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DIVERSITY OF PHYSIOLOGICAL AND BIOCHEMICAL PROPERTIES WITHIN YEAST SPECIES OCCURRING IN ROKPOL CHEESE

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Sixty seven representatives of the seven yeast species existing in Polish blue veined cheese Rokpol were examined for their ability to grow at low temperature and low water activitiy (a_w), to assimilate lactose, lactic and citric acids, produce extracellular proteolytic and lipolytic enzymes and killer toxins. All strains examined were able to grow at 10°C. At the same temperature in the presence of 5% (a_w=0.961) and 10% NaCl (a_w=0.902) the best growth was displayed by the strains of *C. famata* and *C. sphaerica*, but yeasts *G. penicillatum* and *C. lipolytica* were completely inhibited at a_w 0.902. All strains of *C. famata* (32/32), *C. sphaerica* (13/13), *C. intermedia* (5/5) and *C. kefyr* (4/4) but none of *C. lipolytica* (0/5), *S. kluyveri* (0/4) and *G. penicillatum* (0/4) were able to metabolise lactose. Only *C. lipolytica* strains tested utilised both lactic and citric acids. These abilities in the case of other yeast species were strain dependent. The highest activities of both proteolytic and lipolytic extracellular enzymes were demonstrated for *C. lipolytica* strains. Other yeast species investigated varied in the proportion of strains which were positive and the activity level of proteolytic and lipolytic enzymes. Strains of *C. famata* and *C. sphaerica* produced killer toxins which were active exclusively against representatives of *C. lipolytica* species.

INTRODUCTION

Yeasts are a substantial part of non-starter microflora of many cheese varieties. The most numerous yeast populations of 10^6–10^9 CFU/g can develop on the surface of mould and smear cheeses whereas in the interior of the cheeses their levels are 10–100 fold lower [Tempel & Jakobsen, 1998; Eliskases-Lechner *et al.*, 1998; Wojtatowicz *et al.*, 2001].

The high numbers of yeasts in cheeses are linked with their nutritional requirements, certain enzyme activities, especially extracellular proteolytic and lipolytic enzymes and the ability to grow at low temperature and pH but high salt concentration [Roostita & Fleet, 1996; Besancon *et al.*, 1992]. Positive interactions with other microorganisms present in cheese as well as killer activity or resistance are also contributing to the dominance of certain yeast species in cheese environment [Seiler & Busse, 1990; Fleet, 1998; Tornadijo *et al.*, 1998].

Blue veined cheese varieties, like Roquefort, Danablu, Gorgonzola, Valdon, are predominated, by the following species: *Debaryomyces hansenii* (anamorph *Candida famata*), *Kluyveromyces marxianus* ssp. *lactis* (anamorph *C. sphaerica*) and *C. catenulata* [De Boer & Kuik, 1987; Lopez-Diaz *et al.*, 1995; Roostita & Fleet, 1996, 1999; Tempel & Jakobsen, 1998]. Most frequently isolated species from Rokpol cheese were *Candida famata* and *C. sphaerica* followed by *C. intermedia* and *Geotrichum* spp.; other species, such as *C. lipolytica*, *C. kefyr* and *Saccharomyces kluyveri* were found irregularly and at significantly lower levels [Wojtatowicz *et al.*, 2001; Chrzanowska *et al.*, 2003].

Yeasts, depending on their number and composition, can affect the ripening process of cheese positively (de-acidification, aroma formation) or negatively (smell and taste defects) [Roostita & Fleet, 1996; Eliskases-Lechner, 1998; Tornadijo *et al.*, 1998]. It is regarded that the deliberate use of appropriate yeast strains as additional starter cultures in cheese manufacturing could result in obtaining standard quality cheese and developing new cheese varieties. Strains belonging to the species *Debaryomyces hansenii* / *Candida famata*, *Yarrowia lipolytica* / *Candida lipolytica* and *Saccharomyces cerevisiae* seem to be the most promising for these purposes [Besancon *et al.*, 1992; Tempel & Jakobsen, 2000; Hansen *et al.*, 2001; Suzzi *et al.*, 2001; Ferreira & Viljoen, 2003].

The aim of the study was to examine selected, but important from the technological point of view, properties of yeasts occurring in Polish blue veined cheese Rokpol. The examinations carried out included: growth at low temperature and water activity, assimilation of lactose, lactic and citric acids, hydrolysis of tributyrin and casein and killer activity.

MATERIAL AND METHODS

Microorganisms. 67 yeast strains isolated from blue-veined Rokpol cheeses and identified as described by Wojtatowicz *et al.* [2001] were used in this study. They were representatives of seven yeast species associated with the prod-

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uct: *Candida famata* (N=32), *Candida sphaerica* (N=13), *Candida intermedia* (N=5), *Candida lipolytica* (N=5), *Candida kefyr* (N=4), *Saccharomyces kluyveri* (N=4), and *Geotrichum penicillatum* (N=4). The strains were kept on YMPG-agar slopes (0.3% Yeast Extract, Difco; 0.3% Malt Extract, Difco; 0.5% Bacto Peptone, Difco; 1.0% Glucose, POCH, Poland; 1.5% Agar-agar, Merck), at +4°C. For all assays, the yeasts were grown on the YMPG-agar at 28°C for 2 days.

Growth at a low temperature and a low water activity (a_w). MEPS-agar (3% Malt Extract, Difco; 0.3% Soy Peptone, Merck; 1.5% Agar-agar, Merck) supplemented or not with NaCl (0%, 5% and 10% w/v) was used in the experiments. Corresponding water activities (a_w) of resulting media measured with a Novasina Thermoconstanter (Novasina AG, Switzerland) were 0.997, 0.961, 0.902, respectively. The MEPS-agar plates of different a_w were spot inoculated with 1–4 yeast strains and incubated at 20°C and 10°C. Three replicates were prepared for each combination of temperature and a_w. After 14-day incubation, time giant colony diameters were measured manually. The data are shown as the range of mean colony diameters within yeast species.

Assimilation of lactose, lactic acid and citric acid. YNB-agar (0.67% Bacto Yeast Nitrogen Base, Difco; 2% lactose, lactic acid or citric acid; 1.5% Agar-agar, Merck) plates were spot inoculated with 1–4 yeast strains. Three replicates were prepared for each strain and carbon source. After 14-d incubation at 28°C giant colony diameters were measured. The data are shown as the number of positive reaction of strains and the range of mean colony diameters within yeast species.

Determination of extracellular proteolytic and lipolytic activity. The ability of yeasts to produce extracellular proteolytic and lipolytic enzymes was examined by means of agar plates containing Milk-agar and Tributyrin-agar, respectively as described by Harrigan [1998]. The plates (in three replicates) were spot inoculated with 1–4 strains and incubated at 28°C for 5 days. Afterwards the width (d) of the clear zone around colonies was measured. Activity level for both, proteolytic and lipolytic enzymes, was reported as follows: no (0); low ($d<2$ mm); medium ($2\ mm \leq d < 10$ mm); and high ($d \geq 10$ mm).

Determination of killer activity. The capability of yeast strains to kill each other was examined on YEPG agar (0.3% Yeast Extract, Difco; 0.5% Bacto Peptone, Difco; 1% Glucose, POCH, Poland; 1.5% Agar-agar, Merck) supplemented with 30 ppm Methylene blue (MB), Merck, and buffered at pH 4.6 with 0.1 mol/L citric acid-phosphate buffer. On a lawn of potential sensitive strain ($5x10^5$ cells in Petri dishes of 90 mm in diameter), the tested strains were streaked in thick lines and cultivated at 14°C for 3 days. The streaked strain was designated a killer yeast if it was surrounded by a clear zone of inhibition or a region of bluish-coloured cells, or both. All experiments were run in triplicates. The data are shown as the number of positive killer responses divided by the total of mutual testing made.

RESULTS AND DISCUSSION

Rokpol cheese ripens for 6–8 weeks at 10–12°C. During the ripening process, pH is gradually increasing from 4.7 to above 6.0. Salt concentration reaches the level of 2.5–5% [Chrzanowska *et al.*, 2003]. As yeasts, in general, prefer slightly acidic media with optimum pH between 4.5–6.5 their growth in cheese will be affected mainly by a low temperature and a low water activity in the presence of NaCl.

The growth characteristics of all yeast species examined shows that the environmental conditions prevailing in Rokpol during ripening (10°C and a_w up to 0.961 in the presence of 5% NaCl) will support growth of these yeasts (Table 1). At the same temperature in combination with 10% salt (a_w 0.902), a very strong growth was displayed by some strains of *Candida famata* and *C. sphaerica*, followed by *C. intermedia* and *Saccharomyces kluyveri*. The most sensitive were the strains of *Geotrichum penicillatum* and *C. lipolytica*; their growth was restricted by the level of NaCl (a_w=0.902).

Other authors also reported that yeast species *Debaryomyces hansenii* / *Candida famata* and *Kluyveromyces marxianus* ssp. *lactis* / *C. sphaerica* were the most tolerant to combined effects of a low temperature and high salt concentrations [Besancon *et al.*, 1992; Roostita & Fleet, 1996; Tempel & Jakobsen, 1998; Betts *et al.*, 1999]. They confirmed their domination in semi-soft cheeses over other yeast species [Seiler & Busse, 1990; Besancon *et al.*, 1992; Cosentino *et al.*, 2001]. It is worth noting that the strains of *C. lipolytica* investigated in this work appeared to be more halotolerant, compared to the strains of the same species originating from Danablu cheese which were not be able to

TABLE 1. Range of mean giant colony diameters (mm) for strains within tested yeast species at different combinations of temperatures and water activities.

Yeast species (number of strains)	Temperature					
	20°C			10°C		
	Water activity (a_w)					
	0.997	0.961	0.902	0.997	0.961	0.902
C. famata (32)	5.7–19.0	6.4–18.2	4.2–17.4	4.5–10.8	3.5–9.3	2.4–9.1
C. sphaerica (14)	8.0–13.8	5.7–15.0	2.8–12.5	3.9–9.9	2.8–7.9	1.7–7.4
C. intermedia (5)	8.5–11.4	7.3–10.3	4.4–7.3	5.0–7.7	4.0–6.6	2.2–4.6
C. lipolytica (5)	10.5–21.8	8.0–17.8	2.8–8.6	5.5–10.6	2.9–6.0	0
C. kefyr (4)	7.8–10.7	6.6–8.1	3.8–5.1	4.3–6.5	2.7–4.1	0–2.2
S. kluyveri (4)	8.2–12.0	8.3–15.4	5.2–9.6	4.9–8.0	4.8–8.0	3.4–5.6
G. penicillatum (4)	36.8–48.4	10.6–16.0	0	13.4–16.8	3.5–6.5	0

grow at 4.5% NaCl [Tempel & Jakobsen, 2000]. Data in Table 1 clearly demonstrates significant differences at subspecies levels, which is in agreement with the results observed by Besancon *et al.* [1992], Tempel and Jakobsen [1998], Tornadijo *et al.* [1998], and Betts *et al.* [1999].

The assimilation of lactose and lactic and citric acids was a characteristic of yeast species, however differences in growth intensity were found among strains of the same species. All strains of *C. famata*, *C. sphaerica*, *C. intermedia* and *C. kefyr* but none of *C. lipolytica*, *S. kluyveri*, *G. penicillatum* strains that were examined in this study were able to utilise lactose (Table 2). Similar relationships were earlier reported by Besancon *et al.* [1992], Haridy [1993], Tempel and Jakobsen [1998], and Cosentino *et al.* [2001]. The strong ability of *C. famata* and *C. sphaerica* to assimilate lactose is considered to be a key property contributing to their growth and predominance in cheeses and dairy products [Roostita & Fleet, 1996]. Moreover, such strains used as starter cultures could reduce the risk of wild fermentations in cheese, especially in regard of non-starter lactic acid bacteria.

Only *C. lipolytica* strains tested were able to assimilate both, lactic and citric acids. In the case of other yeast species examined those abilities were strain dependent. For example lactic acid was utilised by 23 out of 32 *C. famata* strains and 8 out of 13 strains of *C. sphaerica*, whereas growth on citrate was displayed by 16 out of 32 *C. famata* and by 4 out of 13 *C. sphaerica* strains (Table 2).

Other authors noticed a higher percentage of strains of the above-mentioned species capable of using lactic acid as a source of carbon [Besancon *et al.*, 1992; Haridy, 1993; Tempel & Jakobsen, 1998; Cosentino *et al.*, 2001].

Strains of *C. famata* and *C. sphaerica* metabolising lactose but not lactic acid may be of a great interest in cheese making because they grow well without causing a change in pH value. These strains are quite important for manufacturing of blue veined cheeses in the formation of "morge" – a slimy material partly composed of microbial cells, which allows good sticking of tinfoil to the surface of these cheeses [Besancon *et al.*, 1992]. On the other hand, the assimilation of acids by other strains of these species may lead to an increase in pH value, which in turn creates better conditions for the growth of lactic acid bacteria and activity of their proteolytic and lipolytic enzymes essential for cheese ripening [Pereira-Dias *et al.*, 2000].

Due to proteolytic and lipolytic activities, some yeasts may play an important role in the production of aroma precursors (amino acids, fatty acids, esters) and reduction of cheese ripening period [Suzzi *et al*., 2001; Ferreira & Viljoen, 2003]. Furthermore, they can stimulate the growth of other microorganisms by the excretion of growth factors [Tempel & Jakobsen, 2000].

Of the yeast isolates examined in this study the highest activities of both proteolytic and lipolytic extracellular enzymes (determined on milk agar and tributyrin agar, respectively) were revealed by strains belonging to *C. lipolytica* species (Table 3).

TABLE 3. Extracellular proteolytic (P) and lipolytic (L) activities of strains within tested yeast species.

Species	Type of activity	Activity level			
		high	medium	low	no
C. famata	P	0/32[a]	1/32	4/32	27/32
	L	0/32	6/32	23/32	3/32
C. sphaerica	P	0/13	3/13	2/13	8/13
	L	0/13	1/13	12/13	0/13
C. intermedia	P	0/5	0/5	0/5	5/5
	L	0/5	0/5	5/5	0/5
C. lipolytica	P	5/5	0/5	0/5	0/5
	L	3/5	2/5	0/5	0/5
C. kefyr	P	0/4	4/4	0/4	0/4
	L	0/4	1/4	3/4	0/4
S. kluyveri	P	0/4	0/4	0/4	4/4
	L	0/4	0/4	3/4	1/4
G. penicillatum	P	0/4	0/4	0/4	4/4
	L	0/4	0/4	0/4	4/4

[a] number of positive reactions of strains over the total number of strains examined

Strong abilities of this species to hydrolyse caseins, milk fat and other fat substrates are well documented [Lopez-Diaz *et al.*, 1995; Roostita & Fleet, 1996; Tempel & Jakobsen, 1998; Cosentino *et al.*, 2001; Suzzi *et al*., 2001]. The studies concerning their role in cheese ripening are, however, relatively few [Guerzoni *et al.*, 1998; Ferreira & Viljoen, 2003].

In contrast to *C. lipolytica*, none of 5 strains of *C. intermedia* and 4 strains of *S. kluyveri* possessed extracellular proteinase activity. Strains of *G. penicillatum* (4 out of 4) did not exhibit extracellular lipolytic activity either. Other yeast species investigated varied in the proportion of strains to be positive and the level of each hydrolytic activity. For exam-

TABLE 2. Frequency of strains within tested yeast species able to assimilate lactose (Lac), lactic acid (LA) and citric acid (CA) and range of mean giant colony diameters (mm).

Yeast species	Lac		LA		CA	
C. famata	32/32[a]	(4.2–8.8)	3/32	(4.3–5.6)	16/32	(2.8–5.3)
C. sphaerica	13/13	(4.4–9.4)	8/13	(3.2–7.6)	4/13	(3.9–5.4)
C. intermedia	5/5	(1.6–7.2)	5/5	(3.8–4.4)	3/5	(3.3–4.2)
C. lipolytica	0/5	–	5/5	(10.8–14.4)	5/5	(7.0–11.9)
C. kefyr	4/4	(7.4–9.6)	4/4	(6.3–7.5)	0/4	–
S. kluyveri	0/4	–	3/4	(2.9–3.8)	0/4	–
G. penicillatum	0/4	–	4/4	(40.1–44.8)	0/4	–

[a] number of positive reactions of strains over the total number of strains examined

ple, the majority of *C. famata* (29 out of 32) and *C. sphaerica* (13 out of 13) strains exhibited lipolytic activity but only 5 out of 32 and 5 out of 13 strains, respectively, possessed extracellular proteinase activity and these activities were rather low (Table 3).

Roostita and Fleet [1996] reported the absence of both activities in *D. hansenii* / *C. famata*, although Besancon *et al.* [1992] found a small proportion of strains to be positive for these activities. Our observations are in agreement with the results obtained by Lopez-Diaz *et al.* [1995], who reported significant variations in proteolytic and lipolytic activities between strains within *Debaryomyces hansenii* / *C. famata*, *Kluyveromyces marxianus* ssp. *lactis* / *C. sphaerica* and *Yarrowia lipolytica* / *C. lipolytica* yeast species.

At 14°C and pH 4.6, *C. famata* and *C. sphaerica* species predominating in Rokpol cheeses produced killer toxins which were active exclusively against representatives of *C. lipolytica* species (Table 4). No negative interactions were observed between strains belonging to the same yeast species. Among five tested strains of *C. lipolytica* only one was resistant towards killer proteins secreted by each of the 12 examined strains of *C. famata* and 5 strains of *C. sphaerica*. It may explain the low frequency of yeast *C. lipolytica* in Rokpol cheese.

TABLE 4. Ratio of positive killer responses and the total number of mutual testing made.

Tested species (number of strains)	Lawn seeded on YEPG-MB agar medium				
	C. f. (N=12)	*C. s.* (N=5)	*C. l.* (N=5)	*C. k.* (N=2)	*G. p.* (N=4)
C. famata (n=12)	0/132	0/60	48/60	0/24	0/48
C. sphaerica (n=5)	0/60	0/20	8/25	0/10	1/20
C. lipolytica (n=5)	0/60	0/25	0/20	0/10	1/20
C. kefyr (n=2)	0/24	0/10	0/10	0/2	0/8
G. penicillatum (n=4)	0/48	0/20	0/20	0/8	0/12

In contrast, Addis *et al.* [2001], who studied killer phenomenon among yeasts associated with Camembert and blue-veined cheese varieties, found that a few strains of *D. hansenii* were able to inhibit other strains of this species but no antagonism of *D. hansenii* towards *Y. lipolytica* was observed.

CONCLUSIONS

In conclusion, our study has shown that out of 7 yeast species occurring in Rokpol cheese, *Candida famata* and *C. sphaerica* revealed the best growth at a low temperature of 10°C and a low water activity in the presence of 5% and 10% NaCl. All *C. famata* and *C. sphaerica* strains investigated were capable of utilising lactose and most of them exhibited killer activity, which may explain their predominance in Rokpol cheese [Wojtatowicz *et al.*, 2001; Chrzanowska *et al.*, 2003]. In addition, some strains of the above-mentioned species possessed the ability to metabolise lactic and citric acids and produce lipolytic and proteolytic enzymes, which indicates positive contribution of this microorganisms to the maturation of cheese.

Nevertheless, the most promising – from the technological point of view – appeared to be strain of *C. lipolytica* which revealed the highest lipolytic and proteolytic activities and assimilated both lactic and citric acids. These yeasts may be considered not only as starter cultures for cheese manufacturing but also as a source of enzymes useful in the acceleration of the cheese ripening process.

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ZRÓŻNICOWANIE FIZJOLOGICZNYCH I BIOCHEMICZNYCH WŁAŚCIWOŚCI W OBRĘBIE GATUNKÓW DROŻDZY WYSTĘPUJĄCYCH W SERZE ROKPOL

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Sześćdziesiąt siedem szczepów, będących przedstawicielami 7 gatunków drożdży występujących w serach Rokpol, badano pod kątem ich zdolności do wzrostu w niskiej temperaturze i niskiej a_w, a także do asymilacji laktozy, kwasu mlekowego i cytrynowego oraz produkcji pozakomórkowych enzymów proteolitycznych i lipolitycznych. Ponadto, określano ich aktywność killerową. Wszystkie szczepy były zdolne do wzrostu w 10°C. Przy tej temperaturze w obecności 5% NaCl (a_w=0,961) i 10% NaCl (a_w=0,902) najlepszy wzrost wykazywały szczepy *C. famata* i *C. sphaerica*. Natomiast szczepy *G. penicillatum* i *C. lipolytica* były całkowicie hamowane przy niższej a_w 0,902 (tab. 1). Zdolność metabolizowania laktozy wykazywały wszystkie szczepy gatunku *C. famata* (32/32), *C. sphaerica* (13/13), *C. intermedia* (5/5) i *C. kefyr* (4/4), lecz żaden ze szczepów *C. lipolytica* (0/5), *S. klyveri* (0/4) i *G. penicillatum* (0/4). Drożdże *C. lipolytica* utylizowały zarówno kwas mlekowy jak i cytrynowy; w przypadku pozostałych gatunków zdolności te były związane ze szczepem (tab. 2). Największą aktywność proteolityczną i lipolityczną przejawiały drożdże *C. lipolytica*, zaś żadnej z tych aktywności nie stwierdzono u *G. penicillatum*. Inne gatunki cechowała duża zmienność tak pod względem ilości szczepów pozytywnych jak i poziomu każdej z tych aktywności (tab. 3). Szczepy *C. famata* i *C. sphaerica* produkowały toksyny killerowe aktywne wyłącznie wobec szczepów *C. lipolytica* (tab. 4).