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DEVELOPMENT OF AN AUTOMATED YEAST IDENTIFICATION SYSTEM BY THE APPLICATION OF PCR TECHNIQUE: A COMPARISON OF Inter-LINE PCR WITH REFERENCE METHODS

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1. Introduction

Recently, the identification of yeast strains and their classification by modern methods has been an area of active research in technical microbiology for industrial and medical purposes Yamagishi et al. (1).

Conventional techniques for the differentiation of yeasts, which are based on the analysis of morphological and physiological characters (e.g. auxonographic tests for anabolic processes, or fermentation assays for catabolic conversions), are slow (up to 10 days are required for the growth of certain strains) and are labour-intensive. Moreover, these tests do not give results that are clear and comprehensive enough. In order to improve the reliability and sensitivity in identification, as well as the differentiation of yeasts, biochemical methods based on the analysis of protein profiles or on the comparison of distinct isoenzymes have been developed. Such techniques are more useful but are also expensive and time consuming, besides requiring highly skilled workers.

In the case of yeast taxonomy, the most reliable and modern methods use genetic analysis. The DNA is inert to those interference factors. Some of these techniques are based on the characterization of the complete genomic DNA (as in variants of **p**ulse **f**ield **g**el **e**lektrophoresis, PFGE, as well as in **r**estriction **f**ragment **l**ength **p**olymorphism, RFLP). Other methods such as r-DNA-hybridization techniques or PCR (**p**olymerase **c**hain **r**eaction)-variants, require the analysis of distinct parts of the genome only. One strategy in these PCR-techniques is to characterize specific and typical PCR-fragments on a molecular level, for instance rDNA-spacer-PCR,

single **s**trand **c**onformation **p**olymorphism (SSCP). Another type of method performs the differentiation of yeast by comparison of complex PCR-fingerprints. These are built up by cycles of amplification, starting at distinct primer-binding-sites. The primers anneal in a more or less specific way, followed by extension- and denaturation steps. After 25 - 40 cycles, several different-sized PCR products (AP-PCR, Random Amplified Polymorphic DNA-PCR, Inter-LINE PCR) are generated. This mix of DNA-fragments serves, after suitable gel electrophoresis and staining techniques, as a fingerprint-like identification. The PCR-primers can be derived from arbitrarily chosen sequences, which may be synthetic DNA sequences or else originate from repetitive natural DNA-motifs, and even from mammalian genomes.

The present work examines the use of Inter-LINE PCR (**L**ong **I**nterspersed D**N**A **E**lements) to follow the differentiation of certain yeasts which play a crucial role in biotechnological processes as culture strains, or as contaminants in the beverage industry.

Smida et al. (2) established Inter-LINE PCR conditions for reproducible amplification of LINE sequences. These used the construction of specific primers GF, GR, RF and RR from the DNA of mammalian cells. Reduction of the annealing temperature in the initial five cycles of the PCR, can allow genomic DNA from lower eukaryota, like yeasts, to also serve as a template, see Leibhard and Nickel (3). In this study, some of them primers were used for identification and differentiation of yeasts from the beverage industry. A comparison of Inter-LINE PCR with reference DNA-based techniques (AP-PCR and two variants of PFGE), demonstrates the power of the Inter-LINE PCR method. The value of creating databases on the digitized PCR-fingerprints for an automatization or an identification is demonstrated.

2. Materials and Methods

2.1. Yeast strains

For the characterization of different yeasts by IL-PCR fingerprints, about 80 strains were examined. They were chosen because of their importance in beverage production, and belong to 30 species and 13 genera. They were derived from international or professional collections as well as from industrial laboratories.

Representatives of ten different yeast groups are listed in the Table 1. With these yeasts a general recapitulation of obtained results was performed.

2.2. PCR methods

- *Template DNA isolation*. A modified phenol-chloroform procedure for chromosomal DNA preparation was used (3). Briefly, yeast cells from overnight culture were harvested. After spheroplasting of cells a mixture of DNA and RNA as well as proteins were extracted from the lysate. The pellet, precipitated by absolute ethanol, was dissolved in TE buffer. After hydrolysis of RNA, this solution was at first saturated with an equal volume of phenol and next with an equal volume of chloroform / iso-amyl-alcohol (mixed in ratio as 24:1). After short centrifugation, an equal volume of absolute isopropanol was added to the supernatant to precipitate DNA. The harvested DNA was washed twice with cold 70% ethanol and air-dried. The pellet was dissolved in MiliQ water and stored at -20^{o}C.

- *LINE specific PCR primers*. Specific IL-PCR primers, GF and GR (2) and 01 (3), which are derived from highly conserved regions of LINE sequences, were used:

GF:	GAA GGG TCT TTG CCA AAC TC
GR:	GAG TTT GGC AAA GAC CCT TC
O1:	GGC TGC CTT TAT ATG TTA CTG GCC

- *The IL-PCR method*. The Inter-LINE PCR was carried out in a total volume of 50 μl, as described by Smida et al. (2). About 50 ng template DNA were subjected to amplification by 2 units of Taq polymerase (Gibco-BRL) in 10 mM Tris/HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.005% gelatine, 200 μM of each dNTP and 1 pM of suitable primer. The PCR reaction was performed in a Hybaid heat block (MWG, Ebersberg) with conditions adapted to lower eukaryotes, i.e. initial denaturation at 93^{o}C (for 8 min.), followed by 30 cycles at 93^{o}C (1 min.), annealing at 37^{o}C for 1 min. (first 5 cycles) and at 52^{o}C for 1 min. (next 25 cycles), the extension at 72^{o}C for 1.5 min. with final extension at 72^{o}C for 10 min.

- *AP-PCR - reference method.* PCR fingerprinting was performed by a PCR variant, described by Thanos and co-workers (4). T3B primer (AGGTCGCGGGTTCGAATC) was used for amplification of DNA from 10 strains (listed in Table 1). Results were compared with products of IL-PCR.

- *Detection of PCR products.* PCR fingerprints were separated by PAA-electrophoresis. Non-denaturating 6% polyacrylamide gels were prepared on hydrophilic/hydrophobic support films, according to the specifications of the manufacturer (DIAGEN, Hilden). The electrophoresis was conducted in a temperature gradient gel electrophoresis apparatus (TGGE, DIAGEN) at a constant temperature of 20°C, 4-5 h at 200 V. After staining with silver nitrate, the gels were digitized on a flatbed scanner. Gels were stored at $+4^{o}$ C in the dark for further analysis.

2.3. Pulse Field Gel Electrophoresis (PFGE, CHEF and ZIFE)

Karyotyping of yeast was performed by two variants of pulse field gel electrophoresis, CHEF (Contour-Clamped Homogeneous Electric Field) and ZIFE (Zero Integrated Field Electrophoresis). The 10 representant strains (listed in Table 1) were characterized by the protocols for CHEF (Fig. 1a) and for ZIFE (Fig. 1b) (5, 6).

2.4. Programme for digitalisation of patterns DNA fingerprints

The DNA fragments of the different patterns were sized and compared by the use of scanner-associated software (RFLPscan, version 3.0, Scananalytics CSP Inc., Billerica, Mass.). The band height threshold was set to 10% of the maximal OD within the same lane. The trace smoothing factor, a low pass filter for reducing high frequency noise, was 10. The match tolerance was 1.5%, the value of the smallest gap was 1.8%. Similarity indices, representing the ratio of shared bands to total bands within two lanes being compared during a matching operation, were estimated for the different yeast strains.

3. Results and Discussion

Yeasts are the most important group of microorganisms involved in biotechnological processes, especially for bread and beverage production. Therefore, the correct classification and identification of yeasts has been an area of active research since more than a hundred years. Conventional techniques based on the description of phenotype and biochemical ability do not perfectly and rapidly characterize yeasts which are intended for differentiation.

Therefore, here it is presented a potential application of the Inter-LINE PCR, as a tool for yeast taxonomy and also for identification of incorrect classifications (diagnostic). Two different PFGE techniques were used to attempt to charactrise the karyotypes of ten strains, representing different yeast groups and genera (Table 1).

In Fig. 1a the chromosome separation by CHEF is shown. Only the strains with smaller chromosomes, such as *S. cerevisiae* or *S. carlsbergensis,* can be characterized sufficently (lines 1-3). The karyotypes of the majority of yeast strains relevant for beverage production could not be examined by CHEF. Their chromosomes were stuck in the compression zone and could not be separated electrophoretically (Fig. 1a). Therefore, for these yeasts the ZIFE variant was applied. Fig. 1b shows patterns of separated chromosomes from different species of *Kluyveromyces, Hansenula, Williopsis, Debaromyces, Zygosaccharomyces, Torula* and *Candida*. Notice that in the case of karyotypes including only a few chromosomes (e.g. *Debaromyces hansenii, Candida valida*) the information content of the karyotypes is too poor for a reliable identification and may lead to false classifications. The PFGE-variants have the disadvantage, for the differentiation of yeasts, that either some basic information about the isolate´s karyotype is required, or else it may be necessary to carry out both techniques under optimized conditions.

We have suposed, that PCR-based methods will be able to solve such problems in identification of anonymous isolates, also under standard conditions. The most reliable PCR-variants perform the differentiation of yeasts by analysis of complex PCR-fingerprints. Here, are presented a comparison of Inter-LINE PCR with Arbitrarily-Primed PCR as reference method.

The IL-PCR fingerprints were generated by specific primers (GR, GF and 01). The Fig. 2, 3 and 4 show reproducible PCR-fingerprints which reflect the genetic nature of yeasts strains being analyzed. A comparison of some isolates of the characterized yeast species is presented in Fig. 2. The individual patterns were amplified by IL-PCR primer GR and separated on 6%, non-denaturating horizontal PAA-gels, followed by silver-staining. Fig. 2a illustrates typical fingerprints from different strains of

Saccharomyces cerevisiae, Fig. 2b and 2c show species-specific DNA-pattern of *Saccharomyces carlsbergensis, Williopsis, Hansenula* and *Pichia.*

To a first approximation, only small variations of the typical fingerprint were observed within any given species. The number and size of DNA-bands as well as their location was very similar, however, not identical. This variation was caused by the genetic diversity of the isolates. It is also clear that yeasts which had been incorrectly classified by conventional techniques could be detected easly. That is demonstrated for some species in Fig. 3a (Line 2: this is not *S. carlsbergensis*) and in Fig. 3b (Line 5: this is not *Pichia anomala* but *Williopsis mrakii*).

Each of our Inter-LINE primer (GR, GF and 01) generates a species-specific set of amplified PCR-products. By comparison of the fingerprints from several strains which belong to one species, the typical bands could be identified. Commonly, the products of IL-PCR vary in length between 200 bp and 2000 bp, depend on the species and the IL-PCR primer used. The best results in separation of DNA-fragments within this range were produced by horizontal PAA-electrophoresis on non-denaturating gels, immobilized on support films. All IL-primers tested produced similar results. They generated species-specific fingerprints, with slight variations from strain to strain. However, not every IL-primer gave optimal results for all species. So, one may need to try a set of IL-primers to achieve optimal fingerprints of certain species (data not shown).

Fig. 4 presents PCR-fingerprints of ten strains that had also been subjected to PFGE-techniques (see Tab. 1). Results of IL-PCR, i.e. primer GR and 01 (Fig. 4a, 4b) and of arbitrary PCR (7), primer T3B (4) in Fig. 4c are compared.

Both PCR-variants allow the generation of species-specific fingerprints. However, IL-PCR achieves the best results in combination with PAA-gel electrophoresis, because only this technique produces suitably small DNA-fragments (below 500 bp) within the optimal separation range (200 bp - 1500 bp). Most products of T3B-primer are of higher molecular weight (up to 5 kb), causing trouble in PAA-electrophoresis. Different arbitrarily-PCR primers (AP3, M13-chore, CA) were also tested, but no results comparable to the fingerprints obtained from IL-PCR and PAA-electrophoresis could be achieved (data not shown).

IL-PCR primers were have even been defined for mammalian DNA (2), showing that the IL-PCR method is fully comparable to other established PCR-fingerprinting methods. It is known, that lower Eukaryotes also contain LINE-homologous sequences in their genomes, which could be targeted by IL-primers. Current studies try to evaluate

the mechanism underlying the amplification by IL-PCR. Nevertheless, amplification of fingerprints is reproducible and sensitive. Moreover, typical bands within a fingerprint can serve for development of species-specific DNA-samples, necessary for fast and sensitive hybridization or PCR techniques.

After electrophoresis, the fingerprints were digitalised by scanning of the stained gels, which were supported on a hydrophilic film. Immobilisation of the gel permitted easy handling, with no need for tricky blotting and drying. The digitalisation allows profiling of the fingerprints, sizing of the individual DNA bands and comparison by the use of scanner-associated software (RFLPscan, version 3.0, Scananlytics CSP Inc., Billerica, Mass.). The digitalised DNA-profiles of all analysed isolates, related to beverage industry are being used to build databases, which will facilitate and hasten the identification of new isolates. For example Table 2 shows databases produced by digitizing of IL-PCR fingerprints from three industrial strains of *Saccharomyces cerevisiae* (digitized profiles are shown in the Fig. 5).

The density profiles of the three isolates are quite similar. The 12 peaks are located at nearly the same positions but exhibit some variations in height. The differences are reproducible in independent PCRs and may be due to variations in the composition of template DNA. For example, aneuploidy of distinct chromosomes or amplification of DNA-templates derived not from the nucleus, but from plasmids or mitichondria, will influence the quantity of PCR products. The locations of the peaks are measured automatically by the scanner-associated software and stored in a database (Table 2).

The length-variation of size bands that is tolerated is very small (less than 1.5% of total size in bp). This demonstrates that the PCR and electrophoresis were highly reproducible. It will probably be possible to reduce the number of sized bands of an individual fingerprint, necessary for correct identification of unidentified isolate. Moreover, databasing allows the identification of bands typical for genera, species, groups and even strains. These bands can serve as a tool for the development of specific DNA-probes in more distant applications, such as specific PCR-assays to detect distinct species.

3. Conclusion

- The database methodology allows the identification of bands typical for genera, species, groups and even strains.

- These bands can serve as a tool for the development of specific DNA-samples for further applications, such as specific PCR-assays to detect distinct species.
- This was the first databank component for the development of the automated yeast identification system**.**

4. References

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S u m m a r y

In the Inter-LINE (IL) PCR method, oligonucleotides GF, GR and 01, which originated from mammalian cells led to highly reproducible patterns of amplified template DNA, based on the consensus of LINE-sequences. These were used for the genomic fingerprinting of about 80 strains of yeast, consisting of 30 species from 13 genera). The IL-PCR technique using the above primers is described and compared to reference methods such as **A**rbitrarily **P**rimed-PCR and Pulse Field Gel Electrophoresis (PFGE).

A comparison of the two PCR variants was performed using suitable numbers of digitized PCR-fingerprints. A database for an automated yeast identification system is proposed.

Key words: PCR, yeast identification, industrial strains.

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Figure Legends

Fig. 1. Comparison, by PFGE, of karyotypes from 10 strains from Table 1:
a - CHEF: line 1- S.cerevisiae S-21; line 2- S.diastaticus ATCC 60270,
line 3- S.carlsbergensis 34/70, line 4- K.lactis ATCC 56309,
line 5- H.anomala NCYC 435, line 6- W.mrakii NCYC 500,
line 7- D.hansenii DSM 70244, line 8- Z.rouxii DSM 2531,

line 9- T.delbrückii DSM 70504, line 10- C.valida NCYC 327.

b - ZIFE: line 1- K.lactis ATCC 56309, line 2- H.anomala NCYC 435,
line 3- W.mrakii NCYC 500, line 4- D.hansenii DSM 70244,
line 5- Z.rouxii DSM 2531, line 6- T.delbrückii DSM 70504,
line 7- C.valida NCYC 327, line 8- S.cerevisiae S-21.

Fig. 2. IL-PCR fingerprints of *Saccharomyces* and *Williopsis* species generated by IL-primer GR. The size-standard is a 100 bp-ladder (GIBCO):
a - *Saccharomyces cerevisiae,* industrial strains;
b - *Saccharomyces carlsbergensis,* industrial strains;
c - *Williopsis mrakii,* collection strains.

Fig. 3. Detection, by IL-PCR fingerprints (primer GF), of misclassification of strains. Size standard is a 100 bp-ladder (GIBCO):
a - *Saccharomyces carlsbergensis,* industrial strains;
Line 2: declared strain is not *S. carlsbergensis;*
b - Williopsis/Hansenula mrakii and *Pichia/Hansenula anomala,*collection strains;
Line 5: declared strain is not *Pichia anomala,* but *Williopsis /Hansenula mrakii.*

Fig. 4. Comparison of PCR-based fingerprints of IL-PCR and arbitrarily primed PCR (AP-PCR) from 10 strains according to Table1. Size-standard is a 100bp-ladder (GIBCO):
a - IL-PCR (primer GR);
b - IL-PCR (primer 01);
c - AP-PCR (primer T3B).

Fig. 5. Databasing of DNA-fingerprints. Density proliles of *Saccharomyces cerevisiae* (3 industrial strains) after the digitalisation exhibit minimal length-variation in the location of sized peaks. Amplification primer is GR. Size-standard is 100 bp-ladder (GIBCO):
a - *Saccharomyces cerevisia,* S-21 strain,
b - *Saccharomyces cerevisiae,* S-83 strain,
c - *Saccharomyces cerevisiae*, S-85 strain.

Tables

Table 1. Representative yeast groups important for beverage industry.

Number Application	Yeast species	Number of Collection

1.	Saccharomyces cerevisiae	S-21, Weihenstephan	industrial
2.	Saccharomyces diastaticus	ATCC 60270	laboratory
3.	Saccharomyces carlsbergensis	34/70, Weihenstephan	industrial
4.	Kluyveromyces lactis	ATCC 56309	laboratory
5.	Hansenula anomala	NCYC 435	laboratory
6.	Williopsis mrakii	NCYC 500	laboratory
7.	Debaromyces hansenii	DSM 70244	laboratory
8.	Zygosaccharomyces rouxii	DSM 2531	laboratory
9.	Torula delbrückii H 105	DSM 70504	laboratory
10.	Candida valida	NCYC 327	laboratory

Table. 2. Deviations values of digitalized bands (bp) among *S. cerevisiae* strains. Products of Inter-LINE PCR with GR primer.

Nr of band	***Saccharomyces cerevisiae*, industrial strains**

and	(bp of bands)		
Marker MW (bp)	**S-21**	**S-83**	**S-85**
(1) 1334	1331	1334	1338
(2) 1132	1129	1132	1134
(3) 744	745	734	753
(4) 681	681	680	682
(5) 589	592	586	590
(6) 567	568	565	569
(7) 538	537	539	538
(8) 505	503	505	506
(9) 362	359	359	367
(10) 303	302	303	303
(11) 271	270	271	271
(12) 183	183	183	183