to investigate this aspect.

In milk products, pH is never a limiting factor for *Kluyveromyces* [16]. Conversly, a_w may be important in the elimination of this flora. It is well-known that these species are relatively scarce after the salting phase in some cheeses [9,11].

These yeasts grow from 4 °C for CBS 397 and to 48 °C for IP 513. IP 513 may be used with thermophilic microorganisms (as those in yogourt) at 45 °C to produce drinks or special products [29].

At 12 °C, the growth rates are 0.098 h^{-1} for IP 513 and 0.072 h^{-1} for CBS 397. These values may provide a suitable growth during the ripening phase or the conserving phase of cheese. Consequently, in some cases these strains may grow during cheese making when the water activity is high enough. Finally, the best way to eliminate them is by lowering the a_w.

The bacteriostatic effect of NaCl seems to be essentially due to its ability to reduce a_w.

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