Advanced Alternatives

New technology could allow for easy extraction of sequence-able DNA from medically relevant fungi, as well as the induction of competence in top-fermenting food and beverage strains

By Dr Pablo Castan, Dr Paul Reeves and Dr Jan Rogers at Arcis Biotechnology, Dr Alicia De Pablo and Dr Carlos Toro at Hospital La Paz and Beatriz Lopez-Quintana and Ana Arroyo at Foundation Hospital La Paz

Standard methods of DNA extraction from fungal pathogens are laborious, costly and normally require the use of potentially toxic chemicals. Obtaining DNA from certain fungal organisms is currently a clinical issue due to cell walls or capsules that are strong enough to resist normal lysis. However, a new and rapid method of obtaining DNA from the standard two-step protocol with a novel solution has recently been investigated (1).

This method was tested over seven of the main medically relevant fungi, resulting in good yield, integrity and quality of DNA, as demonstrated by subsequent Sanger sequencing. The seven identifications described in detail within this work illustrate the different levels of strength and resistance, in terms of cell wall integrity, from a wide fungal panel. Parallel testing and slight modification of the protocol was used on top-fermenting fungi, and ale-yeasts (typically required in the brewing industry) resulted in a new procedure for release of DNA with potential for application in recombinant DNA technology.

Extraction Types

Obtaining genomic DNA (gDNA) from medically important fungi is a current major challenge in clinical microbiology laboratories as gDNA is the substrate needed for accurate molecular epidemiologic subtyping and the template for diagnostic polymerase chain reaction (PCR). Likewise, the new direct sequencing solutions need the upstream use of automated systems that facilitate the rapid availability of gDNA (2); this means that a number of different alternatives are currently under development and testing for clinical microbiological labs. Classic methods for gDNA extraction from fungal pathogens may need hours to days to complete, requiring the use of hazardous chemicals needed for disruption of the cell walls. Additionally, the yield of gDNA is impacted in terms of reproducibility, and the result can be poor in those species whose cell walls or capsules are not readily susceptible to lysis.

The novel two-step/high-speed cell disruption system described herein has been validated on a variety of sample types, but not previously on medically important fungi (1,3). Therefore, a series of modifications of this new rapid DNA preparation method were tested from a variety of medically and industrially important yeasts and filamentous fungi (see Table 2, page 34). A set of different inocula (see 'Materials and Methods') were compared for optimisation of obtained yields. The integrity and quality of the extracted genomic DNA were validated by downstream amplification of selected areas through PCR, Sanger sequencing and *in silico* analysis of the sequences obtained from novel protocol extracts.

An additional protocol from this new and rapid DNA preparation method evolved and was tested for induction of competence over two industrially relevant yeasts: *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (1). This was done in collaboration with a corporate research team responsible for production of recombinant enzymes in fungi (4).

Competence is the property that allows microorganisms to undergo transformation: the process of DNA transfer by which some bacteria and fungi take up foreign genetic material. Transformation of certain fungal strains is used industrially – including within the brewing of beer and bread manufacture – to achieve transfer of persistent DNA in the environment that will account for new properties once integrated in

Keywords

DNA/RNA extraction
Fungal pathogens
Sequence-based analysis

Table 1: Primers used for amplification and sequencing of fungal extracts obtained through modification of standard two-step protocol. All products were subjected to standard Sanger sequencing making use of the following sequencing primer: Sequencing primer (Sanger seq. funR

Forward primer used in PCR of sample extracts	funF (5'-ATTGGAGGGCAAGTCTGGTG-3')
Reverse primer used in PCR of sample extracts	funR (5'-CCGATCCCTAGTCGGCAT-3')

the host cell (competent) to undergo stable recombination (transformed). The prerequisite for such strains to undergo transformation is their ability to take up free genetic material – the factor that regulates natural competence varying between various genera. Here, a specific treatment with low amounts of the novel solution induced competence to the two species tested. The resulting transformation efficiency was calculated as the number of transformants generated per µg of DNA used.

Materials and Methods: Extraction Protocol

The novel two-step/high-speed cell disruption method for DNA and RNA extraction was modified, as defined later, for application to medically important fungi. The primary goal was, at this point, to confirm or dismiss compliance with sequencing analysis and identification. A primary solution was obtained through preparation of a +2 McFarland solution of fungal cells in sterile water. After thorough vortex mixing, the sample was added to tube one of the two-step protocol, with a centrifugation step of 10 minutes at 12,000rpm. This modification of the two-step protocol was necessary to generate clean supernatants containing the extracted fungal genetic materials, regardless of the cell wall's thickness. The extracted sample was added to the washing solution by mixing with a ratio 2.5:10 (2.5µl of extracted sample:10µl of tube two washing solution). After mixing, 5µl was transferred into the PCR assay.

Amplification of the fungal extracted DNA targets required for sequence identification was obtained through PCR. The amplification was based on the PCR primers targeting a 500bp region of the 18S rRNA gene (see Table 1) (5).

Competency Protocol

The novel two-step/high-speed cell disruption method for DNA and RNA extraction was modified for induction of competence over two industrially relevant yeasts, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, in collaboration with Parque Cientifico de Madrid, which uses recombinant fungi for biotechnology purposes. This modified protocol required overnight growth of a standard inoculum and verification of cell mass using methylene blue (the tricyclic phenothiazine cationic dye that reversibly adsorbs to anionic biological material) to visualise the increase in the amount of fungi growing in the selective medium (6). After that initial inoculum for exponential growth, 1µl of the novel solution was added to 200µl aliquots. Competence was reached after 15 minutes of incubation at room temperature. Subsequent transformation required the addition of 0.1mcg or 20µl of DNA for transformation through standard thermal shock (7).

Table 2: Summary of PCR results obtained with sample extracts for fungi identified through comparison of nucleotide sequences

Micro and macro identification (microbiology)	PCR	*Sequencing results from sample extraction and BLAST identification*
Candida albicans	+	*C. albicans, Candida dubliniensis, Candida parapsilosis*
Aspergillus fumigatus	+	*Aspergillus sojae, Aspergillus tamarii, A. flavus, A. fumigatus*
Candida spp	+	*C. albicans, C. dublinensis*
Aspergillus flavus	+	*A. sojae, A tamarii, A. flavus, A. fumigatus, Penicillium sp.*
Aspergillus niger	+	*A. niger, Aspergillus terreus, A thuringiensis*
Alternaria	+	*Alternaria alternata*
Trichophyton rubrum	+	*T. rubrum, Trichophyton soudanense, Trichophyton mentagrophytes*

Results from Extraction Protocol

Several batches of PCR products from fungal DNA targets that were obtained from the novel protocol were sequenced for identification using the standard Sanger method. The sequences obtained were positively located within the correct 500bp region of the 18S rRNA gene (5).

Sequence identification was obtained using the basic local alignment search tool (BLAST) to find the regions of similarity between the sequences obtained and the validated databases. The comparison of nucleotide sequences to sequence databases allowed positive identification of the fungal pathogens below, reaching the correct statistical significance (see Tables 2 and 3).

Results from Competency Protocol

Identification of transformed fungi was performed after plating onto a selective medium. The use of the recombinant green fluorescent protein (GFP) as a screenable marker aided the rapid segregation of individual transformation events as the transformed plasmid contained a synthetic GFP gene (sgfpS65T) under the control of the 35S promoter.

Final calculation was obtained for 20µl (0.1µg) of control plasmid containing the GFP gene into 50µl of cells. 30µl of transformed cells

Microbiology identification	Sequence obtained with funR	BLAST result
C. albicans	AGTTAGTCTTCAGTAAATCCAA-GAATTTCACCTCTGACAACTGAATACT-GATACCCCCGACCGTCCCTATTAATCAT-TACGATGGTCCTAGAAACCAACAAAATA-GAACCATAACGTCCTATTCTATTATTCCAT-GCTAATATATTCGAGCAAAGGCCTGCTTT-GAACACTCTAATTTTTTCAAAGTAAAA-GTCCTGGTTCGCCATAAATGGCTACC-CAGAAGGAAAGGCTCGGCTGGGTC-CAGTACGCATCAAAAAGATGGACCG-GCCAGCCAAGCCCAAGGTTCAAC-TACGAGCTTTTTAACTGCAACAACTT-TAATATACGCTTTTGGAGCTGGAATTAC-CGCGGCTGYTGGCACCAGACTTGCCC-CTCCAAWAATA	*C. albicans, C. dublinensis, C. parapsilosis*
A. fumigatus	GCAGTAGTTAGTCTTCAGCAAATCCAA-GAATTTCACCTCTGACAGCTGAATACT-GACGCCCCCGACTATCCCTATTAATCAT-TACGGCGGTCCTAGAAACCAACAAAATA-GAACCGCACGTCCTATTCTATTATTC-CATGCTAATGTATTCGAGCAAAGGC-CTGCTTTGAACACTCTAATTTTTTCACA-GTAAAAGTCCTGGTTCCCCCCACAGC-CAGTGAAGGCCATGAGGTTCCCCAGAA-GGAAAGGTCCAG	*A. fumigatus, A. sojae, A tamarii, A. flavus*
Candida spp	CAGTAGTTAGTCTTCAGTAAATCCAA-GAATTTCACCCCTGAYAACTGAATACT-GATACCCCCGACCGTCCCTATTAATCAT-TACGATGGTCCTAGAAACCAACAAAATA-GAACCATAACGTCCTATTCTATTATTCCAT-GCTAATATATTCGAGCAAAGGCCTGCTTT-GAACACTCTAATTTTTTCAAAGTAAAA-GTCCTGGTTCGCCATAAATGGCTACC-CARAAGGAAAGGCTCGGCTGGGTC-CAGTACGCATCAAAAAGATGGACCG-GCCAGCCAAGCCCAAGGTTCAAC-TACGAGCTTTTTAACTGCAACAACTT-TAATATACGCTTTTGGAGCTGGAATTAC-CGCGGCTGCTGGCACCARACTTGCC-CTCCAAWA	*C. albicans, C. dublinensis*
A. flavus	AGTTAGTCTTCAGCAAATCCAA-GAATTTCMCCTCTKACAGCTGAATACT-GACGCCCCCGACTATCCCTATTAATCAT-TACGGCGGTCCTAGAAACCAACAAAATA-GAACCGCACGTCCTATTCTATTATTCCAT-GCTAATGTATTCGAGCAAAGGCCTGCTTT-GAACACTCTAATTTTTTCACAGTAAAA-GTCCTGGTTCCCCCCACAGCCAGTGAA-GGCCATGAGGTTCCCCAGAAGGAAA-GGTCCAGCCGGACCAGTACTCGCGGT-GAGGCGGACCGGCCAGCCAGACC-CAAGGTTCAACTACGAGCTTTTTAACT-GCAACAACTTTAATATACGCTATTG-GAGCTGGAATTACCGCGGCTGCTGG-MACCAGACTTGCCCTCCAAWAGCGWA	*A. flavus, A. sojae, A tamarii, A. fumigatus, Penicillium sp.*
A. niger	AGTTAGTCTTCAGCAAATCCAA-GAATTTCMCCTCTGACAGCTGAATACT-GACGCCCCCGACTATCCCTATTAATCAT-TACGGCGGTCCTAGAAACCAACAAAATA-GAACCGCACGTCCTATTCTATTATTC-CATGCTAATGTATTCGAGCAAAGGC-CTGCTTTGAACACTCTAATTTTTTCACA-GTAAAAGTCCTGGTTCCCCCCACAGC-CAGTGAAGGCCATGAGATTCCCCA-GAAGGAAAGGTCCAGCCGGACCAG-TACTCGCGGTGAGGCGGACCGGCCAGC-CAGACCCAAGGTTCAACTACGAGCTTTT-TAACTGCAACAACTTTAATATACGCTAT-KGGAGCTGGAATTACCGCGGCTGCTGG-MACCAGACTTGCCCTCCAAWAGCG	*A. niger, A terreus, A thuringiensis*
Alternaria	CAACTGAATACTGATGCCCCCGACT-GTTCCTGTTAATCATTGCGGCGTCTCTA-GAAACCAACAAAATAKAAACGCACGC-CCTATTTTATTATTCCATGCTAACG-TATTCRAGCAAAGGCCTGCTTT-GAACACTCTAATTTTTTCAAAGTAAAA-GTCCTGATTCCCCAGCACGCCCAGTGAA-GGGYATGAGGTTCTTMAGAAGGAA-GGCCCGGCCGGACGAGTGCACGCG-GTGAGGCGGACCCGCCAGCCAGGCC-CAAGTTTCAACTACGAGCTTTTTAACT-GCAACAACTTTAATATACGCTATTG-GAGCTGGAATTACCGCGGCTGCTG-GCACCAGACTTGCCCTCCAATAG	*Alternaria alternata*
Trichophyton rubrum	TCCCTATTAATCATTACGGCGGTCCTA-GAAACCAACAAAATAGAACCACACGTC-CTATTCTATTATTCCATGCTAATG-TATCCGAGCAAAGGCCTGCTTT-GAACACTCTAATTTTTTCACAGTAAAA-GCCCTGGTTCCGCCCACGGCCAGTGAA-GGCCATGGGGTTCCCCAAGAGGAAA-GGCCCAGCCGGACCAGTGCACGC-CGTGAGACGGACCGGCCAGCCAGGCC-CAAGGTTCAACTACGAGCTTTTTAACT-GCAACAACTTTAATATACGCTATTG-GAGCTGGAATTACCGCGGCTGCTG-GCACCAGACTTGCCCTCCAATAG	*T. rubrum, T.soudanense, T. mentagrophytes*

Table 3: Summary of sequences obtained with sample extracts for fungi and BLAST identification within the correct 500bp region of the 18S rRNA gene through comparison of nucleotide sequences

were plated after the addition of 1ml of selective medium (step seven) of transformation protocol and generated an average count of 150 colonies/plate over three repeats. Therefore, as 150 colonies were obtained from 0.1µg of DNA after dilutions were accounted for, transformation efficiency (TE) equalled $1.5x10^7$cfu/µg. Former data from the team that performed these assays proved that commercial strains for industrial generation of recombinant fungi are two logs more efficient, reaching a TE of $1.5x10^9$cfu/µg.

Colonies = the number of fluorescent colonies counted on the plate
Dilution = the total dilution of the DNA before plating
TE = colonies/µg/dilution
µg = the amount of DNA transformed expressed in µg

Considerable added value strains available from molecular biology companies are a few, well-mapped and controlled set of micro-organisms that have been raised and selected for definite uses, such as preparation of complementary DNA and genomic libraries, construction of longer-length genomic libraries, sub-cloning and even methylated DNA cloning. This means that a finite variety of micro-organisms are available that can be used for transformation 'off the shelf'. Meanwhile, having the potential to induce competence through a chemical treatment that has been proven to operate over all types of fungal cell walls opens a potentially endless list of hosts – should the need arise to transform other strains different to those commercially available. Furthermore, commercially available strains cannot be regrown and provide sustained competence. Thus, having the chance to induce competence at any time and over any strain is worthy of further consideration in the generation of wild-type-derived fungal strains that may not be commercially available as competent cells (8).

Discussion

Mycologists have traditionally used morphology and visible phenotypic characters, such as spore-producing structures, as a key means of identifying fungal species. Actually, the use of morphology

in fungal species identification remains the most important aspect in the diagnostic routine. However, morphological approaches to fungal classification can be misleading or problematic even for trained mycologists, as they may not always provide accurate groupings within an evolutionary framework, mainly at the species level. Aiming to solve that situation, the use of molecular data for the identification of fungi began with the description of fungal nuclear ribosomal operon primers (9).

The work presented here is based on the premise that a faster sequencing-based method of identifying fungi is being developed, particularly with the application of a new extraction technology whose ease-of-use and cost are compliant with the widespread function of combatting fungal infections. Standardised systems for clinical use and powerful technologies, such as mass spectrometry, have provided labs with accurate means to identify clinical isolates. However, some systems have proven to be restricted to common clinical isolates (10). The sequencing approach described here is a cost-effective method that overcomes such limitations, allowing identification of potentially any species as long as a reference sequence is deposited in databases. This feature provides the potential to surpass commercial systems for biochemical properties that may also vary according to inoculum and geographic isolate source, but the sequence-based analysis described here would not be affected by such factors.

The fact that the novel solution generated competent strains through the protocol described in this work is also indicative of the potential generated by this chemical treatment. Former papers showcasing uses for the reagent/treatments suggested that varying concentrations of the solution and/or incubation protocols for use may induce different changes in the cell wall structure while retaining compliance with downstream molecular applications (1). This work further supports that assessment if the protection exerted over nucleic acids that has been reported in those articles needs to be further confirmed in this particular area. Should stability data in that sense become available with positive values, immediate application in the diagnosis of candida infections via blood treatment with the novel two-step protocol and RT-qPCR would be achievable within useful limits of detection. This technology has previously been used to boost the sensitivity of malaria diagnostics, and this application will be developed in the yeast model. The rapid protocol can generate stable pools of mRNA from peripheral blood, which facilitates rapid and accurate diagnostics (3). In that sense, it is worth mentioning that cultures for diagnosing invasive candidiasis are limited by poor sensitivity and slow turn-around time. A novel sequence-based solution capitalising on the results presented in this work would be useful to complement cultures, in particular to identify the 'missing 50%' of patients who are blood culture-negative.

Dr Pablo Castan obtained his degree in biology and PhD in molecular biology from Universidad Autonoma de Madrid, Spain, and also has a degree in molecular medicine from the University of East Anglia, UK. He is currently Associate Investigator in the Department of Microbiology at Hospital Carlos III, Spain, and the Research Director for Arcis Biotechnology. Pablo has previously worked for Epistem (now Genedrive) and is a founding member of 2BBlackBio.
Email: pablo.castan@arcisbio.com

Dr Paul Reeves, Development Manager at Arcis Biotechnology, graduated with a degree in genetics and a PhD in molecular biology.
Dr Jan Rogers is Chief Scientific Officer at Arcis Biotechnology, has a PhD in chemistry and also holds an MBA from the University of Bath, UK.
Dr Alicia De Pablo and Dr Carlos Toro are senior researchers in the Department of Microbiology at Hospital La Paz, Spain. Beatriz Lopez-Quintana, Ana Arroyo and all researchers at the Foundation Hospital La Paz have degrees from Universidad Complutense de Madrid, Spain, across fields in pharmacy and medicine.

References

1. Castan P *et al,* Gold Standard, *Innovations in Pharmaceutical Technology* 60: pp28-9, 2017
2. Brunner K and Mach RL, Quantitative detection of fungi by molecular methods: A case study on Fusarium, *Molecular Identification of Fungi:* pp93-105, 2008
3. Iglesias N *et al,* Testing of a novel protocol for processing clinical samples that generates stable pools for both RNA and DNA: mRNA stability and rt-qpcr compliance for one month at room temperature, International Pharmaceutical and Clinical Microbiology Congress: 2016
4. Parque Cientifico de Madrid, UAM
5. Gharizadeh B *et al,* Identification of medically important fungi by the Pyrosequencing technology, *Mycoses* 47 (1-2): pp29-33, 2004
6. Acemioğlu B *et al*, Use of Aspergillus wentii for biosorption of methylene blue from aqueous solution, *Afr J Biotechnol* 9(6): pp874-81, 2010
7. González-Mendoza D *et al*, A rapid method for isolation of total DNA from pathogenic filamentous plant fungi, *Genet Mol Res* 9(1): pp162-6, 2010
8. Fromtling RA *et al,* Taxonomy, classification, and morphology of the fungi, *Manual of Clinical Microbiology* 7: pp1,161-6, 1999
9. Innis MA *et al*, PCR protocols: A guide to methods and application, *San Diego*: 1990
10. Merz WG and Roberts GD, Algorithms for detection and identification of fungi, *Manual of Clinical Microbiology*