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Incubation at Low Temperatures Increases Biomass Yield in Yeasts Isolated from Cold Environments

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Summary

The growth kinetics of several cold-tolerant yeast strains (Candida colliculosa, Candida krusei, Debaryomyces hansenii, Kluyveromyces marxianus and Torulaspora delbrueckii), isolated from refrigerated food, were studied over a range of temperatures (10–30 °C). For comparison, a mesophilic Saccharomyces cerevisiae strain was also included. The specific growth rates at different temperatures were very similar in the cold-tolerant isolates and the mesophilic strain; μ (h^{-1}) increased with temperature, reaching a maximum at 30 °C. Nevertheless, biomass yields did not correlate with the observed growth rates: maximum yields were attained at low temperatures (10 °C), at which the lowest specific growth rates were measured, and low yields were produced at 30 °C where the highest growth rates were observed. On the contrary, Sacch. cerevisiae did not show this behaviour. The variation of glucose consumption rates at the studied temperatures, correlated with the growth rates observed, but again not with the measured yields. Fermentation (mmol ethanol formed/mmol glucose consumed) was not affected by incubation temperature. These data were compared with our data for Candida sake Antarctic strains, which exhibited a similar reponse. Based on that, the enhancement of yield at low temperatures could be considered as a consequence of cold tolerance in yeasts, allowing well adapted strains to develop efficiently, albeit at reduced rates, in low temperature environments.

Keywords: psychrotolerance, low temperatures, spoilage yeasts

Introduction

Food is considered as a habitat for microbial growth, on the base of ecological concepts. Microorganisms from the environment may contaminate raw and processed foods, but only part of this primary microflora will survive under selective pressures exerted by intrinsic and extrinsic food environments *(1)*. In such environments, yeasts are associated with other microorganisms and often are in a minority, especially when compared to bacteria, and consequently play a small role in spoilage. However, conditions favourable to their growth can be a major factor in deterioration, as yeast activity often results in dramatic events and can cause great economic losses in food industry *(2)*.

Temperature is one of the most important factors that determine microflora survival. The range of temperatures for growth of many microorganisms can be characterised by cardinal (minimum, optimum, and maximum) temperatures. While, in general, the temperature range for yeast growth extends from several degrees below 0 °C to a few degrees below 50 °C, the range for individual species or strains does not normally span more than 40 °C and is often much narrower *(3,4)*. However, this range of temperatures for growth of yeasts is not readily applicable to foods.

Psychrophilic and psychrotolerant yeasts have been reported as isolates from natural cold environments (soil, water) and from foods and beverages in cold or frozen storage *(1,2,5)*. In these food products, yeasts can develop and alterate or spil them, particularly under certain circumstances, such as non-continuous cold conditions along the production chain or increased temperature during storage or distribution.

In a previous work we isolated two yeast strains from Antarctic soil. These strains were characterised by their low growth rate but high cell yield at low temperature *(6)*. The aim of the present work was to study the

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physiology and metabolism of several cold-tolerant yeasts isolated from refrigerated foods and compare their behaviour with the psychrophilic Antarctic strains.

Material and Methods

Strains and culture conditions

Seven yeast strains, isolated from different refrigerated food products were used (Table 1). One strain of *Saccharomyces cerevisiae* was also included as control. They were maintained on YMA (Yeast Morphology Agar, 1% (w/v) glucose) slants at 4 °C.

Table 1. Strains used in this work and the source of isolation

Strains/CYC No.	Source/Isolated from
Candida colliculosa 1176	plain yoghurt
Candida colliculosa 1177	fruit yoghurt
Candida krusei 1179	fruit yoghurt
Candida krusei 1180	fruit yoghurt
Debaryomyces hansenii 1157	vacuum packed beet
Kluyveromyces marxianus 1182	liquid fruit yoghurt
Torulaspora delbrueckii 1335	muesli yoghurt

CYC = Complutensis Yeasts Collection

Temperature range for growth

Yeast were inoculated in a rich medium YMB (Yeast Morphology Broth, 1% (w/v) glucose) and incubated at 5, 10, 20, 25, 30, 35, 37, 40 and 45 °C, for 7 days (10–45 °C) or for 30 days (5 °C). Turbidity, as an indication of growth, was considered a positive result.

Survival at –20 °C

Cold tolerance was investigated at –20 °C without cryoprotector on YMB. Viable counts on YMA plates were made after the inoculation and after 30 days incubation.

Determination of the effect of temperature on growth parameters

Growth rate and cell yield studies

The effect of environmental temperature on specific growth rate and biomass yield coefficient was investigated in batch cultures. Strains were grown in 250 mL flasks with 100 mL of a defined culture medium containing 1% (w/v) glucose (7) in a rotatory shaken water bath (150 rpm) at 10, 20 and 30 °C. Appropriate volumes of a logarithmic-phase culture of the strains in the same medium were used as inocula. Growth was monitored by measuring the A_{640} (using a Shimadzu UV-160A spectrophotometer) for determination of specific growth rates (μ, h^{-1}) cfu counts along the growth curves were also carried out in three representative strains. Final yield coefficient (Y) was based on dry weight determinations and consumption of glucose, and calculated as g biomass/100 g consumed substrate. All the experiments were done in duplicate.

Glucose consumption

In order to study glucose consumption as influenced by temperature, the concentration of residual glucose was measured with the corresponding UV-test kit purchased from Boehringer Mannheim. Cells were grown in 1% (w/v) glucose at 10, 20 and 30 °C. Specific rate of glucose consumption (q_{glc}) was calculated as rate of glucose consumed in relation to biomass produced (g/g h).

Ethanol production

Ethanol produced by exponentially growing cells in 1% (w/v) glucose at 10, 20 and 30 °C was measured by using a Boehringer enzymatic kit. Fermentation was calculated as ethanol produced by consumed glucose (mmol EtOH/mmol glucose).

Results

Table 2 shows the maximum temperature for growth of the yeasts studied. *Candida colliculosa* 1176 and *Debaryomyces hansenii* 1157 presented the shortest ranges of temperature for growth (t_{max} = 30 °C). *C. krusei* strains 1179 and 1180, and *Kluyveromyces marxianus* 1182 grew at 45 °C. All the strains (including *Saccharomyces cerevisiae*) were able to grow at 5 °C, the lowest temperature assayed.

Table 2. Maximum temperature for growth of the studied yeasts

Strains	t_{max} / °C
Candida colliculosa 1176	30
Candida colliculosa 1177	35
Candida krusei 1179	45
Candida krusei 1180	45
Debaryomyces hansenii 1157	30
Kluyveromyces marxianus 1182	45
Torulaspora delbrueckii 1335	35
Saccharomyces cerevisiae 1223	40

The studied yeast survived for at least 30 days at –20 °C without cryoprotector. Viable cell counts of isolates from refrigerated foods did not decrease after one month, and decreased only one logarithmic unit in the control yeast *Saccharomyces cerevisiae*.

The specific growth rates (μ) measured for each yeast at 10, 20 and 30 °C are shown in Table 3. As seen, the growth rates increased with temperature, being maximal at 30 °C for all yeasts studied. *Candida krusei* strains 1179 and 1180 presented the highest specific

Table 3. Specific growth rates (μ) of the yeast strains isolated from refrigerated foods and *Saccharomyces cerevisiae* used as control. Data are means of at least 4 determinations. Standard deviations were less than 11.1% of the values

Strains	$\mu\ h^{-1}$		
	10 °C	20 °C	30 °C
C. colliculosa 1176	0.027	0.245	0.327
C. colliculosa 1177	0.033	0.234	0.277
C. krusei 1179	0.021	0.276	0.450
C. krusei 1180	0.018	0.266	0.435
D. hansenii 1157	0.032	0.265	0.390
K. marxianus 1182	0.030	0.235	0.406
T. delbrueckii 1335	0.032	0.250	0.368
Sacch. cerevisiae 1223	0.029	0.225	0.343

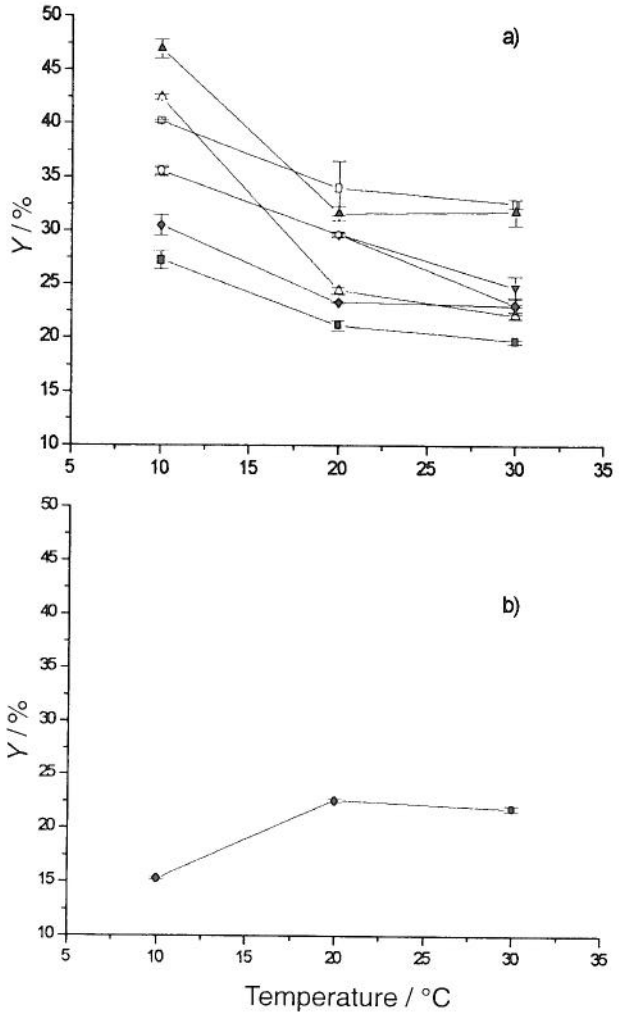

Fig. 1. Cell yield Y at different temperatures: a) strains isolated from refrigerated foods: *Debaryomyces hansenii* 1157 (▲), *Candida colliculosa* 1176 (■), *Candida colliculosa* 1177 (□), *Candida krusei* 1179 (Δ), *Candida krusei* 1180 (▼), *Kluyveromyces marxianus* 1182 (○), *Torulaspora delbrueckii* 1335 (◆); b) *Saccharomyces cerevisiae* 1223 (●) used as a control.

growth rates at 20 and 30 °C, their μ at 10 °C being similar to those attained by the rest of the yeasts.

The response of the cell yield coefficient (Y) to temperature at starting concentration of substrate (1%) is shown in Fig. 1. Cell yield seemed to be dependent on temperature over the range tested (10 to 30 °C) for yeasts isolated from refrigerated foods. In contrast with results of μ previously described, cell yields increased when temperature decreased, maximum Y values being obtained at 10 °C. As observed, the plot of *Sacch. cerevisiae* (Y_{min} at 10 °C, Y_{max} at 20–30 °C) was completely different from the correspondent plot of cold-tolerant yeasts that exhibited higher yields at low temperature.

Fig. 2. shows the specific rates of glucose consumption (q_{glc}) as a function of temperature. Minimal rates were obtained at 10 °C, corresponding to the lowest specific growth rates observed. In accordance, glucose was consumed at higher rates at more elevated temperatures (20, 30 °C).

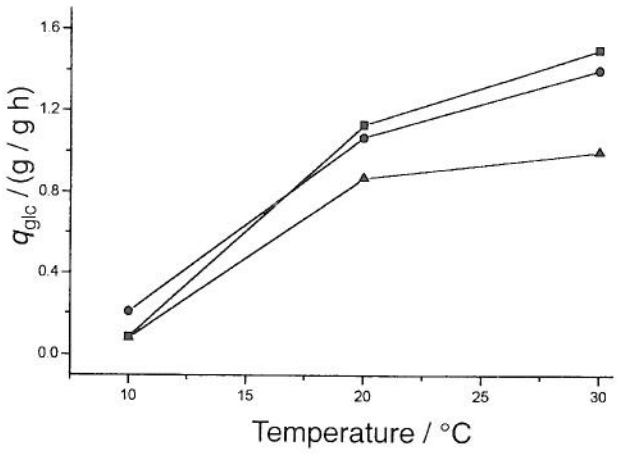

Fig. 2. Specific rate of glucose consumption of (q_{glc}) at different temperatures of *Debaryomyces hansenii* 1157 (▲), and *Candida colliculosa* 1176 (■), isolated from refrigerated foods, and *Saccharomyces cerevisiae* 1223 (●) used as control.

Table 4. Fermentation expressed as ethanol produced per glucose consumed (mmol EtOH/mmol glucose) of selected strains isolated from refrigerated foods: *Debaryomyces hansenii* 1157, and *Candida colliculosa* 1176, and *Saccharomyces cerevisiae* 1223 used as control.

Strains	mmol Ethanol/mmol Glucose		
	10 °C	20 °C	30 °C
C. colliculosa 1176	0.42	0.28	0.32
D. hansenii 1157	0.12	0.17	0.20
Sacch. cerevisiae 1223	0.95	1.12	0.93

Fermentation expressed as mmol of ethanol produced per mmol of consumed glucose was not influenced by incubation temperature as shown in Table 4 for strains *C. colliculosa* 1176, *D. hansenii* 1157 and *Sacch. cerevisiae* 1223.

Discussion

Refrigerated food and beverages are susceptible to contamination and subsequent spoilage by cold-tolerant yeasts, especially when additional factors contribute, such as low pH, medium or high sugar content, or vacuum or low oxygen concentration. The yeast strains studied in the present work were isolated from spoiled yoghurts and vacuum packed beet, characterised by some of the mentioned features.

Cold-tolerant yeasts are grouped in two categories, psychrophilic and psychrotolerant yeasts, according to cardinal temperatures needed necessary for growth. Specifically, psychrophiles are those with optimum and maximum temperatures for growth below 25 °C *(3)*. Based on that, our isolated strains would be considered as psychrotolerant mesophilic yeasts, with t_{max} at or above 30 °C, with optimum specific growth rates at temperatures around 30 °C, and with the ability to grow at low temperatures, *e.g.* 5 °C (Tables 2 and 3).

Nevertheless, when growth yields were calculated as grams of biomass formed per 100 grams of consumed

substrate at different temperatures, the results did not appear to correlate with the observed growth rates: *i.e.* the maximum yields were attained at low temperatures (10 °C) at which the lowest specific growth rates were measured (Table 3 and Fig. 1). As shown in Fig. 1, the cell yield of the cold-tolerant isolates was dependent on temperature over the range studied (10–30 °C), and increased as temperature decreased. We found a similar behaviour in two cold-tolerant *Candida sake* strains isolated from Antarctic soil. These yeasts could be grown in a tempeerature range of 0–30 °C, with maximum specific growth rates between 18 and 25 °C. The logarithm of biomass yield decreased linearly with temperature: yield was 50.1% at 5 °C, but only 19.7% at 30 °C *(6)*. However, *Sacch. cerevisiae* exhibited a completely different response to temperature changes, with lower μ and *Y* as temperature dropped from 30 °C to 10 °C. This latter result is in agreement with the common knowledge that biomass yield is largely independent of temperature up to a characteristic value, beyond which the yield falls rapidly *(8,9)*.

The study of glucose consumption of representative strains isolated from refrigerated foods revealed that growth was balanced at each of the tempereatures assayed (10, 20 and 30 °C). As shown in Fig. 2, the specific rates of glucose consumption were minimal at low incubation temperature (μ was also minimal) and higher at 20 and 30 °C, *i.e.* temperatures at which maximum growth rates were observed.

Occurrence of alcoholic fermentation has a negative effect on the biomass yield on sugar. Several environmental factors can trigger alcoholic fermentation in yeasts, depending on their physiological type. The most common of these is oxygen deprivation. Nevertheless, in the experimental conditions used in this study (non-airtight flasks, incubation in rotatory shaken bath) oxygen limitation is much less likely to occur at any of the temperatures studied. As shown in Table 4, alcoholic fermentation was not influenced by incubation temperature in the selected strains *D. hansenii* 1157, an oxidative yeast, *C. colliculosa* 1176, an oxidative/fermentative species, and in a typically fermentative mesophilic yeast *Sacch. cerevisiae* 1223 used as control. Consequently, the observed changes in biomass yields could not be explained on the basis of a modification in the fermentation patterns.

In conclusion, growth rate and specific glucose consumption rates exhibited a typically mesophilic response to temperature, but biomass yield did not. A mechanism for this could involve the evolution of different sensitivities of anabolic and catabolic reactions to temperature.

Cold tolerance and psychrophily could be defined from our results as a better adaptation to growth at low temperatures, this not being a function of μ (growth rate), but by considering the cell yield as a measure of the efficiency of growth. The specific growth rate attained by any strain depends on the rate of substrate consumption and the efficiency at which this substrate is transformed into cell biomass. If the studied cold-tolerant strains grew at low temperature with the same yield that they presented at high temperature, their growth would be irrelevant in cold environments. They could also compete with mesophilic microorganisms growing rapidly at higher temperatures, if the storage conditions were inadequate. Thus, cold-tolerant yeasts contaminating refrigerated foods seem to be well adapted to their environments and should be specifically controlled in food industries, as it has been demonstrated that chill storage by itself cannot be relied upon to prevent yeast development in these commodities *(5)*.

To control spoilage of foods caused by yeasts, it is essential to develop basic knowledge about the physiology of yeasts and the expression of their potential as influenced by ecological parameters associated with various foods.

Acknowledgments

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References

1. T. Deák, L. R. Beuchat: *Handbook of Food Spoilage Yeasts*, CRC Press. Inc., Boca Raton, Florida (1996).
2. T. Deák, Foodborne Yeasts. In: *Advances in Applied Microbiology, Vol. 36*. Academic Press, London (1991) pp. 179–277.
3. N. van Uden, *Adv. Microbiol. Physiol. 25* (1984) 195.
4. K. G. Watson, Temperature relations. In: *The Yeasts, 2nd ed., Vol. 2. Yeasts and the Environment*, A. H. Rose, J. S. Harrison (Eds.), Academic Press, London (1987) pp. 41–72.
5. E. A. Tudor, R. G. Board, Food-Spoilage Yeasts. In: *The Yeasts, 2nd ed., Vol. 5*, A. H. Rose, J. S. Harrison (Eds.), Academic Press, London (1993) pp. 435–516.
6. A. Santos, M. J. Valderrama, J. M. Peinado, *2ª Jornadas de levaduras Nicolau van Uden* (1994).
7. N. van Uden, *Arch. Microbiol. 58* (1967) 155.
8. S. J. Pirt: *Principles of Microbe and Cell Cultivation*, Blackwell Sci. Publ., Oxford (1985).
9. B. A. Atkinson, F. Mavituna: *Biochemical Engineering and Biotechnology Handbook, 2nd ed.*, Stockon Press, New York (1991).

Inkubacija pri niskim temperaturama povećava udjel biomase u kvascima izoliranim iz hladnih staništa

Sažetak

Kinetika rasta psihrotolerantnih sojeva kvasaca (Candida colliculosa, Candida krusei, Debaryomyces hansenii, Kluyveromyces marxianus and Torulaspora delbrueckii) izoliranih iz zamrznute hrane, istraživana je pri temperaturama od 10 do 30 °C. Usporedno s njima ispitivan je mezofilni soj Saccharomyces cerevisiae. Arrheniusov prikaz specifične brzine rasta prema temperaturi pokazao je slične profile za izolate psihrotolerantnih sojeva i za mezofilni soj. Maksimalna specifična brzina rasta (μ) postignuta je pri 30 °C. Međutim, povećanje biomase ne ovisi o opaženim brzinama rasta. Maksimalni prinosi postignuti su pri niskim temperaturama (10 °C), pri kojima je izmjerena najniža specifična brzina rasta, a mali je prinos dobiven pri 30 °C, gdje je opažena najveća brzina rasta. Nasuprot tome, soj Sacch. cerevisiae nije se tako ponašao. Promjene brzine utroška glukoze pri ispitivanim temperaturama ovisile su o opaženim brzinama rasta, ali ponovno ne i s izmjerenim prinosom biomase. Ovi su rezultati uspoređeni s podacima autora dobivenim sa Candida sake, antarktičkim sojevima koji su se slično ponašali. Prema tome, povećanje prinosa kvasaca pri niskim temperaturama moglo bi se smatrati mehanizmom tolerancije na hladnoću, omogućavajući uspješan razvoj dobro adaptiranim sojevima, ali uz smanjenu brzinu rasta pri niskim temperaturama okoliša.