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Proteolytic, lipolytic and molecular characterisation of *Yarrowia lipolytica* isolated from cheese

G. Suzzi [a], M.T. Lanorte [a], F. Galgano [a], C. Andrighetto [b], A. Lombardi [b], R. Lanciotti [c], M.E. Guerzoni [c,*]

[a] *Dipartimento di Biologia, Difesa e Biotecnologie Agroforestali, Università degli Studi della Basilicata, Via Nazzario Sauro 85, 85100 Potenza, Italy*

[b] *Veneto Agricoltura, Istituto per la Qualità e le Tecnologie Agroalimentari, Via San Gaetano 74, 36016 Thiene (Vicenza), Italy*

[c] *Dipartimento di Protezione e Valorizzazione Agroalimentare, Università degli Studi di Bologna, Via S. Giacomo 7, 40126, Bologna, Italy*

Abstract

This work studied the qualitative and quantitative proteolytic and lipolytic activities of *Yarrowia lipolytica* strains isolated from two cheese types. Randomly amplified polymorphic DNA-PCR (RAPD-PCR) analysis was used to compare the cheese strains of *Y. lipolytica* with strains isolated from other food products and with the type strain of the species in order to investigate the genetic diversity and occurrence of specific environmental groups.

Diversity of proteolytic and especially lipolytic activity within *Y. lipolytica* strains isolated from dairy products was observed. In particular, the degree of specificity for saturated or unsaturated fatty acids as well as for even- or odd-numbered carbon free fatty acids (FFAs) varied among the strains. The RAPD-PCR profiles showed low genetic relatedness between many of the food isolates and the type strain of the species. Such genetic variability needs to be further evaluated. Most of the *Y. lipolytica* strains appeared to be specific to the particular environment from which they were isolated. However, phenotypic characteristics having technological importance in dairy products and, particularly, lipolytic activities did not correspond to the genetic differences observed by RAPD-PCR analysis. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: *Yarrowia lipolytica*; Lipolytic and proteolytic activities; RAPD-PCR analysis

1. Introduction

Yeasts can be a substantial part of the microflora of different cheeses such as mould-, smear-, soft-, semi-hard and brine-ripened cheeses (Fleet, 1990; Eliskases-Lechner and Ginzinger, 1995; Rhom et al., 1992). The role of yeasts depends on the type of cheese: in some varieties they are responsible for spoilage, causing fruity flavours, gassiness, slime formation and discoloration (Fleet, 1990; Vilijoen and Greyling, 1995; Welthagen and Viljoen, 1999), while in other they are involved in the ripening process and contribute to microbial interactions, texture changes and biosynthesis of flavour compounds (Rossi et al., 1998; Roostita and Fleet, 1996a; Welthagen and Viljoen, 1999). In particular, their main contribution to the maturation process is the utilisation of lactic acid, which in turn increases the pH, favouring bacterial growth and initiates the second stage of cheese ripening (Rhom et al., 1992).

* Corresponding author. Tel.: +39-51-2099784; fax: +39-51-2099782.

E-mail address: guerzoni@foodsci.unibo.it (M.E. Guerzoni).

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Moreover, their contribution to flavour and aroma is generally attributed to the ability of some species to ferment lactose and, as a result, to produce ethanol, acetaldehyde, ethyl acetate and ethyl butyrate (Vilijoen and Greyling, 1995). Due to features such as high proteolytic and lipolytic activities, some yeast species play an important role in the production of aroma precursors (amino acids, fatty acids and esters); furthermore, they stimulate the growth of other microorganisms by the excretion of growth factors (Lenoir, 1984). In particular, the dynamics of release of free fatty acids (FFAs) in cheeses, which largely depends on the milk fat as well as on the milk and microbial lipase selectivity and activity, determines the flavour of many dairy products (Kim and Lindsay, 1992).

The acceleration of flavour development in blue cheese by yeast lipase addition (Farag et al., 1992) and the reported production of cheese-like flavours by yeasts also support the use of yeasts as starter cultures for cheese making (Jakobsen and Narvhus, 1996). Despite the frequent occurrence of different yeast species in cheeses and the well-known species' effect on the final product (Marcellino and Benson, 1992), no special attention has been given to the effects of the intraspecific variability which can have a technological impact on cheese characterisation.

Yarrowia lipolytica is recognized as one of the most frequent species associated with milk products and, due to its enzymatic activities, it has been regarded as a good candidate as a ripening agent (Guerzoni et al., 1998). However, the studies concerning the role of this microorganism in cheese ripening are relatively few. Some patents have proposed the use of this species to accelerate ripening and Guerzoni et al. (1998) have studied some biochemical changes induced in cheese and on caseins by different, deliberately inoculated, *Y. lipolytica* strains.

The aim of this work was to study the qualitative and quantitative proteolytic and lipolytic activities of *Y. lipolytica* strains isolated from two cheese types. In addition, randomly amplified polymorphic DNA-PCR (RAPD-PCR) analysis was used to compare cheese isolates of *Y. lipolytica* with strains isolated from other food products and with the type strain in order to investigate their genetic diversity and occurrence of specific environmental groups.

2. Materials and methods

2.1. Micro-organisms

Twenty-four strains of *Y. lipolytica*, from the Collection of the Dipartimento di Protezione e Valorizzazione Agroalimentare of Bologna University and Dipartimento di Biologia, Difesa e Biotecnologie Agroforestale, of Basilicata University, were employed. The strains were isolated from the following habitats: commercial light butter (strains labelled RO); water-buffalo mozzarella cheeses (strains labelled PZ); goat cheeses (strains labelled LF); salami (strains labelled S). The type strain of *Y. lipolytica* was obtained from the Industrial Yeast Collection (Dipartimento di Biologia Vegetale, Perugia University, Italy). All the strains were maintained on yeast extract–malt extract–glucose–peptone agar at 4 °C.

2.2. Proteolytic activity

The proteolytic activity was evaluated in skim milk (Oxoid) inoculated with about 10^5 CFU/ml of the different *Y. lipolytica* strains over 14 days of incubation at 10 and 25 °C, following the method described by Folkerstma and Fox (1992). The data are the average of at least five replications. The variability coefficients, expressed as the percentage ratio between the standard deviation and the mean value, were lower than 5%.

2.3. Lipolytic activity

Y. lipolytica strains were inoculated in butter agar medium containing yeast extract (1%), peptone (1%), NaCl (0.5%), agar (2%) and butter (5%) (Oxoid Basingstoke, UK) and incubated at 28 °C for 3 and 6 days . The inoculum level for each strain was about 10^6 CFU/ml.

Lipid extraction and quantification of FFAs were carried out on 1 g of the homogenised culture medium, following the method proposed by Lencioni et al. (1998). In particular, 2 ml of culture medium was put in a screw-capped glass centrifuge tube to which 2 ml of ethanol and 0.3 ml of 0.5 M H_2SO_4 were added. The mixture was extracted three times with 3 ml of a diethyl ether/hexane (1:1) mixture. Each time the slurry was heated at 40 °C, shaken for 5 min using a Vortex mixer and clarified by a short

centrifugation (2 min, 2000 *g*, 4 °C). After centrifugation, the upper solvent layer was transferred to a screw-capped glass centrifuge tube containing 1 g of anhydrous Na_2SO_4, in order to adsorb the residual water. Before the successive step of FFA purification, the lipid extract (c.a. 9 ml) was transferred to a 50 ml volumetric flask and brought up to volume with hexane.

The separation of the FFA fraction from the other lipid classes was conducted utilising SPE aminopropyl columns (500 mg) (Supelco, Milan, Italy). After conditioning the column with 10 ml of hexane, the whole lipid extract was loaded onto the column and eluted drop by drop. The neutral lipids were washed out by using 4 ml of a chloroform/hexane (1:1) mixture, and the FFA fraction was then eluted using 4 ml of diethyl ether containing 2% formic acid. After the recovery of the FFA fraction, the column was washed with 10 ml of methanol and reconditioned with 10 ml of hexane before being reused.

The sample was then spiked with 250 μl of a C7:0 solution (1 mg/ml) as internal standard.

2.4. Gas chromatographic analysis

FFA analysis was carried out using a Carlo Erba Mega 5160 gas chromatograph (Carlo Erba, Milan, Italy), equipped with a flame ionisation detector and a cold on column injector. The sample (1 μl) was injected into a Nukol wide-bore column (15 m × 0.53 mm ID) having a film thickness of 0.5 μm (Supelco). The carrier gas (helium) flow rate was set at 20 ml/min, the detector temperature at 220 °C and detector attenuation at 10. The oven temperature was programmed from 90 to 195 °C at 10 °C/min. The data are the average of at least three replications. The variability coefficients, expressed as the percentage ratio between the standard deviation and the mean value, were lower than 5%. The identification of gaschromatographic peaks was performed using standard mixes such as Rapesed for long chain fatty acids and GLC 30 for short chain ones (Supelco).

2.5. DNA extraction and RAPD-PCR characterization

Yeast cells were grown overnight in 5 ml of YEPD medium (1% yeast extract, 2% peptone, 2% glucose). The DNA was isolated using the method of Querol et al. (1992), except that the lytic enzyme from *Rhizoctonia solanii* (Sigma, St. Louis, MO, USA) was used to digest the cell wall.

Amplification reactions were performed with primers M13 and RF2 as described by Andrighetto et al. (1999). RAPD-PCR profiles were analysed with the pattern analysis software package Gel Compare Version 4.0 (Applied Maths, Belgium). The final dendrogram was obtained by means of the Unweighted Pair Group using Arithmetic Average (UPGMA) clustering algorithm.

3. Results and discussion

3.1. Proteolytic activity

Eight strains isolated from goat cheese (labelled LF) or from water-buffalo mozzarella cheese (labelled PZ) were inoculated in skim milk and incubated at 10 and 25 °C and their proteolytic activities were measured over the 14 days of incubation. The proteolytic activity of *Y. lipolytica* strains was considered, because for most cheese varieties, proteolysis is a commonly used index of ripening (Farkye and Fox, 1990). Moreover, it is also useful in evaluating starter culture performances (Fox, 1989) because this lytic activity strongly contributes to flavour and texture development (Freitas et al., 1997). In fact, the proteolysis or the conversion of casein to peptides of different molecular weight and free amino acids have a direct bearing on cheese flavour or are precursors of flavour components (Parra et al., 1996).

After 8 days of incubation at 10 and 25 °C, the proteolytic activity (expressed as mg of leucine/100 ml of skim milk) was very weak for all the considered strains, while from the 8th to the 14th day a great increase of proteolytic activity was observed. As shown in Fig. 1, the proteolytic activity at the end of incubation was markedly affected by temperature. At 25 °C all the strains produced more than 300 mg leucine/100 ml skim milk while at 10 °C, all the considered strains showed a limited activity. However, proteolytic activity at temperatures lower than 10 °C is a rare feature among yeasts. The minimum temperature for producing efficient proteinases, for a great number of *Y. lipolytica* strains isolated from different habitats, was reported to be 0–3 °C by

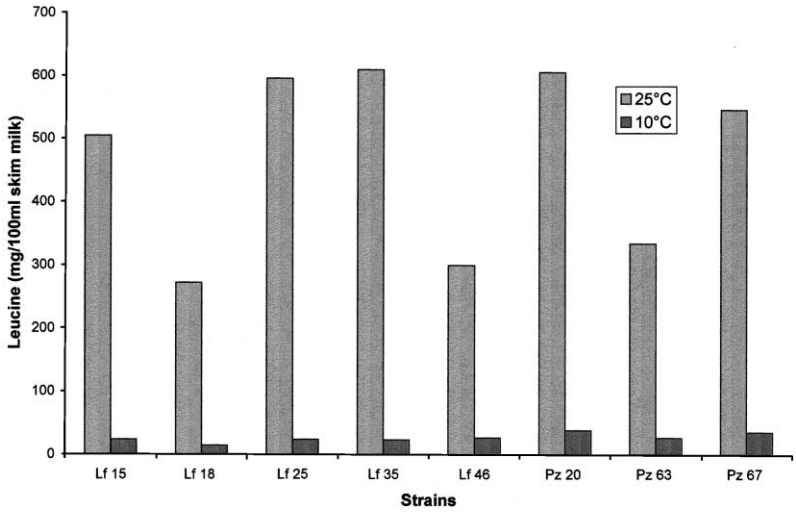

Fig. 1. Proteolytic activity, expressed as mg of leucine/100 ml of skim milk, of *Yarrowia lipolytica* strains in relation to incubation temperature after 14 days of incubation.

Sinigaglia et al. (1994) and Guerzoni et al. (1998). At 25 °C, the strains showed two different proteolytic behaviours. In particular, the strains LF15, LF25, LF35, PZ20 and PZ67 produced in 100 ml of skim milk more than 500 mg of leucine while the strains LF18, LF46 and PZ63 had a lower lytic capability, producing amounts of free amino acids ranging between 300 and 350 mg in the same medium. According to Sinigaglia et al. (1994), the proteolytic activity at 25 °C exhibited high discriminatory power among *Y. lipolytica* strains having different origin.

The quantitative method used in this work was unable to identify possible different protein breakdown profiles. However, a relevant diversity in patterns of α_{s1} and β caseins hydrolysis among *Y. lipolytica* strains of different origin was observed by Guerzoni et al. (1998).

3.2. Lipolytic activity

In Fig. 2, the total FFA produced by the different strains after 3 and 6 days of incubation at 25 °C in butter fat are reported. In terms of total lipolytic activity, two different behaviours were observed. In fact, some strains, such as LF25, LF35, LF46 and PZ67, showed very high lipolytic activity over the first 3 days of growth, producing the highest amounts of total FFA. However, continued incubation of these strains resulted in a significant decrease of total FFA concentrations. In the samples inoculated with the other strains, characterised by a lower lipolytic activity at 3 days of growth, the total FFA contents increased at the end of experiment. However, the specificity of the individual fatty acid release, and presumably their subsequent metabolism, were not associated to this preliminary grouping.

Table 1 reports the FFAs released after 3 and 6 days in the medium by the inoculated strains. It can be noted that the short-chain FFAs (C4–C10) were produced by all the strains at low levels, corresponding to only about 1–2% of the total FFA. Short-chain FFAs were generally released during the first days of incubation and later, with the exception of PZ20, they disappeared. A decrease in previously released longer chain FFA, such as palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0), oleic (C18:1) and linoleic (C18:2) acids, was also observed after 6 days of incubation in the samples inoculated with the strains LF25, LF35, LF46 and PZ67. The linolenic acid (C18:3), present after 3 days of incubation, tended to disappear in all the samples. In regard to the specificity of lipases, all the strains hydrolysed both saturated and unsaturated fatty acids from milk fats. In agreement with previous results of Roostita and Fleet (1996b), in all the strains, after 3 days of

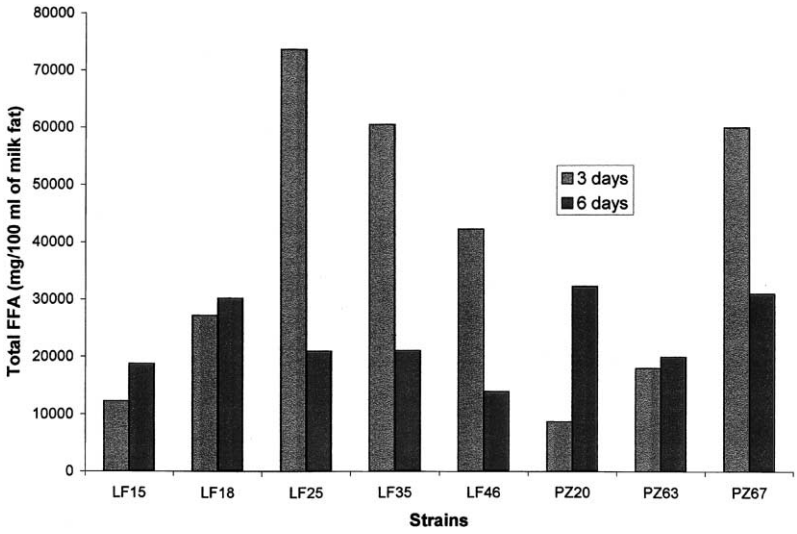

Fig. 2. Total FFA produced by the different *Yarrowia lipolytica* strains after 3 and 6 days of incubation at 25 °C in milk fats.

incubation, the major FFA released was C18:1 followed by C16:0, with the exception of PZ67 and LF25 in which C16:0 represented the most relevant fatty acid released, followed by C18:1. After 6 days of incubation, C16:0 became the major FFA accumulated for the strains PZ63, LF46, LF35 and LF18. The other relevant FFA were myrystic acid (C14:0) and stearic acid (C18:0). All the strains hydrolysed the fats with the liberation of high concentrations of even-numbered carbon FFA, while the odd-numbered FFA represented a limited proportion of FFA. The odd numbered FFA are present in milk in traces (Mietton et al., 1994). The LF25 strain presented, with respect to the other strains, metabolic peculiarity which appeared evident after 6 days. In fact, the fatty acid profile observed was totally modified with respect to that observed after 3 days. A relevant increase of lauric acid (C12:0), pentadecanoic acid (C15:0) and margaric acid (C17:0), presumably at the expense principally of C14:0, C16:0 and C18:1, was observed. The results suggest that the strain LF25 metabolised the FFA released from triglycerides or in cellular components or in shorter chain compounds, such as C12:0, C15:0 and C17:0. Free unsaturated fatty acids can be transformed by the microbial enzymes (lipoxygenase, epoxidase and hydratase) in the relative hydroxy-acids (Maga, 1976). The hydroxy-acids can be transformed in shorter chain molecules, including lactones, by means of α, β and δ oxidation. The yeasts can oxidise fatty acids by a conventional process of β oxidation to yield fatty acids having two atoms of carbon less than the parent (Ercoli et al., 1992). On the other hand, one α oxidation cycle reduces the chain length of a carbon atom. The pathway for δ oxidation of saturated fatty acids yields to even shorter fatty acids. The occurrence of high proportion of C15:0 in the samples inoculated with LF25 suggests the involvement of an α oxidation process. Due to the late accumulation of C15:0 accompanied by a decrease of C16:1, C18:1, C18:2 and C18:3, this hypothesis seems to be more persuasive than a specific lipase. A mechanism of β oxidation can be involved in the increase of C12:0. It has been shown that fungi can oxidise fatty acids by an abortive β oxidation sequence to yield alkane-2-one containing one carbon less than the parent acids (Coppock et al., 1928; Hocking et al., 1998). The diminution of total FFA occurring in the samples inoculated with some strains suggests that these molecules were transformed to alcohols, ketones and lactones. As reported by Wong et al. (1975) and Maga (1976), cheese-related microorganisms could possibly produce, from long chain fatty acids, shorter chain hydroxy acids which could lactonize to their corresponding lactones. Moreover, Amstrong (1989) and Ercoli et al. (1992)

Table 1
Free fatty acids released in agar-butter inoculated with different *Yarrowia lipolytica* strains (mg FFA/100 g of milk fat)

FFA	LF 15[a]		LF 18[a]		LF 25[a]		LF 35[a]		LF 46[a]		PZ 20[b]		PZ 63[b]		PZ 67[b]	
	3 days	6 days	3 days	6 days	3 days	6 days	3 days	6 days	3 days	6 days	3 days	6 days	3 days	6 days	3 days	6 days
C4:0	96	–	142	–	118	–	54	–	379	–	228	137	91	–	91	–
C6:0	95	–	99	–	112	–	77	–	346	–	139	159	76	–	99	–
C8:0	–[c]	–	–	–	–	–	–	–	90	–	80	114	–	–	72	–
C10:0	92	–	178	–	331	123	132	–	420	–	102	148	48	–	107	–
C12:0	493	384	92	156	1773	2965	1827	86	1294	301	345	811	676	452	990	–
C14:0	1272	1930	5092	2108	6162	139	7591	2490	3618	1395	735	2500	2664	3014	5075	1508
C15:0	126	298	185	432	874	7946	599	128	536	234	24	377	167	79	846	388
C16:0	3980	6349	7986	11552	27000	456	18658	8227	12396	5581	2719	9225	5154	6610	23426	17197
C16:1	407	839	100	699	1872	154	2303	205	1904	524	305	1438	660	890	373	–
C17:0	81	275	305	451	627	2402	642	231	560	123	–	440	220	193	480	155
C18:0	1313	2040	2709	3875	8140	6309	6751	2935	4805	1707	904	3562	1693	1884	7133	5170
C18:1	4257	6490	8754	9131	23040	409	19165	6400	14036	4063	3006	13015	5678	6177	20568	6501
C18:2	–	157	1387	1694	3073	–	2478	334	1846	–	58	422	797	628	640	–
C18:3	–	–	104.2	–	400		293	–	140	–	–	–	–	–	112	–
Total	12212	18762	27133.2	30098	73522	20903	60570	21036	42370	13928	8645	32248	17924	19927	60012	30919

[a] Isolated from goat cheese.
[b] Isolated from mozzarella cheese.
[c] Not detectable.

showed that some *Y. lipolytica* strains were particularly effective in converting, by β oxidation, unsaturated fatty acids, such as oleic, palmitoleic and linoleic acids, in C10 and C12 gamma lactones. The lactones are widely diffused in food and beverages and their presence contributes to specific dairy product (including butter) flavours (Dufossé et al., 1994). It can also be mentioned that under conditions of limited moisture, lipases also catalyse the reverse reaction (Miller et al., 1988).

The lipase specificity and the subsequent metabolism of FFAs are important for the aromatic characterisation of cheeses. It is well known that long-chain FFAs play a major role in the cheese flavours, given their high perception thresholds, while short and moderate-chain even-numbered FFAs have much lower perception thresholds, each one having a characteristic note (Molimard and Spinnler, 1995). The qualitative presence and the quantitative concentrations of FFA, which are largely dependent on the milk fat and lipolytic strain specificity and activity, are reported to contribute to the flavour characterisation of many dairy products (Kim and Lindsay, 1992). Consequently, diversity among the strains in the fat hydrolysis could be exploited to obtain products having different aromatic features.

3.3. RAPD-PCR characterisation

In order to evaluate whether the variability of proteolytic and lipolytic activities of *Y. lipolytica* strains isolated from cheeses corresponded to some genetic diversity, the strains isolated from goat and water-buffalo mozzarella cheese were analysed by means of RAPD-PCR. Strains isolated from light butter (labelled RO) and from fermented meat products (labelled S) were also included in the RAPD-PCR analysis in order to investigate the occurrence of specific environmental groups. In the resulting dendrogram (Fig. 3), four major clusters were detected, which indicated the presence of great diversity among *Y. lipolytica* strains and reflected their different origins. Cluster 1 grouped all the strains from cheese, one strain isolated from sausages as well as the type strain of the species. The strains isolated from butter were all included in cluster 2, while clusters 3 and 4 contained all the remaining strains from sausages and strain PZ 20 from mozzarella cheese. The four clusters well corresponded to the origin of the isolates, except in the case of strains S1 and PZ 20. Among the isolates derived from cheeses, small differences could be found in the RF2 profile of strains PZ67, PZ63, LF18 with respect to LF25, LF46, LF15, while strain PZ 20 showed M13 and RF2 profiles very different from those of the other cheese strains. However, the metabolic peculiarity in terms of lipolytic activity of strain LF25 was not reflected at the genetic level, as revealed by the RAPD-PCR profile of this strain, which was identical to the profile of the other strains of dairy origin. Similarly, the grouping of strains on

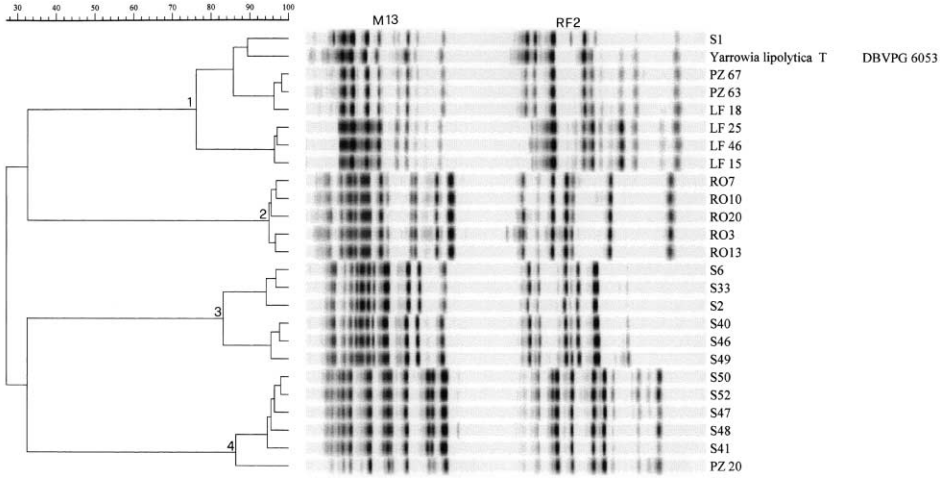

Fig. 3. Cluster analysis of RAPD-PCR patterns generated with M13 and RF2 primers for strains of *Yarrowia lipolytica*. (For information on the origin of the blue strains see Materials and methods.)

the basis of their proteolytic activity was not related to the grouping of strains obtained by cluster analysis of their RAPD-PCR profiles.

4. Conclusions

The biochemical activities of *Y. lipolytica* strains can play an important role in the organoleptic features of cheeses, due to the production of aromatic compounds and/or their precursors such as methyl ketons, alcohols, lactones and esters. In addition, nutritional and anti-nutritional properties of the substances produced have to be investigated intensively. In fact, it is well known that a large number of bioactive peptides are produced by milk protein hydrolysis (Xu, 1998). Although the physiological role of these peptides is not yet clear, they offer a great potential for developing functional foods.

Lipase activity from various strains of *Y. lipolytica* showed interesting variations. The degree of specificity for saturated or unsaturated fatty acids as well as for even- or odd-numbered carbon FFAs also varied among the strains. The *Y. lipolytica* strains can show different routes, and, consequently, different enzymatic systems, for FFA metabolism.

The relative abundance of palmitic, lauric and oleic acids, which are precursors of methyl ketones by successive β-oxidation cycles, emphasises the role of this yeast in the aroma of several cheeses. A great diversity concerning proteolytic and especially lipolytic activity within a group of strains isolated from dairy products was observed. RAPD-PCR with primer M13 and RF2 was previously described as a powerful approach for the identification of yeast species commonly found in dairy products (Andrighetto et al., 1999). In this paper, the method was applied to detect the intraspecific diversity of strains of *Y. lipolytica* isolated from cheese and other food products. The UPGMA dendrogram of the RAPD-PCR profiles showed a low genetic relatedness between many of the food isolates and the type strain of the species. Such genetic variability needs to be further evaluated. Furthermore, most of the *Y. lipolytica* strains appeared to be specific to the particular environment from which they were isolated. The genetic relatedness among strains of different origin remains to be elucidated on a larger number of strains but may reflect the exposition to different food environments, processing technologies and geographic areas of production. However, phenotypic characteristics having technological importance in dairy products, and particularly lipolytic activities, did not reflect the genetic differences observed by RAPD-PCR analysis.

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