POLISH JOURNAL OF FOOD AND NUTRITION SCIENCES

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Pol. J. Food Nutr. Sci.
2007, Vol. 57, No. 2, pp. 221–226

COMPARISON OF PHYSIOLOGICAL AND PCR-RFLP rDNA IDENTIFICATION OF YEAST SPECIES COMMONLY FOUND IN CHEESE

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Key words: *C. famata*, *C. sphaerica*, *C. lipolytica*, cheese, assimilation tests, PCR-RFLP rDNA

The identification of twenty four yeast isolates from cheese performed with API ID 32C simplified assimilation test and restriction fragments length polymorphism (PCR-RFLP) of ITS1-5.8S rDNA-ITS2 region was compared. The accordance of the results of both methods for eleven strains identified as *Candida famata*, five strains as *C. lipolytica* and four strains as C. *sphaerica* was observed. However, the other four yeast isolates classified with API as *C. sphaerica* revealed amplification products as well as restriction patterns characteristic for *C. famata* species.

INTRODUCTION

Yeasts are frequently found in many cheese varieties [Viljoen & Greyling, 1995; Jakobsen & Narvhus, 1996; Wojtatowicz *et al*., 2001]. Although, previously mainly identified as spoilage agents of dairy products causing defects of smell and texture [Nichol & Harden, 1993] recently, some yeast species are recognized as very important in cheese technology and even necessary for proper cheese quality [Tempel & Jakobsen, 2000].

Some strains belonging to species *Debaryomyces hansenii* (teleomorph of *Candida famata*) or *Geotrichum candidum* are utilized in smear or mould ripened cheese technology (Tylzit, Limburger, Gorgonzola) due to their ability to assimilate organic acids, especially lactic acid, formed during fermentation. The growth of those yeasts on the curd surface leads to the pH increase, which enables the development of smear bacteria as well as farther growth of lactic acid bacteria population [Eliskases-Lechner & Ginzinger, 1995; Bockelmann & Hoppe-Seyler, 2001]. Yeast are also recognized as the source of amino acids and vitamins (riboflavin, thiamine, biotin *etc.*) indispensable for the growth of lactic acid bacteria [Roostita & Fleet, 1996]. Some yeast species can also play the role of antagonistic factor against undesirable microorganisms *e.g.* moulds, enteropathogens, clostridia or other yeast species due to the competition for nutritional compounds, excreting antibacterial metabolites or in case of enteropathogens – binding them on the cell surface [Fatichenti *et al.*, 1983; Gedek, 1991; Bockelmann, 2002; Zarowska *et al.*, 2004]. Some species like *Yarrowia lipolytica* / *C. lipolytica* with very strong proteolytic and lipolytic activities [Gdula et *al.*, 2003] or *Kluyveromyces lactis* / *Candida sphaerica* and *K. marxianus* / *C. kefyr* due to the fermentation of sugars present in cheese [Tempel & Jakobsen, 2000] can contribute to the formation of sensory properties of cheese.

The increasing interest in the use of certain yeasts as starter cultures in cheese production as well as the analysis of contaminating yeast microflora demand quick and reliable methods for the identification of isolates from cheese samples.

Traditional techniques are based on morphological and physiological characteristics. Full identification requires determination of: reproduction type, hyphae or pseudohyphae formation, fermentation and assimilation of different carbon and nitrogen sources, vitamin demands, antibiotics resistance *etc.* [Kurtzman & Fell, 2000]. Recently, some simplified commercial tests have also been available, for example API systems (bioMérieux, Marcy-l'Etoile, France), which are based on shortened assimilation profiles. API systems such as: Candida ID 32C or 20C Aux, were designed mainly for the identification of medical yeast isolates [Fricker-Hidalgo *et al.*, 1996].

Traditional identification methods are more and more frequently substituted by molecular methods which mainly utilize Polymerase Chain Reaction (PCR) to amplify some fragments of genomic or mitochondrial DNA [Esteve-Zarzoso *et al.*, 1999; Tempel & Jakobsen, 2000; Hall *et al.*, 2003]. Ribotyping, an analysis of genes coding different rRNA subunits and some variable regions within them, is an example of methods used for the identification of yeast species. An analysis can be based on genes coding small (18S rRNA) or large (26S rRNA) subunits of ribosomal RNA containing variable regions D1 and D2 [Corredor *et al.*, 2000; Hall *et al.*, 2003] as well as the region between 18S and 26S of rRNA which

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contains 5.8S rRNA gene flanked by two internal transcribed spacers (ITS1 and ITS2). Amplified fragment can be either sequenced [Chen *et al.*, 2000, 2001; Hall *et al.*, 2003] or cut by restriction endonucleases and analysed electrophoretically [Esteve-Zarzoso *et al.*, 1999; Povhe Jemec *et al.*, 2001].

The aim of the present work was to compare the identification of yeast isolates from Polish blue-mould ripened cheese – Rokpol performed with API ID 32C system and restriction fragment length polymorphism (PCR-RFLP) of rDNA.

MATERIALS AND METHODS

Yeast strains. Twenty four yeast isolates (Table 1) from Polish blue mould ripened cheese – Rokpol were used in this study. They were representatives of the three phenotypic groups of yeasts occurring in the cheese [Wojtatowicz *et al.*, 2001]. All strains are deposited in the culture collection of Biotechnology and Food Microbiology Department, Wroclaw University of Environmental and Life Sciences, Poland. *D. hansenii* NCAIM 898 (National Collection of Agricultural and Industrial Microorganisms, Hungary), *D. hansenii* CCY 41-3-3 (Culture Collection of Yeasts, Slovakia), *K. lactis* CLIB 384 (Collection de Levures d'Interet Biotechnologique, France) and *Y. lipolytica* ATCC 20460 (American Type Culture Collection, USA) were used as reference strains. Cultures were grown on YM agar slants (0.3% yeast extract, 0.3% malt extract, 0.5% bactopeptone, 1% glucose, 1.5% agar) at 25°C.

Physiological identification procedure. Assimilation tests were performed with API ID 32C strips (bioMérieux, Marcy-l'Etoile, France) consisting of 29 cupules with different carbone sources, two additional tests for cycloheximide resistance and reaction with esculine, and one cupule as a control. All cupules on the strip were inoculated according to manufacturer procedure. Results were read after 48-h incubation at 30°C and analysed with APILAB Plus ver. 3.2.2 software (bioMérieux, Marcy-l'Etoile, France).

DNA extraction. Gene Matrix DNA purification kit for yeast (EUR_x, Poland; www.eurx.com.pl) was used for genomic DNA isolation. Extracted nucleic acid was suspended in TE buffer.

ITS1-5,8S-ITS2 rDNA amplification and electrophoresis. ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3')

TABLE 1. Identification of yeast isolates from cheese with API ID 32C.

Yeast isolate	API 32C identification[a]	Identification type[b]	Conformity (%id)	T index[c]	Incompatible tests (no./30)	Incompatible tests[d]			
BI6a	*C. famata*	acceptable	89.8	0.26	3	XYL (-)	GLN (-)		ARA (-)
BII3c	*C. famata*	acceptable	89.8	0.26	3	XYL (-)	GLN (-)		ARA (-)
KI2a	*C. famata*	acceptable	99.3	0.21	3	XYL (-)	GLN (-)	LAC (+)	
AI4a	C. famata	good	99.6	0.37	2	XYL (-)	GLN (-)		
AI4c	*C. famata*	good	99.6	0.37	2	XYL (-)	GLN (-)		
DNIIP1b	*C. famata*	good	99.6	0.37	2	XYL (-)	GLN (-)		
EII2b	*C. famata*	good	99.6	0.37	2	XYL (-)	GLN (-)		
PI1a	*C. famata*	good	99.6	0.37	2	XYL (-)	GLN (-)		
SII2c	*C. famata*	good	99.6	0.37	2	XYL (-)	GLN (-)		
AII4b	*C. famata*	very good	99.8	0.50	2	XYL (-)		LAC (+)	
MI1a	*C. famata*	very good	99.6	0.65	1		GLN (-)		
MI4a	*C. sphaerica*	acceptable	84.1	0.32	2		AGLN (+)		2KG (+)
OI3a	*C. sphaerica*	acceptable	88.1	0.33	2		AGLN (+)		ACT (-)
BI6c	*C. sphaerica*	good	98.7	0.30	3		AGLN (+)	LAC (-)	ACT (-)
DNIIW2b	*C. sphaerica*	excellent	99.9	0.98	0				
EII1a	*C. sphaerica*	excellent	99.9	1.00	0				
EII3a	*C. sphaerica*	excellent	99.9	0.98	0				
FII5a	*C. sphaerica*	excellent	99.9	0.96	0				
FII7a	*C. sphaerica*	excellent	99.9	0.96	0				
JII1a	*C. lipolytica*	excellent	99.9	0.99	0				
JII1c	*C. lipolytica*	excellent	99.9	1.00	0				
PII6a	*C. lipolytica*	excellent	99.9	1.00	0				
PII6b	*C. lipolytica*	excellent	99.9	1.00	0				
PII6c	*C. lipolytica*	excellent	99.9	1.00	0				

[a] *C. famata*– anamorph *D. hansenii*; *C. sphaerica*- anam. *K. lactis*; *C. lipolytica*- anam. *Y. lipolytica*; [b] According to APILAB Plus manual identification can be described as: excellent (% id > 99.9 and T index > 0.75), very good (% id > 99.0 and T index > 0.50), good (% id > 90.0 and T index > 0.25), acceptable (% id > 80.0 and T index > 0.0); [c] T index – conformity with the most typical set of reactions for the stated taxon; [d] XYL- D-xylose; GLN- glucosamine; AGLN- N-acetyl-glucosamine; LAC- DL-lactate; ARA- L-arabinose; 2KG- 2-keto-D-gluconate; ACT- cycloheximide (actidion) resistance.

and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') primers were used to amplify the repeated region of DNA coding 5.8S rRNA flanked by two internal transcribed spacers ITS1 and ITS2 [White *et al.*, 1990]. The amplification mixture contained: 5 μL of DNA template, 0.8 μL of 10 × PCR buffer, 1.6 μL of 25 mmol $MgCl_2$, 1.6 μL of 2 mmol dNTPs mix, 0.1 μL of 100 μmol of each ITS1 and ITS4 primers, 0.1 μL of 10 U/μL Taq DNA polymerase in the final volume of 20 μL. All chemicals were purchased from Fermentas company (Lithuania) with exception of primers, which were synthesized in Biomers.net laboratories (Germany). PCR reaction was performed in a Tpersonal thermocycler (Biometra, Germany) under the following conditions: initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 94°C for 1 min, primers annealing at 55.5°C for 2 min, extension at 72°C for 2 min; and final elongation at 72°C for 10 min.

PCR products were separated by electrophoresis in 1% agarose gel (Fermentas, Lithuania) in 0.5 x TBE buffer, 125V, 1 h. DNA fragments were stained with 6 μL of 10 mg/mL ethidium bromide solution (Sigma). Gene Ruler 100 bp DNA Ladder Plus (Fermentas, Lithuania), Gene Ruler 50 bp DNA Ladder (Fermentas, Lithuania) and Marker X (Roche Diagnostics, Germany) were used as molecular standards. Results were documented and analysed with BioCapt and BioGene software (Vilber Lourmat, France).

Restriction analysis. Three endonucleases: *Bsu*RI (isoschizomer *Hae*III), *Hinf*I, *Hin*6I (isoschizomer *Cfo*I) from Fermentas (Lithuania); were used to digest amplification products. The reaction mixture containing: 4 μL of amplified DNA, 0.4 μL of 10 U/μL restriction enzyme, 1 μL of 10 × proper buffer and 4.6 μL of distilled water, was incubated at 37°C through the night. The restriction was stopped by the addition of 2 μL of 6 × loading dye solution. The whole mixture was transferred into 1% agarose gel prepared with 0.5 × TBE buffer. Electrophoresis conditions and analysis were as described above.

RESULTS AND DISCUSSION

Physiological identification

On the basis of assimilation profiles performed with API ID 32C and APILAB Plus software yeast isolates were classified into three species: *C. famata* / *D. hansenii* (11/24), *C. sphaerica* / *K. lactis* (8/24) and *C. lipolytica* / *Y. lipolytica* (5/24) (Table 1).

All *C. lipolytica* strains were identified with 99.9% of conformity in relation to all other taxa in the database and in four cases with T index equal to 1.0, which means that those strains showed 100% of results concordance with the most typical set of reactions for the stated taxon. Such classification is referred to as "excellent" according to the producer's manual.

The identification of yeast strains as *C. famata* was not so precise. None of the eleven *C. famata* strains was recognized with "excellent" conformity. The identification of only two strains was "very good", while classification of five yeast isolates was only "good". Three strains were even identified at an "acceptable" level, which was connected with lower conformity (89.8÷99.3) and very low T index (0.21÷0.26). All *C. famata* strains revealed at least one contrary reaction: the lack of ability to utilize D-xylose or glucosamine, or both of those carbon sources (Table 1). Other incompatible tests were as follows: the lack of L-arabinose assimilation or the ability to utilize DL-lactate. According to the identification key of Meyer *et al.* [2000], all incompatible tests are strain dependent with exception of D-xylose assimilation. In the description of the species Nakase *et al.* [2000] indicate that those yeasts are able to utilize this saccharide. On the other hand, the research conducted by Prillinger *et al.* [1999] demonstrated that even that characteristic can be variable; only two out of six *D. hansenii* strains isolated from Austrian dairies could assimilate D-xylose. Nevertheless, more studies report about strains possessing that ability, *e.g.* strains of *D. hansenii* as well as their imperfect forms *C. famata* isolated from Bulgarian cheese [Savova & Nikolova, 2000–2002]. However, they revealed delayed growth on that carbon source. Similarly,

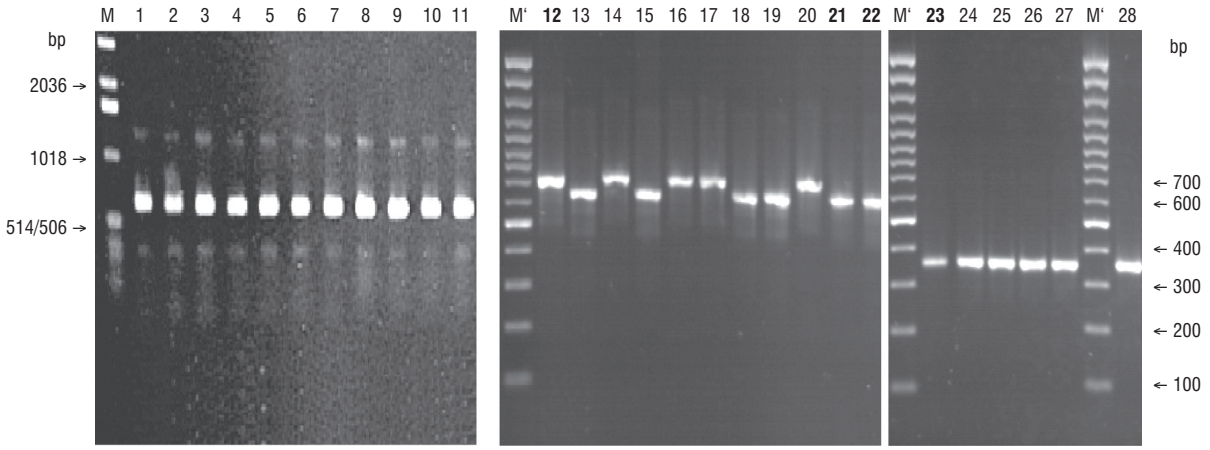

FIGURE 1. Yeast amplification products of ITS1–5,8S rDNA–ITS2 region obtained with ITS1 and ITS4 primers.
M: marker X (Roche), M': Gene Ruler 100bp DNA Ladder Plus (Fermentas). Lanes 1-11 *C. famata strains* (API identification): lane 1: AI4a, lane 2; AI4c, lane 3: AII4b, lane 4: BI6a, lane 5: BII3c, lane 6: DNIIP1b, lane 7: EII2b, lane 8: KI2a, lane 9: MI1a, lane 10: PI1a, lane 11: SII2c. **Lane 12: *K. lactis* CLIB 384**. Lanes 13–20 *C. sphaerica strains* (API identification): lane 13: BI6c, lane 14: EII1a, lane 15: EII3a, lane 16: FII5a, lane 17: FII7a, lane 18: MI4a, lane 19: OI3a, lane 20: DNIIW2b. **Lanes 21–22 *D. hansenii* strains: lane 21: NCAIM 898, lane 22: CCY-41-3-3. Lane 23 *Y. lipolytica* ATCC 20460**. Lanes 23–28 *C. lipolytica* strains (API identification): lane 24: JII1a, lane 25: JII1c, lane 26: PII6a, lane 27: PII6b, lane 28: PII6c.

Table 2. Size of amplification products of ITS1-5,8S-ITS2 region and their restriction with three endonucleases.

Species[a,b]	Strain	PCR product size (bp)	Restriction fragments (bp)		
			*Bsu*RI (*Hae*III)	*Hinf*I	*Hin*6I (*Cfo*I)
D. hansenii	NCAIM 898[r]				
D. hansenii	CCY 41-3-3[r]				
C. famata	AI4a, AI4c, AII4b, BI6a, BII3c, DNIIP1b, EII2b, KI2a, MI1a, PI1a, SII2c	630 ±18	413 ±18 142 ±10 83 ±16	304 ±19	285 ±15 51 ±1
C. sphaerica	BI6c, EII3a, MI4a, OI3a				
K. lactis	CLIB 384[r]				
C. sphaerica	EII1a, FII5a, FII7a, DNIIW2b	706 ±15	654 ±9 81±1	297 ±4 195 ±5 128 ±3 91 ±1	286 ±0 198 ±3 177 ±2 108 ±4
Y. lipolytica	ATCC 20460[r]				
C. lipolytica	JII1a, JII1c, PII6a, PII6b, PII6c	350 ±9	353 ±2	185 ±1	208 ±2 171 ±2

[a] API ID 32C identification except reference strains; [b] *D. hansenii* – teleomorph of *C. famata*; *K. lactis* – teleomorph of *C. sphaerica*; *Y. lipolytica* – teleomorph of *C. lipolytica*; [r] reference strain; ± standard deviation

nineteen strains of *D. hansenii* isolated from Danish smear cheese and tested by Petersen *et al.* [2001] could grow in the presence of D-xylose as a sole C compound.

Identification of yeast isolates as *C. sphaerica* was more precise. It was "excellent" in five out of eight cases (% id =99.9, 0.96 < T index < 1.00) and "good" for one strain (BI6c; 3 contrary tests and T index equal 0.30). Two other strains were identified with only "acceptable" conformity because of 2 incompatible reactions. One contrary test, common for all those strains, was the ability to assimilate N-acetyl-glucosamine, while only four percent of *C. sphaerica* strains from the APILAB Plus database has got such ability. Other contrary tests, incompatible also with species description of Lachance [2000] were as follows: the ability to grow on 2-keto-gluconate, the lack of ability to utilize DL-lactate and sensibility to cycloheximide.

Identification with PCR-RFLP of rDNA method. The amplification products obtained with ITS1 and ITS4 primers contained a small fragment of 18S rDNA, two internal transcribed spacers (ITS1 and ITS2) flanking the 5.8S rDNA region and a short fragment of 26S rDNA [White *et al.*, 1990]. Amplification profiles obtained for all tested strains are presented in Figure 1.

All strains identified with API as *C. lipolytica* and *C. famata* produced characteristic amplification products (approx. 350 bp and 630 bp, respectively) identical to reference strains used in the study (Table 2). Such results are in agreement with sequence data reported by Chen *et al.* [2000; 2001] who determined the length of the mentioned fragment for *Y. lipolytica* as 352 bp and for *C. famata* as 630 bp. Similar sizes of products were obtained by Petersen *et al.* [2001] for *Y. lipolytica* strains from CBS collection (360 bp) and for *D. hansenii* CBS 789 (type strain of *D. hansenii var. fabryi*) and *D. hansenii* CBS 767 (type strain of *D. hansenii var. hansenii*) - 639 bp.

Surprisingly, four isolates classified with API to *C. sphaerica* species (BI6c, EII3a, MI4a, OI3a) gave amplification products with size similar to that of *D. hansenii* reference strains as well as all *C. famata* isolates. It would confirm the earlier assumptions, formed after the analysis of contrary tests obtained with API for three of the mentioned strains. Although API identification of the fourth one (EII3a) was "excellent" the size of the amplification product indicates classification of that strain rather as *C. famata*.

Other *C. sphaerica* strains yielded bigger products (app. 706 bp) with size similar to that obtained for the perfect form – *K. lactis* CLIB 384 used as a reference strain. Esteve-Zarzoso *et al.* [1999] and Petersen *et al.* [2001] reported slightly bigger length of that fragment (740 bp), yet it can be the fault of gel accuracy. Simultaneous analysis of reference strains and yeast isolates appear to be helpful in diminishing electrophoresis inaccuracy (Figure 1).

The use of 3 endonucleases confirmed the results of amplification. All *C. lipolytica* strains gave restriction patterns similar to the reference strain *Y. lipolytica* ATCC 20460 and compatible with data reported by Esteve-Zarzoso *et al.* [1999]. It is also in agreement with theoretical digestion profiles calculated from *Y. lipolytica* ATCC 18942 type strain sequence (GeneBank accession number AF335961 and AF218983). Restriction enzymes *Hinf*I and *Hin*6I (*Cfo*I) have one restriction site in mentioned fragment while *Hae*III are not able to cut that fragment.

All isolates classified with API to *C. famata* species as well as four questionable strains (BI6c, EII3a, MI4a, OI3a) produced characteristic profiles similar to those of *D. hansenii* CCY-41-3-3 and *D. hansenii* NCAIM 898 (CBS 767) (Figure 2, Table2). *Hae*III and *Hinf*I endonucleases have two restric-

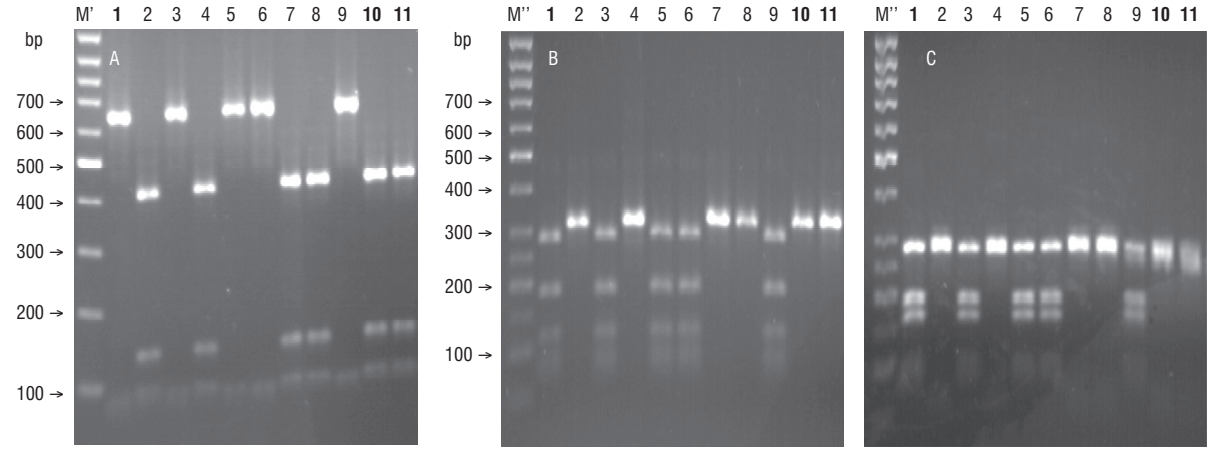

FIGURE 2. Restriction profiles of ITS1–5,8S rDNA–ITS2 region obtained with *Bsu*RI / *Hae*III (A), *Hinf*I (B) and *Hin*6I / *Cfo*I (C) endonucleases. M': Gene Ruler 100bp DNA Ladder Plus (Fermentas). M'': Gene Ruler 50bp DNA Ladder (Fermentas). **Lane 1 *K. lactis* CLIB 384**. Lanes 2–9 *C. sphaerica* strains (API identification): lane 2: BI6c, lane 3: EII1a, lane 4: EII3a, lane 5: FII5a, lane 6: FII7a, lane 7: MI4a, lane 8: OI3a, lane 9: DNIIW2b. **Lanes 10–11 *D. hansenii* strains: lane 10: NCAIM 898, lane 11: CCY-41-3-3**.

tion sites in the amplified product of *D. hansenii*. The first enzyme produces three separated fragments while the second gives only one band on agarose gel (Figure 2B, Lanes 10–11). Sequence analyses demonstrated that with the mentioned enzyme three bands are produced, two of approximately the same size (315 bp and 316 bp) and the third one 8 bp-long which is not visible on agarose gels [Petersen *et al.*, 2001].

Restriction patterns of 4 other isolates confirmed their identification as *C. sphaerica* when compared to the reference strain as well as to data obtained by Esteve-Zarzoso *et al.* [1999].

CONCLUSIONS

Summarizing, PCR-RFLP analysis of rDNA enabled more precise identification of strains belonging to species most frequently isolated from cheese such as *C. famata* and *C. sphaerica* than the API ID 32C test based on assimilation profiles.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. Huu-Vang Nguyen from the Collection de Levures d'Interet Biotechnologique (INRA, Thiverval-Grignon, France) for the kind donation of *K. lactis* CLIB 384 strain.

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Received May 2006. Revision received October and accepted March 2007.

PORÓWNANIE IDENTYFIKACJI GATUNKÓW DROŻDŻY POWSZECHNIE SPOTYKANYCH W SERACH W OPARCIU O CECHY FIZJOLOGICZNE ORAZ PCR-RFLP rDNA

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Porównano identyfikację dwudziestu czterech izolatów drożdży z serów wykonaną przy pomocy testu asymilacyjnego API ID 32C (tab. 1) oraz metodą polimorfizmu długości fragmentów restrykcyjnych (PCR-RFLP) regionu ITS1-5.8S rDNA-ITS2 (tab. 2). Zaobserwowano zgodność wyników otrzymanych obiema metodami w stosunku do jedenastu szczepów zidentyfikowanych jako *Candida famata*, pięciu jako *C. lipolytica* i czterech jako *C. sphaerica.* Jednakże pozostałe cztery izolaty drożdży, sklasyfikowane przez system API jako *C. sphaerica* wykazały produkty amplifikacji, jak również profile restrykcyjne charakterystyczne dla gatunku *C. famata* (rys. 1 i 2).