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Molecular Techniques for Yeast Identification in Food Processing

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Summary

Yeasts are crucial agents in many important natural and industrial bioprocesses as well as in spoilage of foods and in some diseases in humans and animals. Quick and reliable methods for yeast identification and classification are highly appreciated. Classical methods include morphological and physiological-biochemical tests. They are time and material consuming; reliability and distinctive capacity of closely related isolates are low.

Besides phenotypic characterization many molecular biology analyses of yeast nucleic acids have recently been developed. They can give stable and unique electrophoretic profiles independently of the microbial cultivation conditions. In this paper the application of nuclear and mitochondrial DNA analyses for identification of food-borne yeasts is reviewed – electrophoretic karyotyping, study of the restriction fragment length polymorphism (RFLP) of mtDNA, and two techniques based on PCR amplification of yeast DNA: the amplification with non-specific primers (AP-PCR, arbitrarily primed PCR, RAPD, random amplified polymorphic DNA analysis) and PCR ribotyping – restriction analysis of amplified ribosomal RNA genes of tested yeasts.

The advantages and limitations of the application of molecular methods based on DNA analysis for yeast characterization in research and routine industrial laboratories are discussed. Generally, nucleic acid-based assays offer sensitivity and specificity that could not be achieved by conventional identification methods. However, the described methods still rely on cultural enrichment and DNA isolation. In perspective, specific DNA probes/primers and direct sequencing of PCR amplified DNA fragments will likely replace the currently used techniques as a uniform method for identification of yeasts in general.

Keywords: yeasts, identification, classification, molecular methods, food processing

Phenotypic and Genotypic Identification of Food-borne Yeasts

Yeasts are unicellular fungi which reproduce by budding or fission. This definition covers a wide variety of organisms, among them ascomycetes, basidiomycetes and imperfect yeasts, *Fungi imperfecti (1).* A border between yeasts and other fungi is not strict – from the morphological point of view yeasts may be a stage in the life cycle of the hyphal fungi; under certain conditions some yeasts form pseudo- or true mycelia. Regardless of the taxonomic definitions of yeasts, biological properties of these microorganisms have well-known beneficial and also harmful effects in food processing:

a) Yeasts are the most important group of microorganisms that are exploited for commercial purposes *(2).* The main contribution is made by traditional biotechnologies, like fermentation of alcoholic beverages, bakers' yeast production etc.

b) Yeasts may cause spoilage of foods. Their role as spoilage agents is becoming more evident in recent processing and preservation techniques, where food products tend to contain less alcohol, salt, acid and sugar, less preservatives or none at all, or have passed milder heat treatments *(3–5).*

Fast and reliable methods are needed for characterization of industrial yeasts as well as for detection of contamination routes and accurate intervention procedures in food processing. Our present yeast classification

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system and the identification schemes, which arise from it, still rely on phenotypic characters, such as physiological reactions and morphology *(6,7)*. Some of these complex characters still have an unknown genetic basis. Variability in phenotypic characters could be due to different physiological states of the cells. Consequently, the interpretation of sometimes doubtful results depends on subjective factors. Moreover, these methods are generally labour-intensive, time and material consuming, hence limited in their applicability. Although many tests are included, a distinctive capacity of closely related strains is low. As many of the industrial yeast strains belong to a group of closely related *Saccharomyces sensu stricto* yeasts, they cannot be readily distinguished and identified by classical biochemical methods *(8)*.

In addition to phenotypic methods, different molecular biology techniques have been developed recently and applied also in food mycology. The methods based on DNA analysis have the advantage of being independent of the gene expression, the DNA sequences are not influenced by the environmental conditions of the cells *(9)*. Unlike multidimensional phenotypic characterization, genotypic (sequence) information has a single dimension. There are just a few elements (nucleotides) which can be objectively judged in mathematically defined relationships *(10)*. However, nucleotide sequence determination, the basic source of genotypic information, is technically still an inappropriate method for quick identification of industrial microbial isolates. This is the reason for the development of different molecular-genetic methods avoiding direct sequencing but with the ability to provide fast information about microbial isolates at the level of their DNA structure. They could be used for the identification of microorganisms on generic, species or strain levels. Methodological principles are different. The analytical object could be the whole genome (total genomic DNA, nuclear DNA (nDNA), mitochondrial DNA (mtDNA), plasmid DNA (pDNA) etc.), or very limited regions (i.e. ribosomal DNA (rDNA) or even more specialized sequences). The DNA could be isolated directly from the microbial cells or amplified enzymatically in the laboratory, on the basis of the microbial target DNA, by means of polymerase chain reaction (PCR).

In this paper four molecular biology techniques, which have recently been introduced and applied for the identification of food-borne yeasts are presented through own experience and in terms of cited literature.

Yeast Mitochondrial DNA Restriction Analysis

Analyses of restriction fragment length polymorphism (RFLP) of mitochondrial DNA have the longest tradition among molecular yeast characterization techniques presented here. Yeast mitochondria contain their own, relatively short (*c.* 20.0–80.0 kb), usually circular DNA molecules, which are present in many identical copies in each cell and in each mitochondrion. Growing on a non-fermentable substrate, a yeast cell contains *c.* 50 molecules of mtDNA, representing 5–25% of the total cell DNA *(11)*. Consequently, with the application of the restriction endonucleases, it is possible to get a few restriction fragments with a high DNA concentration, ensuring clear electrophoretic restriction patterns. Their polymorphisms were early employed for distinction of closely related beer and wine yeast strains *(12–15)*. However, technically complicated mtDNA isolation in the ultracentrifuge did not permit routine industrial application of mtDNA analysis. To avoid this problem, a simplified procedure was developed on the basis of the standard miniprep isolation of the total DNA, with the use of selected restriction endonucleases *(16)*. The procedure was employed for the distinction of *Saccharomyces cerevisiae* wine strains and for simplified yeast population kinetics studies of spontaneous and induced alcoholic fermentations on a strain level *(17–19)*.

Recently, a mitochondrial molecular marker has been reported for distinction of yeast species *(20,21)*. Diagnostic differences were based on the presence/absence of *ori-rep-tra*, a special class of mitochondrial intergenic sequences, in industrially important *Saccharomyces* yeasts and in some other yeast genera (*Zygosaccharomyces, Torulaspora, Debaryomyces, Kluyveromyces* etc.). *Ori-rep-tra* sequences participate actively in cellular metabolism and play a role in determining a mode of mtDNA inheritance *(20,22)*. However, a molecular probe containing an *ori-rep-tra* sequence was employed specifically for the distinction and identification of isolates belonging to the species complex *Saccharomyces sensu stricto (20)*. With the hybridization of restriction patterns of the enzymes which cut specifically in GC clusters, three to eight *ori-rep-tra* sequences were found in *S. cerevisiae, S. bayanus, S. carlsbergensis, S. douglasii, S. monacensis, S. pastorianus, S. paradoxus* and *S. uvarum* strains *(21)*.

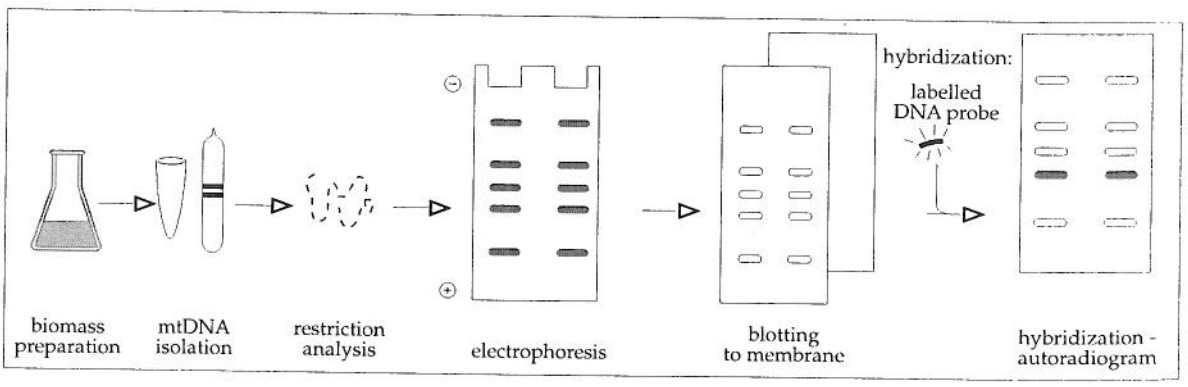

Fig. 1. Schematic presentation of mtDNA RFLP analysis

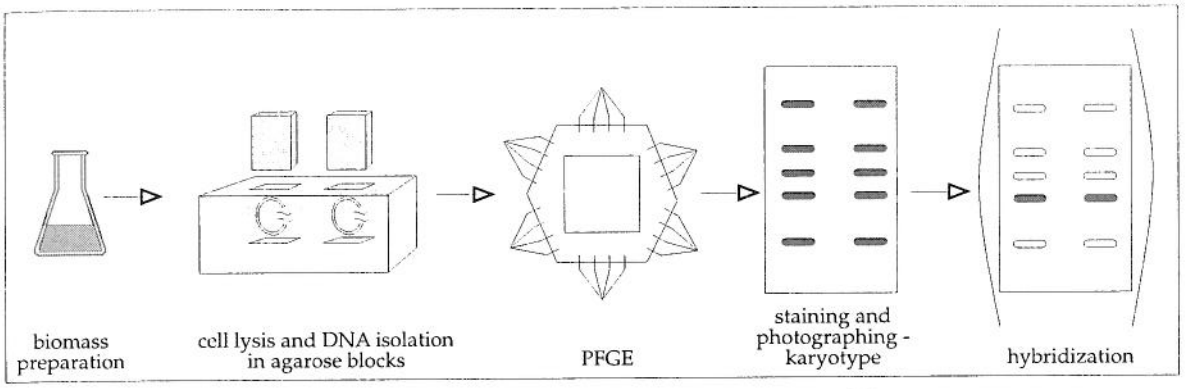

Fig. 2. Schematic presentation of yeast karyotyping with PFGE

Electrophoretic Karyotyping of Yeasts with Pulsed Field Gel Electrophoresis

Another approach for yeast DNA fingerprinting was given by the development of a special type of agarose gel electrophoresis enabling separation of very large DNA fragments (*c.* 200 kb–10 Mbp, i.e. the size range of intact yeast chromosomes). The study of yeast chromosomes by light microscopy has been hampered by their small size. After application of different techniques of pulsed field gel electrophoresis (PFGE) like PFGGE, pulsed field gradient gel electrophoresis *(23)*, OFAGE, orthogonal field alternation gel electrophoresis *(24)*, FIGE, field inversion gel electrophoresis *(25)*, CHEF-PFGE, contour-clamped homogeneous electric field gel electrophoresis *(26)*, RGE, rotating gel electrophoresis *(27)*, etc., yeasts small chromosomal size has become an advantage which enables their electrophoretic karyotyping. Chromosomal DNA fragments are reoriented in the gel due to periodic changes of direction of the electric field. The time of reorientation is in correlation with the molecular size. This is the basic principle for the separation of large DNA fragments with all types of PFGE *(28,29)*.

The first applications of PFGE have been made with yeasts *(23,24)*. Carle and Olson *(24)* separated 15–17 electrophoretic fragments of *S. cerevisiae*, which have been confirmed to be yeast chromosomes by hybridization with gene- or chromosome specific DNA probes. In the past ten years a high level of polymorphism of electrophoretic karyotypes of industrial yeasts has been revealed on interspecific and also on intraspecific levels, especially for *Saccharomyces sensu stricto* yeasts. A multivariate analysis of polymorphisms in the electrophoretic karyotypes of many certified authentic strains from this group revealed that they could be separated into four subgroups *(30)*, corresponding to the taxa distinguished by classical genetic *(31)* and molecular methods *(32)*. However, direct determination of very closely related sibling species of *Saccharomyces sensu stricto* group remains difficult (i.e. aneuploidy of the industrial strains, the size differences of homologous chromosomes make identification impossible without specific probes etc).

We used the electrophoretic karyotyping of yeasts for another purpose – a very high distinctive capacity of the method gives an opportunity for studying population dynamics of yeasts during spontaneous alcoholic fermentations on a strain level. We found great heterogeneity of *Saccharomyces cerevisiae* and also of non-*Saccharomyces* yeasts involved in spontaneous and induced alcoholic fermentations in some wine producing regions in Slovenia. Distinction of *S. cerevisiae* strains was possible on the basis of polymorphism and/or different number of copies of short (I,VI, III, IX, VIII) and middle sized chromosomes (XI, X, XIV, II, XIII) *(33)*. The frequency, i.e. population dynamics of the strains involved in a particular fermentation process was followed through different fermentation phases (fresh must at the start of fermentation, tumultuous fermentation, young wine before racking). Sequential substitution of some less frequent *S. cerevisiae* strains was observed, but some dominating strains present in all fermentation stages were recognized *(34)*. Fig. 3 provides an example of the heterogeneity of the electrophoretic karyotypes of the fifteen yeast strains of *S. cerevisiae* that were isolated from the same sample of young wine »Kraski teran« after alcoholic fermentation was over, i.e. just before racking. Electrophoretic karyotyping of natural isolates of wine yeasts could find practical application in selection of strains naturally adapted to environmental conditions that are formed during specific wine fermentations, and in this way are suitable for starter culture application. On the other hand, comparative analyses of results could reveal new information about the relationship between ecological/geographical origin and genetic relationships among natural yeast populations.

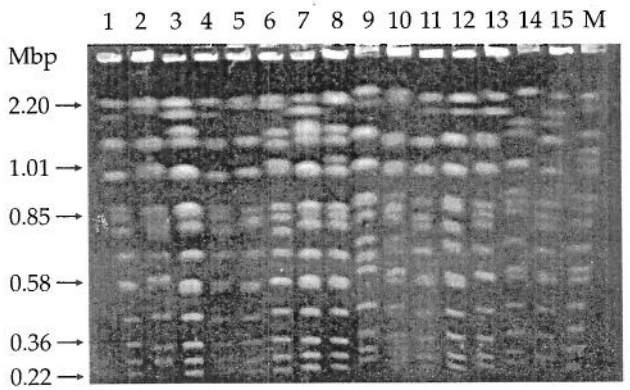

Fig. 3. Heterogeneity of 15 electrophoretic karyotypes of yeasts isolated in the late stage of alcoholic fermentation of »Kraski teran« (Vinakras, Sežana, autumn 1995). (M: PFGE marker II (*S. cerevisiae*), Boehringer), CHEF PFGE (Pulsaphor LKB Pharmacia) conditions: pulse time: 60 s (15 h), 90 s (8 h), 100 s (1 h); total running time: 24 h, constant voltage 180 V, 1% NA-LKB agarose, 0.5 × TBE buffer, t = 12 °C

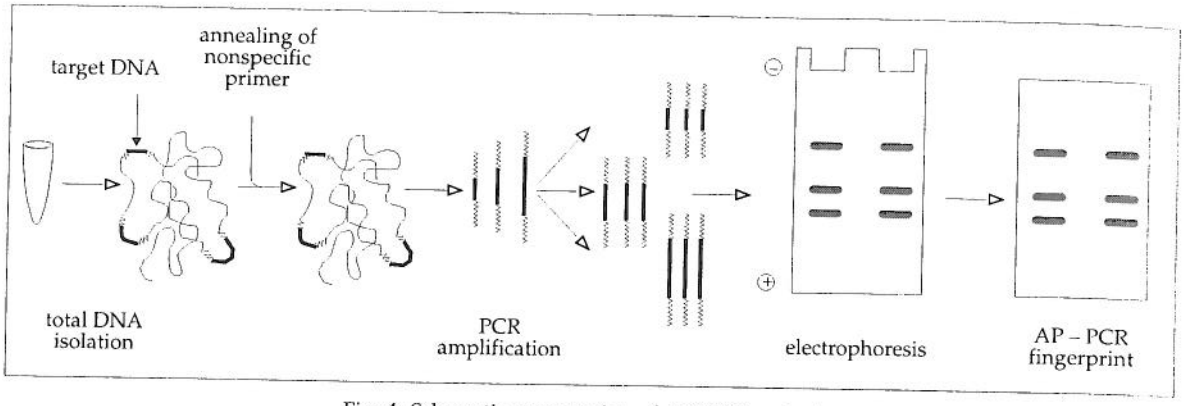

Fig. 4. Schematic presentation of AP-PCR analysis

However, the application of mtDNA analysis including hybridization as well as electrophoretic karyotyping of yeasts require a few days to obtain results. Quicker techniques are essential when an appropriate decision must be taken as soon as possible (e.g. in the case of contamination control during technological process).

PCR Amplification of Yeast DNA with Non-specific Primers (AP-PCR, RAPD Analysis)

A possibility for quicker analysis was offered with the application of polymerase chain reaction (PCR) *(35)* in the identification tests of yeasts. Two techniques have been developed and applied recently, the amplification of microbial DNA with non-specific primers (AP-PCR, arbitrarily primed polymerase chain reaction *(36)*, RAPD, random amplified polymorphic DNA analysis *(37)*) and PCR ribotyping – restriction analysis of amplified ribosomal DNA of yeasts *(38)*.

Since 1990, when two independent groups of researchers published papers on the basic principle of AP-PCR, i.e. the amplification of DNA segments with a single primer of arbitrary nucleotide sequence (schematically presented in Fig. 4.), this simple and rapid genotyping method has gained many applications for the differentiation of organisms at the species and particularly at the subspecies levels. It has many advantages compared to other genotyping techniques. It is less labour intensive, and it requires very small amounts of the template DNA and almost no knowledge about the molecular background of the strains examined. Comparison with the PCR typing with specific primers shows that no sequence information is needed, but more polymorphisms can be detected as a result of mutations of the priming sites and/or insertions/deletions between them *(37)*.

In industrially important yeast genera, *Saccharomyces* and *Kluyveromyces* yeasts associated with wines, beers, distilling and baking products, polymorphism in microsatellite DNA primed sequences was reported *(39)*. In the past two years, RAPD assay with the selected decamer and microsatellite DNA primers was used for distinction of *Zygosaccharomyces*, *Saccharomyces* and *Candida* spoilage yeasts *(5,40)*, different genera of wine yeasts, such as *Saccharomyces*, *Candida*, *Pichia*, *Torulaspora*, *Hansenula* and *Rhodotorula (41,42)*, and *Saccharomyces* yeasts isolated from different foods *(43–45)*. Although comparative analysis of AP-PCR with other phenotypic and genotypic methods for yeast characterization reveals a high distinctive capacity of AP-PCR, it seems that the choice of the primer(s) has a strong influence on the discriminatory power of the technique. A species- and/or strain specificity should be evaluated in each particular case and confirmed with a larger number of the type strains, because the reaction does not give an easily identifiable marker (like karyotyping or PCR ribotyping of yeasts, described in the next paragraph, see Figs. 5. and 7. for comparison). There is another problem associated with the technique, which relies on the use of non-specific primers and low annealing temperatures of primers to the target DNA. Non-stringent annealing conditions may result in lower reproducibility and unclear background of the fingerprints because of different intensity of the amplified fragments. According to this, a major criticism of the method is due to hardly comparable results of AP-PCR fingerprinting. The technique could be used for distinction of very closely related strains (of the same species), but it is almost without any value for comparison of isolates on the species level or even on higher levels. However, to some extent still contradictory literature data indicate that more comparative analyses of AP-PCR with different primers are needed to confirm the usefulness of this simple and rapid method for direct identification of yeasts from foods and other sources on the species and strain levels *(40,42,45)*.

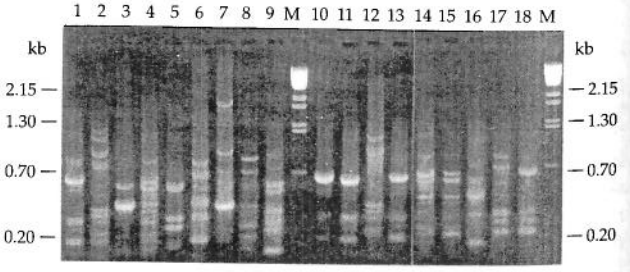

Fig. 5. AP-PCR fingerprints of *Saccharomyces* type strains (indicated by ^{T}) and some other isolates. Primer: $(GACA)_{4x}$ (MWG Biotech), $t_{annealing}$ = 38 °C; 1: *S. cerevisiae*^{T}; 2: *S. paradoxus* ^{T}; 3: *S. bayanus*^{T}; 4: *S. pastorianus*^{T}; 5: *S. exiguus*^{T}; 6: *S. servazzii*^{T}; 7: *S. castellii*^{T}; 8: *S. dairensis*^{T}; 9: *S. kluyveri*^{T}; 10, 11: *S. cerevisiae*; 12: *S. douglasii* 13: *S. diastaticus*; 14: *S. monacensis*^{T}; 15: *S. carlsbergensis*^{T}; 16: *S. uvarum*^{T}; 17, 18: *S. carlsbergensis*; M: λ DNA/*Bst*E II *(44)*

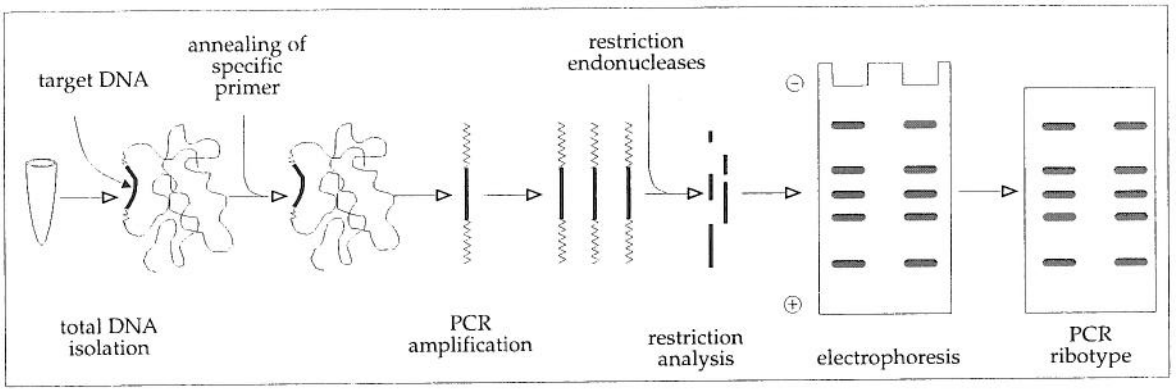

Fig. 6. Schematic presentation of PCR ribotyping of yeasts

PCR Ribotyping of Yeasts

Yeast ribosomal RNAs (18S, 25S, 5.8S and 5.0S rRNA) are encoded by the genes organized in the rDNA unit that is repeated 100–200 times on the chromosome XII. Mitochondrial RNAs (15S, 21S rRNA) are encoded by mtDNA. If the PCR primers with the sequences complementary to nuclear or mitochondrial yeast ribosomal RNA genes are used, it is possible to amplify the whole nuclear or mitochondrial rDNA in just two PCR reactions. If the amplified fragments are subjected to RFLP analysis with the selected restriction endonucleases, specific ribotyping patterns, i.e. ribotypes can be generated after electrophoretic separation of restriction fragments (Fig. 6).

Because of the presence of both highly conserved and variable regions the restriction fragment length polymorphisms (RFLPs) of yeast ribosomal DNA (rDNA) repeats have been used successfully to show inter- and intraspecific relationships of different yeast taxa *(46–49)*. With the long range ribotyping of the *Saccharomyces* species, where the amplicons of the whole chromosomal rDNA were cut with different endonucleases, the results were in complete accordance to the rRNA sequence analysis. Characteristic ribotyping patterns of the *Saccharomyces* species type strains were shown *(49)*. In addition, we studied different combinations of amplified rDNA fragments (PCR primers from 18S and 25S rDNA) and restriction enzymes to check the species specificity of the restriction patterns for a larger number of well characterized synonym type strains of *Saccharomyces sensu stricto* and *Torulaspora* yeasts *(50)*. We tried to find species-specific riboprinting patterns that could be used for quick identification of the yeast isolates from foods, natural and industrial fermentation processes etc.

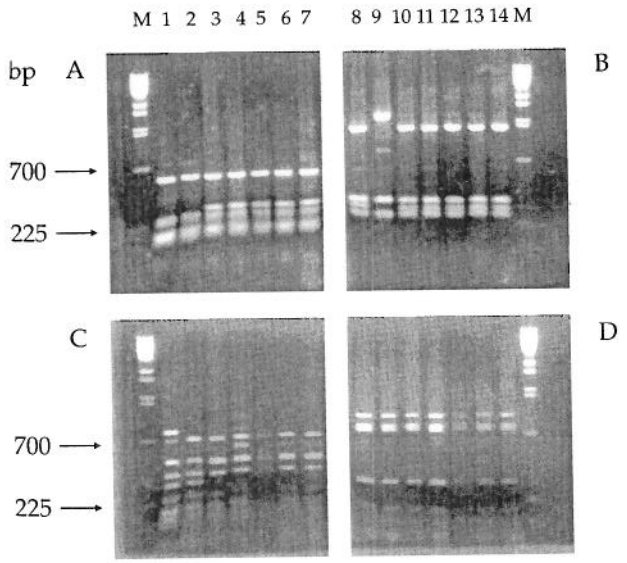

Fig. 7. PCR ribotyping of the type strains and the synonym type strains of *Saccharomyces sensu stricto* yeasts. 1, 8: *S. cerevisiae*^{T}; 2, 9: *S. paradoxus*^{T}; 3, 10: *S. bayanus*^{T}; 4, 11: *S. pastorianus*^{T}; 5, 12: *S. monacensis*^{T}; 6, 13: *S. carlsbergensis*^{T}; 7, 14: *S. uvarum* ^{T}. **A**: 18S rDNA+ITS1/*Hae*III, **B**: 18S rDNA+ITS1/*Msp*I, **C**: 25S rDNA/ /*Hae*III, **D**: 25S rDNA/*Msp*I, (the amplified DNA fragment/the endonuclease used, ITS1: internal transcribed spacer, a variable part of rDNA unit), M: λ DNA/*Bst*E II *(51)*

It was shown that the restriction rDNA analysis offers a convenient tool for quick identification of yeasts, i.e. distinction of isolates on the species level (Fig. 7). Owing to the good reproducibility of ribotypes, they are suitable for computerized databases development. However, to apply such databases to minimize time and material expenses in yeast identification schemes, some limitations of the method should be resolved. Firstly, because of highly conserved regions, distinction capacity should be checked with a larger number of restriction endonucleases, and secondly, species specificity of riboprinting patterns should be tested with a larger number of strains from the same and from other taxa to avoid misidentification because of strain-specific riboprints of the species type strains. Some applications have been published recently for identification of food-borne yeasts *(40, 45)*, but more results are needed, especially for quick identification of non-conventional, i.e. non-*Saccharomyces* industrial yeasts.

Future Perspectives

Molecular methods based on DNA analysis have been applied for direct identification of yeasts quite recently. They have certain advantages over phenotypic identification but some limitations as well. The methods presented in this paper require isolation of pure microbial cultures and also material and time consuming isolation of DNA. This is especially true of mtDNA isola-

tion and of the isolation of intact chromosomal size DNA fragments, the basic requirement for electrophoretic karyotyping. This continued reliance on quite extensive microbiological and molecular-biological manipulation (including labelling of DNA probes and membrane hybridization (Figs. 1 and 2) is probably the biggest drawback. With recent advances in automation of nucleic acid sequencing and accumulation of the knowledge of species specific PCR primers development it seems likely that direct sequencing of PCR amplified DNA fragments could replace the currently used techniques. In fact, DNA sequence analysis of PCR products could be accepted as a uniform method for identification of yeasts and other microorganisms. All the assays could be configured in a single format using identical reagents, protocols and equipment *(52)*. Databases already formed on DNA sequence analysis of partial or complete 18S and/or 25S rRNA/DNA present an indispensable source of information for the development of specific PCR primers also for yeast genera including industrially important yeast species *(53–56)*. However, a development of species- or strain-specific PCR primers is still required for most of the industrial yeast strains. Considering the intensity of research in this field of applied science it is realistic to expect one-day procedures for identification of the most important industrial yeasts directly from food in the very near future. However, on the basis of up-to-date technology, direct transfer of these techniques from research to routine industrial laboratories remains very limited, due to technical pretentiousness, limited education of employees and the price for such type of routine analysis.

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Molekularne tehnike identifikacije kvasovk v živilstvu

Povzetek

Kvasovke imajo važno vlogo v številnih naravnih in industrijsko vodenih bioloških procesih, pa tudi pri nezaželjenih procesih, kot so kvar živil in nekatera obolenja ljudi in živali. Hitra identifikacija in taksonomska uvrstitev izolatov kvasovk je pomembna na vseh omenjenih področjih, vendar v praksi mnogokrat problematična. Klasične metode, temelječe na morfoloških in fiziološko-biokemijskih testih, so dolgotrajne, materialno zahtevne, možnost razločevanja zelo sorodnih sevov in zanesljivost sta majhni. Zato se vpeljujejo molekularno-biološke tehnike, kot so analiza celičnih proteinov, maščobnih kislin, predvsem pa analize dednine mikroorganizmov. Takšna karakterizacija je neodvisna od ekoloških pogojev kultivacije in omogoča filogenetsko osnovano klasifikacijo organizmov.

V prispevku so opisane tehnike elektroforetske kariotipizacije oz. uporabe pulzne elektroforeze za ločevanje intaktnih kromosomov kvasovk, analize raznolikosti restrikcijskih vzorcev mitohondrijske DNK (mtDNK), in tehniki, ki vključujeta namnoževanje mikrobne DNK z verižno reakcijo s polimerazo: PCR ribotipizacija, t.j. restrikcijska analiza namnoženih fragmentov ribosomske DNK po uporabi specifičnih začetnih oligonukleotidov in študij raznolikosti elektroforetskih vzorcev namnožene DNK z nespecifičnimi, naključno izbranimi začetnimi oligonukleotidi.

Prednosti in pomanjkljivosti predstavljenih tehnik na današnji stopnji razvoja so obravnavane v luči zahtev za hitro identifikacijo kvasovk v živilih oz. živilstvu. Specifičnost, občutljivost, čas, enostavnost oz. uniformiranost izvedbe in cena analize so ključnega pomena za nadaljnji razvoj in uporabo identifikacijskih metod, temelječih na analizi nukleinskih kislin. Perspektivo za odpravo pomanjkljivosti predstavljenih tehnik daje razvoj specifičnih DNK sond oz. začetnih oligonukleotidov PCR, ki bi omogočili direktno identifikacijo kvasovk iz živil na osnovi sekvenciranja specifično pomnoženih PCR produktov.