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Original article

Evolution of the microflora of Kopanisti cheese during ripening. Study of the yeast flora

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Summary — The microflora of Kopanisti cheese during its ripening was studied. For this, samples were taken from the surface and the interior of the cheese 1, 8, 16, 32 and 46 days after its manufacture and the total streptococci, lactobacilli, yeast and molds counts as well as coliforms were determined. The results showed the changes in these microbial parameters during cheese ripening as well as the differences between the surface and the interior of the cheese. 86 strains of yeast from the cheese samples were also isolated and identified out of which the following species were found : *Trichosporon cutaneum* (*T. beigelii*), *Kluyveromyces lactis* (*Kl. marxianus* var. *lactis*), *Saccharomyces exiguus*, *Saccharomyces cerevisiae*, *Rhodotorula rubra*, (*Rh. mucilaginosa*), *Trichosporon penicillatum* (*Geotrichum penicillatum*), *Candida lusitaniae* (*Clavispora lusitaniae*) and *Debaryomyces hansenii*.

Kopanisti cheese — maturation — identification — yeasts

Résumé — Evolution de la flore microbienne du fromage Kopanisti au cours de la maturation. Étude des levures. *Nous avons étudié la flore microbienne qui se développe à la surface et à l'intérieur du fromage Kopanisti durant sa maturation.Dans ce but, des échantillons ont été examinés, 1, 8, 16, 32 et 46 j après la fabrication. Nous avons dénombré la flore microbienne totale, les streptocoques, les lactobacilles, les levures, les moisissures et les bactéries coliformes. Les résultats obtenus ont démontré, d'une part l'évolution de ces groupes microbiens durant la maturation du fromage, d'autre part la différence entre la surface et la partie interne. De ces échantillons, nous avons isolé 86 souches de levures parmi lesquelles nous avons identifié les espèces suivantes :* Trichosporon cutaneum *(*T. beigelii*)*, Kluyveromyces lactis *(*Kl. marxianus *var.* lactis*)*, *S*accharomyces exiguus, Saccharomyces cerevisiae, Rhodotorula rubra, *(*Rh. mucilaginosa*)*, Trichosporon penicillatum *(*Geotrichum penicillatum*)*, Candida lusitaniae *(*Clavispora lusitaniae*) et* Debaryomyces hansenii.

fromage Kopanisti — maturation — identification — levures

INTRODUCTION

Kopanisti is a well known traditional Greek cheese made of cow's, ewe's and goat's milk or mixtures of them (Zygouris, 1952; Davis, 1976; Anifantakis, 1987). It is soft, with good spreadability and a strong flavour similar to that of blue cheeses. These interesting properties of the cheese are attributed to the intensive changes during its ripening. The procedure of manufacture and ripening promotes an abundant growth of a great variety of microorgan-

isms which are considered responsible for these changes. The present work, which is part of a wider project, was carried out at the Agricultural University of Athens in order to study the microflora of Kopanisti cheese and its changes during ripening as well as the yeast flora.

MATERIAL AND METHODS

Milk

Cow's milk was utilized without antibiotics and pasteurised at 68 °C for 10 min.

Starters

Lyophilized starters of *Streptococcus lactis* and *Lactobacillus casei* (Chr. Hansen's Laboratory, Copenhagen, Denmark) were used at 2% and 0.5%, respectively. These starters were grown overnight at 30 °C in skim milk three times before use.

Rennet

Calf rennet powder (trade name HA-LA, Chr. Hansen's Laboratory, Copenhagen, Denmark) 0.3 g for 100 kg of milk was used. The rennet was added to the milk at 30 °C after an increase in its acidity of 2—3 °D produced by the starters. Under these conditions the curd was ready for cutting 3 h after the addition of the rennet.

Ripe Kopanisti cheese

Good quality of mature Kopanisti cheese was used at 1%. This ripe cheese was well mixed with fresh curd after it had been drained.

Cheese manufacture

The cheese was manufactured according to the method described by Anifantakis (1987). The main stages of this method are the addition of 4% sodium chloride to drained acid curd (pH 4.9, moisture 53.5%), mixing it with 1% of ripe Kopanisti cheese of good quality, as usually practiced and keeping the mixture in open basins at room temperature for ripening. During the ripening the mass of the cheese is mixed 3—4 times in order to distribute the microflora grown on the surface of the cheese into the whole mass. This practice permits the growth of an abundant microflora to which the flavour of the cheese is attributed. Five cheese preparations were made in total.

Sampling

Samples from the 2-cm thick surface layer and the interior of the cheese were taken under aseptic conditions 1, 8, 16, 32 and 46 days after manufacture of cheese. The samples of the 1st day were taken before salting and mixing the curd with the ripe Kopanisti.

Microbial examination of samples

The total streptococci, lactobacilli, yeast and mold counts were determined using the methods of the American Public Health Association (1967). The coliforms were enumerated according to the International Dairy Federation (1974). The media and conditions used were, for total count : milk agar (Oxoid), incubation for 3 days at 30 °C; for streptococci : M-17 agar (Merck), incubation for 2 days at 37 °C under $CO_2 + H_2$ atmosphere (Gas-Pak system); for lactobacilli : MRS Agar at pH 5.7 (Oxoid), incubation for 2 days at 37 °C under $CO_2 + H_2$ atmosphere; for yeasts and molds : chloramphenicol yeast extract glucose agar (IDF 1980), incubation for 2 days at 37 °C.

Yeasts were isolated from chloramphenicol yeast extract glucose agar medium, purified by streaking on the same medium and maintained

at 4 °C on the surface of potato dextrose agar (Oxoid).

The idenfication of yeasts was made according to the taxonomy of Barnett *et al.* (1979) and Lodder & Kreger-van Rij (1970) on the basis of their morphological and biochemical characteristics. Microscopic appearance of yeasts on slide cultures, anaerobic fermentation of 10 sugars (D-glucose, D-galactose, D-maltose, sucrose, α-D-trehalose; milibiose, lactose, D-cellobiose, melezitose and raffinose), resistance to actidione (0.01% and 0.1%) and assimilation of ethanol were carried out as described by Harrigan & Mc Cance (1976), while formation of ascospores or other spores in Gorodkowa agar (Van Der Walt, 1970), with growth at 37 °C and assimilation of nitrate in Bacto yeast base (Difco) were performed according to Wickerham (1951). Also, assimilation of carbon sources was investigated using the API system * 50 CH.

The enzyme pattern of the yeasts was done using the rapid miniaturized system API-ZYM.

RESULTS AND DISCUSSION

Evolution of the microflora of Kopanisti cheese during ripening

The evolution of the total streptococci, lactobacilli, yeast and mold counts grown on the surface and in the interior of the Kopanisti cheese after 1, 8, 16, 32 and 46 days of ripening is presented in Figures 1, 2, 3 and 4 respectively. It can be easily seen that the values at each stage of the ripening for the total streptococci and lactobacilli counts were similar for the surface and the interior of the cheese. By contrast, yeasts and molds were found to grow significantly faster on the surface.

Figure 1 shows that the total colony counts for the surface and the interior of the cheese during ripening ranged from 10^7—10^9/g of cheese and that the maximum values were obtained at the 1st day of the ripening. Between the 1st and the 8th day, a small decrease of these values was observed. Afterwards the counts remained nearly constant. Similar results were also obtained for Camembert (Kikuchi, 1966) and Roquefort (Devoyod *et al.*, 1968).

The number of the streptococci and their changes during ripening of Kopanisti cheese were similar to that of the total colony counts (Fig. 2). This was also observed in Camembert cheese (Lenoir, 1962).

Contrary to the total colony and streptococci counts, the numbers of lactobacilli showed a significant increase from the 1st to the 8th day and remained nearly constant thereafter throughout the ripening.

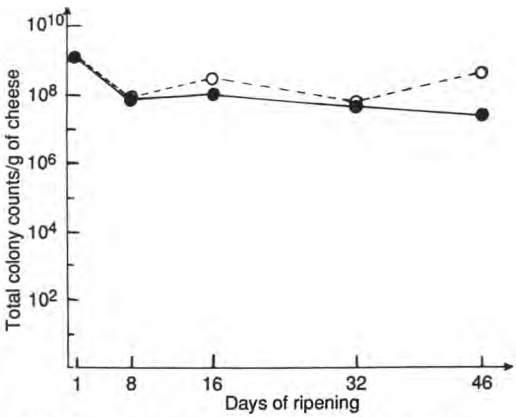

Fig. 1. Changes in total counts of microorganisms during ripening of Kopanisti cheese (●—● interior of cheese, ○—○ surface layer of cheese).
Evolution de la flore microbienne totale du fromage Kopanisti durant la maturation (●—● intérieur du fromage, ○—○ extérieur du fromage).

* API system : La Balme-les-Irattes, 38390 Montalieu-Vercieu, France.

Significant increase of lactobacilli during the first days of ripening was also observed in Manchego (Ordonez *et al.*, 1978) and Kefalotyri (Anifantakis & Kaminarides, 1987) and was attributed to the higher resistance of the lactobacilli to the low acidities (Harrigan & McCance, 1976).

The changes in yeasts and molds counts of Kopanisti cheese during ripening are shown in Figure 4. Comparing these results to those presented in the Figures 1, 2 and 3 it can be seen that yeasts and molds grow in a different way from the other groups of microorganisms. Their number was significantly higher on the surface than in the interior of the cheese and increased gradually up to the 16th and 32nd day, respectively. Then a small decrease was observed. This group of microorganisms was found to show a similar growth tendency in Camembert cheese. In this case, the maximum values were observed at the 10th day for the surface and 15th day for the interior of the cheese (Schmidt & Lenoir, 1978).

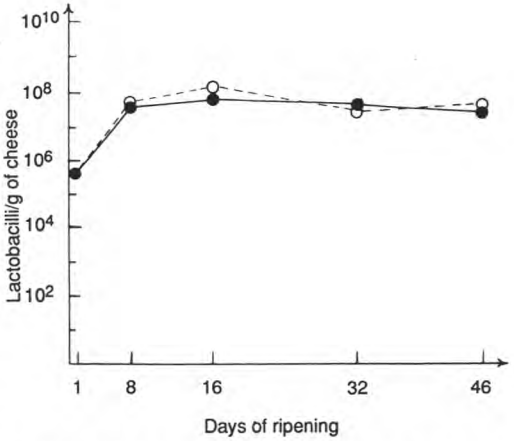

Fig. 3. Changes in lactobacilli counts during ripening of Kopanisti cheese (●—● interior of cheese, ○—○ surface layer of cheese).
Evolution des lactobacilles du fromage Kopanisti durant la maturation (●—● intérieur du fromage, ○—○ extérieur du fromage).

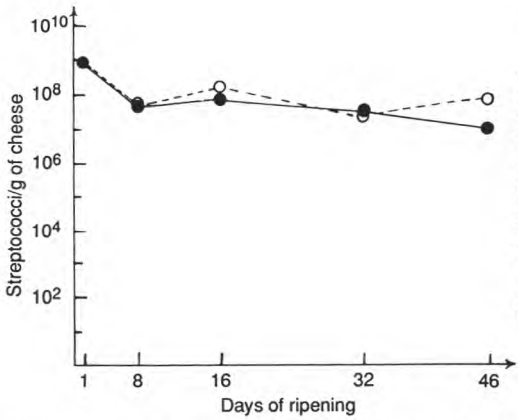

Fig. 2. Changes in streptococci counts during ripening of Kopanisti cheese (●—● interior of cheese, ○—○ surface layer of cheese).
Evolution des streptocoques du fromage Kopanisti durant la maturation (●—● intérieur du fromage, ○—○ extérieur du fromage).

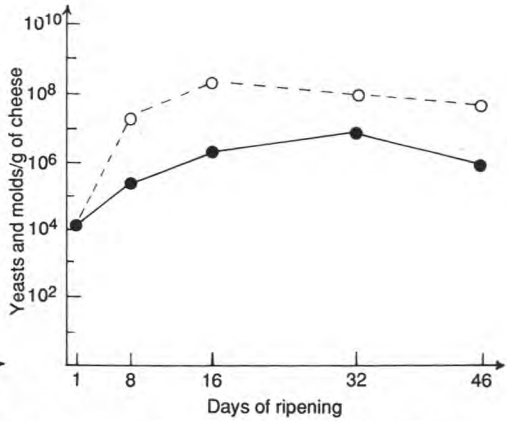

Fig. 4. Changes in yeasts and molds counts during ripening of Kopanisti cheese (●—● interior of cheese, ○—○ surface layer of cheese).
Evolution des levures et des champignons du fromage Kopanisti durant la maturation (●—● intérieur du fromage, ○—○ extérieur du fromage).

A small number of coliforms in Kopanisti cheese was observed on the surface at the beginning of ripening. They may be considered as contaminants. These microorganisms disappeared during the process of ripening and were not found in ripe cheese. The presence of coliforms in fresh cheese as contaminants, and their decrease or disappearance in ripe cheese was also observed in other types of cheeses such as Cottage, Roquefort, Cheddar, Feta, Camembert, Manchego, Telemes, Gruyère, Halloumi, Kefalotyri, etc. (Schultze & Olson, 1960; Devoyod & Bret, 1966; Dommet, 1970; Veinoglou *et al.*, 1974; Mourgues *et al.*, 1977; Ordonez *et al.*, 1978; Veinoglou *et al.*, 1980; Zerfiridis *et al.*, 1984; Kaminarides & Anifantakis, 1985; Anifantakis & Kaminarides, 1987). The low pH of the cheese inhibits the growth of the acid sensitive coliform bacteria and leads to their disappearance from ripe cheese (Kosikowski & Fox, 1968; Reamer *et al.*, 1974).

Study of the yeast flora

The yeasts represent a substantial part of the flora of Kopanisti cheese mainly attributed to the special handling during the cheese manufacture.

The study of the yeast flora was carried out to identify the major species of yeasts and their enzymatic activities.

Eighty-six strains of yeasts taken from samples of different ages were isolated and identified. The results of yeast identification as well as some biochemical activities which were not included in taxonomy system used and that could be new useful characteristics are given in Table I.

It is obvious that *Trichosporon cutaneum (T. beigelli)* is the yeast which dominates in the cheese. From the 86 identified strains of yeasts, 61 were found to belong to this species. Out of these 61 strains, 47 were found in ripe and 14 in unripe cheese. The high frequency of *Trichosporon cutaneum* in Kopanisti cheese could be attributed to the wide occurrence of this species in nature and to the fact that it is frequently associated with humans (Lodder & Kreger-Van Rij, 1970). *Kluyveromyces lactis* (*Kl. marxianus* var. *lactis*) was also isolated from the cheese samples taken at the middle and the end of ripening. This yeast, which was also found to dominate at the end of ripening in Camembert (Schmidt & Lenoir, 1978) and *Trichosporon cutaneum* seem to be the most important microbes for the ripening of Kopanisti cheese.

The other species of yeasts that were isolated, in order of frequency, were : *Saccharomyces exiguus, Rhodotorula rubra (Rh. mucilaginosa), Saccharomyces cerevisiae, Trichosporon penicillatum (Geotrichum penicillatum), Candida lusitaniae (Clavispora lusitaniae) and Debaryomyces hansenii.* The yeasts *Kluyveromyces marxianus* var *lactis* were also isolated from Camembert (Schmidt & Lenoir, 1978; 1980), Roquefort (Devoyod & Sponem, 1970), Saint-Nectaire (Vergeade *et al.*, 1976), Cantal (Millet *et al.*, 1974) blue-veined cheeses (De Boer & Kuik, 1987), and Cabrales (Nunez *et al.*, 1981), *Saccharomyces cerevisiae* from Camembert (Schmidt & Lenoir, 1978; 1980), Saint-Paulin (Ducastelle & Lenoir, 1965) and blue-veined cheeses (De Boer & Kuik, 1987), *Debaryomyces hansenii* from Telemes (Georgantas, 1979), Camembert (Schmidt & Lenoir, 1978; 1980), blue-veined cheeses (De Boer & Kuik, 1987), Roquefort (Devoyod & Sponem, 1970) Saint-Nectaire (Dale, 1972; Vergeade *et al.*, 1976) and Cabrales (Nunez *et al.*, 1981), *Rhodotorula* sp. from Telemes (Georgantas, 1979), Cantal (Millet *et al.*, 1974), Roquefort (Devoyod & Sponem, 1970) and Camembert (Lenoir, 1962) and

Tableau I. Classification of 86 strains of yeasts isolated from Kopanisti cheese during ripening and some of their biochemical characteristics.
Classification des 86 souches de levures isolées du fromage Kopanisti durant l'affinage, et quelques-unes de leurs caractéristiques biochimiques.

Species of yeasts	*No. of isolates*	*Enzymatic activity*					*Utilization of carbon source*				
		Esterase (C_4)	Esterase—lipase (C8)	Lipase (C14)	Leucine amino-peptidase	Valine amino-peptidase	Cystine amino-peptidase	l-Methyl-β D-xyloside	l-Methyl-α D-Mannoside	N-Acetyl glucosamine	Adonitol
Trichosporon cutaneum (Trichosporon beigelii)	61	+	+	–	++	V	V	–	V	+	–
Kluyveromyces lactis (Kl. marxianus var. lactis)	12	+/–	+	–	++	+/–	–	–	–	–	–
Saccharomyces exiguus	6	+	+	–	+	+/–	–	–	–	–	–
Rhodotorula rubra (Rhodotorula mucilaginosa)	2	+/–	+	–	++	+	–	–	–	–	+
Sacch. cerevisiae	2	+/–	+	–	++	+/–	–	–	–	–	–
Trich. penicillatum (Geotr. penicillatum)	1	+/–	+	–	++	+/–	–	–	–	–	–
Candida lusitaniae (Clavispora lusitaniae)	1	+/–	+/–	–	++	+/–	–	–	–	+	+
Debaryomyces hansenii	1	+/–	+/–	–	+	–	–	–	–	+	–
Total	86										

Species of yeasts	*Utilization of carbon source*																
	D-Fruc-tose	D-Man-nose	Dulci-tol	D-Sor-bitol	Amyg-dalin	Escu-lin	Glyco-gen	Xyli-tol	Gentio-biose	D-Tura-nose	D-Ly-xose	D-Taga-tose	D-Fu cose	-L-Fu-cose	D-Arabi-tol	L-Arabi-tol	Glu-conate
Trichosporon cutaneum (Trischosporon beigelii)	+	+	V	–	–	V	–	–	V	V	–	–	–	V	–	–	+
Kluyveromyces lactis (Kl. marxianus var. lactis)	+	+	–	+	–	–	–	+/–	–	+	–	–	–	–	–	–	–
Saccharomyces exiguus	+	+	–	–	–	+/–	–	–	–	–	–	–	–	–	–	–	–
Rhodotorula rubra (Rhodotorula mucilaginosa	+	+	–	+	–	–	–	–	–	+	+	–	–	+/–	+	–	–
Sacch. cerevisiae	+	+	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–
Trich. penicillatum (Geotr. penicillatum	+	+	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–
Candida lusitaniae (Clavispora lusitaniae)	+	+	–	+	+	+	–	+	+	+	–	–	–	–	+	–	–
Debaryomyces hansenii	+	+	–	+	–	–	–	–	–	+	–	–	–	–	–	–	–

– : Negative reaction; +/– : weak reaction; + : positive reaction; ++ : intense reaction; V : variable reaction.
– : réaction négative, +/– : réaction faible; + : réaction positive; ++ : réaction intense; V : réaction variable.

Candida sp. from Camembert (Lenoir, 1962; Schmidt & Lenoir, 1978), blue-veined cheeses (De Boer & Kuik, 1987), Saint-Nectaire (Dale, 1972; Vergeade *et al.*, 1976), Saint-Paulin (Ducastelle & Lenoir, 1965) and Roquefort (Devoyod & Sponem, 1970).

The microscopic appearance and reproduction of isolated yeasts are given in Figure 5.

The enzymatic activities of the isolated microorganisms were examined by using the miniaturized API-ZYM test that allows the determination of 19 characteristics. The most interesting activities that might play an important role during ripening — esterase, lipase and aminopeptidase — are given in Table I. The following conclusions can be summarized :

Trichosporon cutaneum (T. beigelii), Trichosporon penicillatum (Geotrichum penicillatum), Kluyveromyces lactis (Kl. marxianus var lactis), Rhodotorula rubra (Rh. mucilaginosa), Saccharomyces cerevisiae and *Candida lusitaniae (Clavispora lusitaniae)* showed an intensive production of leucine aminopeptidase and a weak to remarkable production of valine aminopeptidase.

Trichosporon cutaneum (T. beigelii) and *Saccharomyces exiguus* presented a remarkable production of esterase C_4 while the remaining species of yeasts had a weak production. Also *Trichosporon penicillatum (Geotrichum penicillatum), Kluyveromyces lactis (Kl. marxianus* var. *lactis), Rhodotorula rubra (Rh. mucilaginosa), Saccharomyces exiguus* and *Saccharomyces cerevisiae* presented a remarkable production of lipolytic esterase C_8.

CONCLUSION

From the results given so far it can be concluded that the microflora of Kopanisti cheese changes during cheese ripening. Lactobacilli and streptococci were found to be the predominant groups, especially at the beginning of ripening. Yeasts and molds were also found in high numbers in Kopanisti, especially on its surface and therefore their contribution to the chemical and organoleptic characteristics of the cheese may be important. From the yeasts which were isolated from the cheese, *Trichosporon cutaneum (T. beigeli)* and *Kluyveromyces lactis* (*Kl. marxianus* var. *lactis*) were the predominant species.

The hygienic quality of the experimental Kopanisti cheese, judged by the coliform presence, was found to be satisfactory. On the contrary, Kopanisti cheese made from raw milk, without using starters and uncontrolled inoculum of ripe Kopanisti was not always satisfactory (Kaminarides, 1986).

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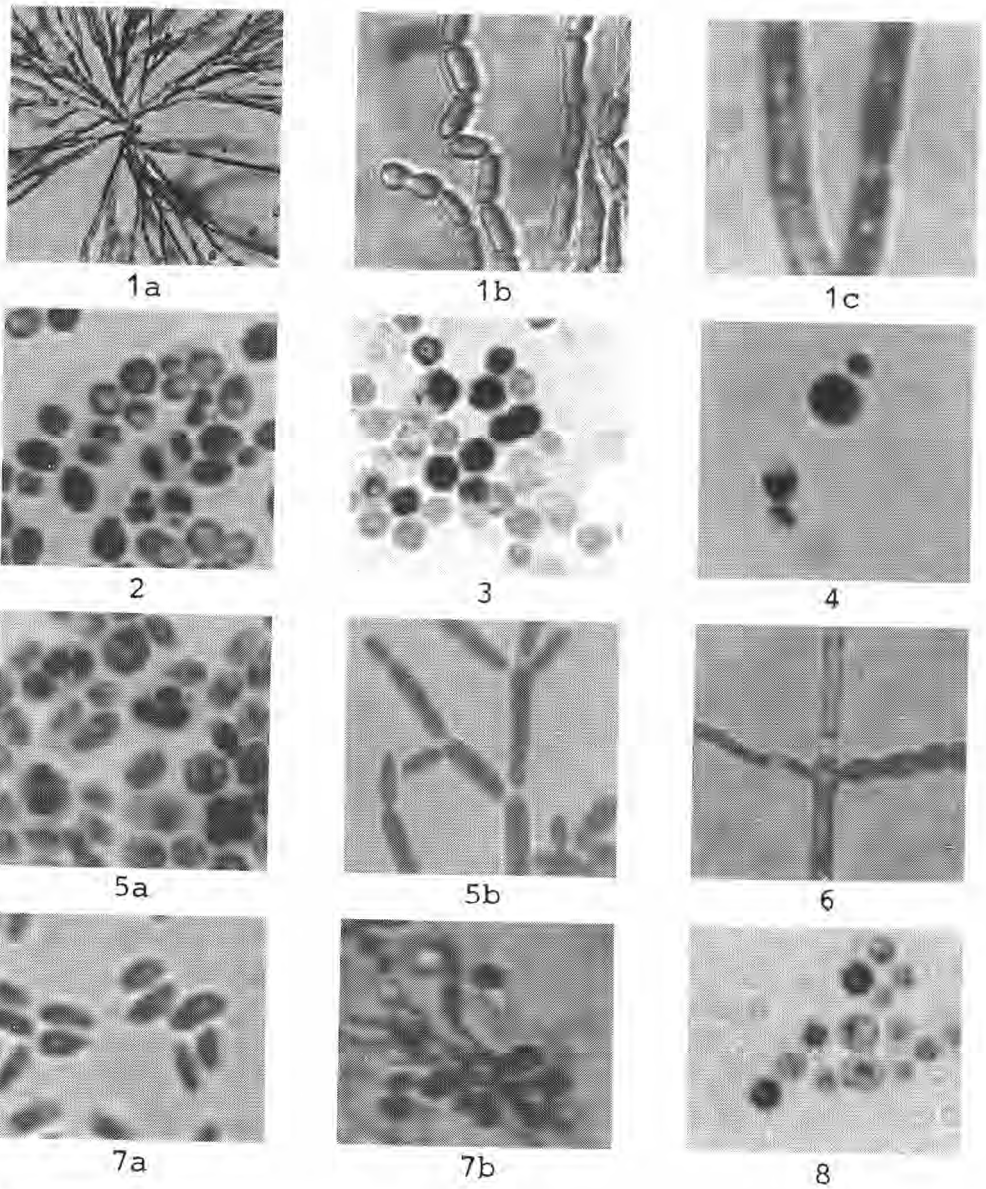

Fig. 5. Microscopical appearance of isolated yeasts.
1 = *Trich. cutaneum (Trich. beigelii);* 2 = *Kluyv. lactis* x 1300 *(Kl. marxianus* var. *lactis*); 3 = *Sacch. exiguus* x 1300; 4 = *Rhodot. rubra* x 1300 (*Rh. mucilaginosa*); 5 = *Sacch. cerevisiae* x 1300; 6 = *Trich. penicillatum* x 260 *(Geotrichum penicillatum);* 7 = *Cand. lusitaniae* x 1300 (*Clavispora lusitaniae*); 8 = *Debar. hansenii* x 1300.
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