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DNA Isolation from *Aspergillus flavus*: Optimal method selection

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ABSTRACT
The methods for fungal genomic DNA isolation for PCR amplification, including commercially available kits, must often be adapted in order to produce sufficient amounts of high-quality DNA from specific fungal species. The aim of this study was to select an optimal method for the isolation of DNA from *Aspergillus flavus* suitable for PCR reaction. Four different methods were compared according to their efficiency in isolating pure DNA, their price and time consumption. DNA quantification and purity estimation were performed using the NanoDropTM 1000 UV/VIS spectrophotometer and DNA integrity and PCR products were determined by gel-electrophoresis. DNA quantity ranged from 92.77 ± 11.52 to 5477.4 ± 22.03 ng/µL, with $A_{260}/_{280}$ from 1.14 ± 0.10 to 1.94 ± 0.16, and $A_{260}/_{230}$ 0.37 ± 0.05 to 1.91 ± 0.17. There were also great differences in time consumption per sample, ranging from 1 hr 15 min to 7 hr 5 min. The determined costs per sample were ranging from 0.12 € to 2.29 € per sample. All tested methods were suitable for the isolation of *A. flavus* genomic DNA and subsequently for PCR reaction.

Introduction

The fungus *Aspergillus flavus* is a known opportunistic pathogen in crops, animals and humans (Klich, 2007). Food crops such as maize and peanuts, as well as immunocompromised humans are particularly sensitive to infection by *A. flavus* (Amaike and Keller, 2011; Yu, 2012). Also, this fungus produces extremely toxic secondary metabolites - mycotoxins such as aflatoxin B_1, one of the most potent carcinogens (IARC, 1993; IARC, 2002). Dietary exposure to aflatoxin-contaminated food has been associated with serious health-related issues including liver cancer, growth retardation, suppression of immunity and death (Miller et al., 1994; Šarkanj et al., 2018). Aflatoxin contamination occurs due to dumping climate, inappropriate crop storage and exposure to insect damage, frequently in sub-developed countries. It also poses a serious hurdle to international crop trade due to strict market regulations on mycotoxins in Europe and in the USA (European Commission, 2006; van Egmond et al., 2007). For those reasons, *A. flavus* is an object of up-to-date investigation, with the purpose of contamination and infection by the minimisation of aflatoxins (Fountain et al., 2015; 2016; Kovač et al., 2017). The genetic and phenotypic responses of the fungus, provoked by different environmental conditions (i.e. global climatic changes), in many ways resulted in global transcription changes of this fungus, which define the lifestyle - from saprobic to pathogenic (Battiliani et al., 2012; 2016; Reverberi et al., 2012; Reverberi et al., 2013).
Throughout the 1990s, the polymerase chain reaction (PCR) has complemented conventional culture-based methods for fungal determination and has become the cornerstone of detection and identification for a whole range of fungal species. The PCR methods enabled direct sample testing, they are rapid and highly specific (Brunner et al., 2007). So far, PCR

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has been the most powerful method for the detection of *A. flavus*, due to its almost single-molecule sensitivity and speed. Due to a rapid increase in the size of the *A. flavus* DNA database and the ability to design PCR primers specific for *A. flavus* genes, PCR allows the precise detection of aflatoxigenic species and provides a useful tool for their early determination in naturally contaminated samples (Payne and Brown, 1998; Shapira et al., 1996).

The bottleneck of PCR based detection of filamentous fungal pathogens is the resistance of their cell walls to traditional DNA extraction protocols causing inefficient isolation of genomic DNA, a prerequisite for efficient PCR (Goltapeh et al., 2007). The other difficulties in the extraction of DNA from filamentous fungi include fungal nucleases and high polysaccharide content (Muller, 1998). A large number of DNA extraction methods for filamentous fungi have been described in the scientific literature (Goltapeh et al., 2007; Muller, 1998; Liu et al., 2000; Roche, 2007; Yeates et al., 1998). The methods with bead-vortexing or grinding with the use of liquid nitrogen were used for the initial breaking up of mycelia, but it was found that these methods can be unsuitable for the turnover of a large number of samples. In addition, a detergent such as SDS and toxic chemicals like phenol and chloroform were used, and they are well-known inhibitors of nucleic acids purification. Although these methods of DNA extraction from filamentous fungi were time-consuming, labour-intensive and required highly toxic chemicals, they often resulted in poor DNA quality and quantity. The choice of the DNA extraction protocol in laboratories is often very subjective and closely associated with cost, time consumption, skilled laboratory staff and available laboratory equipment. Nevertheless, their applicability for different fungal species and/or sources remains questionable (Yeates et al., 1998).

Most of the DNA isolation protocols described in the literature are highly organism-specific, whereas no single extraction method is optimized for DNA extraction from *A. flavus*. Similarly, their commercial counterparts are often unspecific and produce low DNA yields. Considering the huge impact of molecular identification of *A. flavus* on mycology research, fungal DNA purity and integrity are of critical importance for the subsequent efficiency of PCR amplification. For these reasons, four recent DNA extraction methods, with respect to their efficiency, time consumption, and cost per sample, were compared. The methods were chosen so they represent the most common DNA isolation methods used in laboratories. Kits for isolating DNA are more expensive, but also more convenient for use than conventional methods. Despite that, some conventional methods are used rapidly in laboratories for fungal DNA isolation. They require more skills, but give higher DNA yields and consume less time in some cases.

Materials and methods

The growth of fungi in culture media

Aspergillus flavus NRRL 3251 was used in this study as a model fungus. Conidia suspension (10^6 CFU/mL) used for inoculation was prepared according to Šarkanj et al. (2013). The fungus was grown in the GMS medium (Yu et al., 2012) inoculated with 20 μL of prepared spore suspension, and incubated for 96 hours at 29 ± 1 °C in a rotary shaker (KS 260 basic, IKA, Germany) at 150 rpm. After incubation, mycelium was separated from the medium by filtration through sterile filter paper and weighed. Mycelium was stored at -80 °C until DNA isolation.

DNA isolation

Cells were disrupted with liquid nitrogen and ground with a sterile mortar and pestle. 100 mg of disrupted mycelium was used for all isolations. All isolations were carried out in a triplicate with an addition of Proteinase K (Roche, Germany). All methods conducted without the RNase treatment were marked as methods ***a***, and those with the RNase treatment (Sigma Aldrich, Germany) were marked as methods ***b***. For all methods, the RNase treatment was performed during incubation in order to remove the co-extracted RNA. Purified DNA samples were resuspended in 100 μL of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The compared DNA isolation methods are described hereafter.

Method #1

The protocol for the isolation of nucleic acids from bacteria or yeast, from the High Pure PCR Template Preparation Kit (Roche, Germany) was used (Roche, 2007). The 100 mg of disrupted mycelium was centrifuged for 5 min at 3000 x g and resuspended in 200 μL of PBS (100 mM, pH 7.4). Also, 5 μL of lysozyme (10 mg/mL in 10 mM Tris-HCl, pH 8) was added to the suspension and the 15 min incubation period at 37 °C followed. Method ***b*** included the addition of 5 μL of RNase (20 mg/mL) at this point. Finally, 200 μL of binding buffer and 40 μL of proteinase K were added, and the 10 min incubation period at 70 °C followed. Prior to washing and elution, 100 μL of isopropanol

was added and the standard washing and elution protocol described by the manufacturer's instructions was followed (Roche, 2007).

Method #2
The procedure described by Liu et al. (2000) was closely followed. We must note that the original paper did not prescribe the starting sample weight (the term used was 'a small lump'). The 100 mg of disrupted mycelium was mixed with 500 µL of lysis buffer (400 mM Tris-HCl (pH 8.0); 60 mM EDTA (pH 8.0), 150 mM NaCl, 1% SDS) in 1.5 mL nuclease-free microcentrifuge tubes. In the modification (method ***b***), 15 µL of RNase (20 mg/mL) was added during this step. The tubes were incubated at 25 °C for 10 min. The 150 µL of potassium acetate (pH 4.8) was added to the tube was subsequently vortexed and centrifuged (10 000 x g for 1 min). The supernatant was transferred into a new tube and diluted with an equal volume of isopropanol, mixed by inversion, and centrifuged at 10 000 x g for 2 min. The settled DNA pellets were mixed with 300 µL of 70% ethanol and centrifuged at 10 000 x g for 1 min. After discarding supernatant, DNA was dried in a sterile cabinet for 60 min at RT, and after that resuspended in 100 µL of TE buffer.

Method #3
The purport of this method was a modified CTAB procedure described by Goltapeh et al. (2007). 100 mg of mycelium was mixed with 600 µL extraction buffer modified according to Kawata et al. (2003) (1 M Tris-HCl (pH 8.0), 5 M NaCl, 0.5 M EDTA (pH 8.0) 2% (w/v) CTAB, 28.6 mM 2-mercaptoethanol) in 1.5 mL nuclease-free microcentrifuge tubes. In the modification (method ***b***) 15 µL of RNase (20 mg/mL) was added during this step. After the 60 min incubation period a t 60 °C and centrifugation (6 minutes at 10 000 x g), supernatants were separated and diluted with an equal volume of chloroform-isoamyl alcohol (24:1). The tube was gently mixed by inversion and centrifuged for 20 min at 10 000 x g. The supernatant was precipitated with 0.6 volume of cold isopropanol (-20 °C), and 0.1 volume of 3 M sodium acetate (pH 5.2), and centrifuged for 15 min at 12 000 x g. DNA pellets were washed with 200 µL of 70% ethanol, dried and resuspended in 100 µL of TE buffer.

Method #4
A protocol using sonication, described by Yeates et al. (1998), for microbial DNA extraction from soil for PCR amplification was used. The 200 µL of extraction buffer (100 mM Tris-HCl (pH 8.0), 100 mM EDTA (8.0), 1.5 M NaCl) was mixed with 100 mg of disrupted mycelia. The mixture was sonicated using a Labsonic M (Sartorius, Germany) ultrasound processor with a 2 mm titan probe for 5 min, with 1 min intervals of cooling in an ice bath between every 30 sec of sonication. The 20 µL of SDS and 40 µL of proteinase K (Roche) were added to the suspension and incubated for 60 min at 65 °C. After centrifugation at 6000 x g for 10 min, a supernatant was diluted with a half-volume of polyethylene glycol (30%)/NaCl (1.6 M) in new tubes and the 2 h incubation period at 25 °C followed.
In method ***b***, 5 µL of RNase (20 mg/mL) was added to the mixture. Samples were centrifuged at 10 000 x g for 20 min and the partially purified nucleic acid pellet was resuspended in 400 µL of Tris-EDTA buffer (TE). Potassium acetate (7.5 M) was added to the final concentration of 0.5 M. The samples were ice-cooled for 5 min and after that centrifuged at 16000xg for 30 min at 4 °C. The aqueous phase was extracted with phenol/chloroform/isoamyl alcohol (25:24:1), and DNA was precipitated by a 0.6 volume of isopropanol. After 2 hours of incubation at 25 °C, DNA was settled by centrifugation (16000xg for 30 min at 4 °C) and resuspended in 100 µL of TE buffer.

NanoDrop™ 1000 UV/VIS spectrophotometer measurements

The absorbance measurements at 260 nm, 280 nm, A_{260}/A_{280} and $A_{260}/_{230}$ ratios were performed using the NanoDrop™ 1000 UV/VIS spectrophotometer (Thermo Scientific, USA), according to the manufacturer's instructions.

Electrophoresis of isolated DNA

Electrophoresis of genomic DNA was performed using 2% agarose gel with SYBR safe DNA gel stain 10 000 x concentrate (Invitrogen, USA). The running buffer was 1 x TBE (0.5 M Tris, 0.5 M boric acid, 10 mM EDTA, pH 8). The 5 µL of isolated DNA was mixed with 1.5 µL of loading dye (6x mass ruler by Fermentas, USA) and applied to individual wells. Gels were run at 25 °C for 120 min at 60 V, 50 mA and visualized with UV illumination at 254 nm.

PCR reaction

The PCR reaction was conducted according to Degola et al. (2007). The primers AflR-R and AflR-F1 (Metabion, Germany) were used to amplify the specific region of *an afl*R gene. PCR was performed

in a 20 µL PCR buffer containing 50 ng of extracted DNA as template, 50 mM of $MgCl_2$, 10 mM of dNTPs, 10 µM of primers, and 0.5 U of *Taq* DNA polymerase (PlatinumTaq®, Invitrogen, USA). The PCR cycling parameters as described previously (Degola et al., 2007) were performed using the GeneAmp PCR system 9700 instrument (Applied Biosystems, USA).

Electrophoresis of PCR products

Amplification products of 321 bp were analyzed by electrophoresis in 1.5% agarose gel, stained with SYBR Safe DNA stain (Invitrogen, USA) and visualized with 254 nm transillumination. The O'Gene Ruler 100 bp DNA ladder (Fermentas, USA) was included in each electrophoresis. The electrophoresis conditions were 25 °C for 40 min at 100 V and 80 mA.

Statistical analysis

Data were expressed as mean ± SD of three independent DNA isolations. All analyses were performed using Statistica 12.0 (Dell, 2015) and Microsoft Office Excel 2016 (Microsoft, USA). The differences were considered significant at the $p < 0.05$ level.

Results and discussion

The assessment of nucleic acids purity by determining the ratio of spectrophotometric absorbance of each sample at 260 nm and at 280 nm, commonly referred to as the A_{260}/A_{280} ratio, was described for the first time by Warburg and Christian. Because nucleic acids (DNA, RNA and nucleotides) absorb light at 260 nm and proteins, especially aromatic ring structures, absorb light at 280 nm, the $A_{260}/_{280}$ nm ratio is the most commonly used procedure for DNA purity assessment today. A ratio lower than 1.8 for DNA and lower that 2.0-2.3 for RNA indicates contamination, usually with proteins. The values of the secondary ratio A_{260}/A_{230} are also used as a signal of contamination and should be above 1.9. Lower values indicate the presence of organic contaminants, especially phenolic solutions, thiocyanates, carbohydrates and other, which might inhibit the PCR reaction.

The purity and quantity of genomic DNA isolated using the four different methods compared in this study were estimated by measuring their A_{260}/A_{280} and A_{260}/A_{230} ratios (Table 1). Method #2 (both with and without the RNase treatment) gave the best A_{260}/A_{280} ratio (#2a 1.94±0.16 and #2b 1.84±0.15). On the contrary, methods #1, #3 and #4 had the A_{260}/A_{280} ratios below 1.7, suggesting high amounts of co-purified proteins or other contaminants absorbing at 280 nm. The RNase treatments did not affect the A_{260}/A_{280} ratios in any of the samples, indicating that four DNA extraction protocols used in this research successfully eliminated the RNA without additional enzyme treatment.

Also, lower A_{260}/A_{280} ratios by methods #1, #3 and #4, with the application of proteinase K treatment, point out the inadequate removal of protein contaminants and/or protein precipitation. It should be noted that DNA isolated by method #1 (commercially available Roche kit) which uses solid phase extraction, also resulted in an A_{260}/A_{280} ratio below 1.8 (#1a 1.14±0.10 and #1b 1.20±0.05). This method uses the solid matrix to bind DNA, RNase, and the proteinase K treatment to remove contaminating RNA and proteins, isopropanol for DNA precipitation, inhibitor removal buffer, DNA washing steps and finally the elution of DNA from the column. The results indicated that method #1 is inefficient in removing protein contamination.

Table 1. Comparison of DNA purity, cost and time needed for the genomic DNA isolation from *Aspergillus flavus* using four different protocols without (a) or with (b) RNase treatment

Isolation protocol	A_{260}/A_{280}	A_{260}/A_{230}	DNA [ng/µL]	Cost per sample [€]	Isolation time [Time/hours]
#1a	1.14 ± 0.10	0.37 ± 0.05	92.77 ± 11.52	1.95	1.25
#1b	1.20 ± 0.05	0.40 ± 0.03	101.60 ± 27.02	2.29	1.25
#2a	1.94 ± 0.16	1.91 ± 0.17	4143.87 ± 1530.03*	0.12	1.75
#2b	1.84 ± 0.15	1.35 ± 0.49	650.73 ± 247.07	0.46	1.75
#3a	1.60 ± 0.02	1.37 ± 0.02	3096.9 ± 74.39	0.16	4.75
#3b	1.56 ± 0.03	1.35 ± 0.06	2590.57 ± 81.01	0.50	4.75
#4a	1.16 ± 0.01	1.08 ± 0.01	5475.5 ± 10.27*	0.41	7.12
#4b	1.22 ± 0.02	1.11 ± 0.02	5477.4 ± 22.03*	0.75	7.12

*calculated after dilution of isolated DNA, since NanoDrop™ precision was declared up to 3700 ng/µL. Methods: #1a Roche kit (Roche, 2007); #1b Roche kit with RNase (Roche, 2007); #2a (Liu et al., 2000); #2b (Liu et al. with RNase) (Liu et al., 2000); #3a (Goltapeh et al., 2007); #3b (Goltapeh et al. with RNase) (Goltapeh et al., 2007); #4a (Yeates et al.); (Yeates et al. with RNase) (Yeates et al., 1998).

Thiocyanate, present in the method #1 lysis buffer absorbs near 230 nm and may have also contributed to the low A_{260}/A_{280} ratio. Because the phenolic solution absorbs with peaks at 270 nm and 230 nm, while polysaccharides absorb at 230 nm, it is not surprising that the phenol used in the method #4 can cause overestimation of the DNA concentration and a low A_{260}/A_{230} ratio. Therefore, only the method #2a showed A_{260}/A_{280} (#2a 1.94±0.16 and #2b 1.84±0.15), and A_{260}/A_{230} (#2a 1.91±0.17 and #2b 1.354±0.49) ratio values in the appropriate range for DNA purity.

All four examined methods for DNA extraction from filamentous fungus gave satisfactory amounts of DNA for the PCR reaction (Table 1). Method #4 by Yeats et al. (1998), produced the largest amounts of extracted DNA, greater than 37 kb (#4a 5475.5 ± 10.27 ng/µL and #4b 5477.4 ± 22.03 ng/µL), followed by methods #2a, #3a and #1. At the same time, the agarose gel electrophoretic analysis of DNA integrity showed that method #4 generates high quantities of degraded DNA in comparison to the other three methods (Fig. 1). An aggressive approach to DNA isolation by method #4, consisting of a combination of cell disruption techniques (grinding, sonicating and cell wall digestion with SDS detergent), prevention of RNA contamination by the RNase treatment, and protein extraction and DNA precipitation by a mixture of organic solvents, expectedly yielded low DNA quality. Although this procedure generates degraded DNA, this DNA is still effective as a template for further PCR amplification.

According to literature data, the advantage of high molecular weight DNA during PCR amplification, in comparison with low molecular weight DNA, is in having less potential for the formation of chimeric molecules
(Liesack et al., 1991). The DNA obtained by method #2 following the RNase treatment showed great discrepancies in DNA quantity. The reason for this may be in the fact that RNA and DNA absorb with a peak at 260 nm.

Regarding the costs, the Roche kit (method #1) was the most expensive one (#1a 1.95 € and #1b 2.29 €), while the other methods were considerably cheaper (Table 1). Method #2 was the cheapest (10 times cheaper than the kit) (#2a 0.12 € and #2b 0.46 €), followed by methods #3 and #4. The use of liquid nitrogen significantly increases the cost of each method.

According to the time needed for DNA isolation, methods #1 and #2 were the fastest (#1-1.25 h and #2-1.75 h) and required less than 2 hours of work (Table 1). Time consumption analysis for DNA isolation methods presented here was determined without the time required for the preparation of chemicals. Finally, PCR was conducted to confirm the identity and the PCR applicability of the isolated genomic DNA, using the primers specific for the *A. flavus* *afl*R gene (Degola et al., 2007). The estimated size of the PCR product (321 bp) correlated with the size of the PCR bands on the gel (Fig. 2).

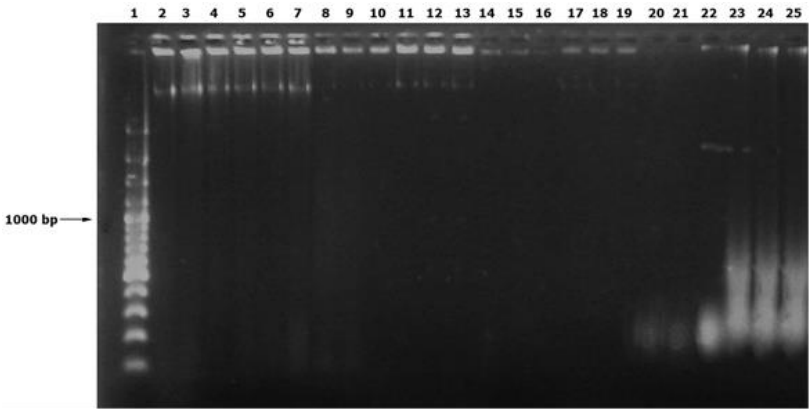

Figure 1. Electrophoresis of isolated genomic DNA from *A. flavus*.
DNAs were separated on a 2% agarose gel in 0.5X TBE buffer. Lane 1 shows low range DNA ladder (60.8 ng/µL). Lanes 2-4 show DNA obtained by method #1a. Lanes 5-7 show DNA obtained by method #1b. Lanes 8-10 show DNA obtained by method #2a. Lanes 11-13 show DNA obtained by method #2b. Lanes 14-16 show DNA obtained by method #3a. Lanes 17-19 show DNA obtained by method #3b. Lane 20-22 show DNA obtained by method #4a. Lanes 23-25 show DNA obtained by method #4b.

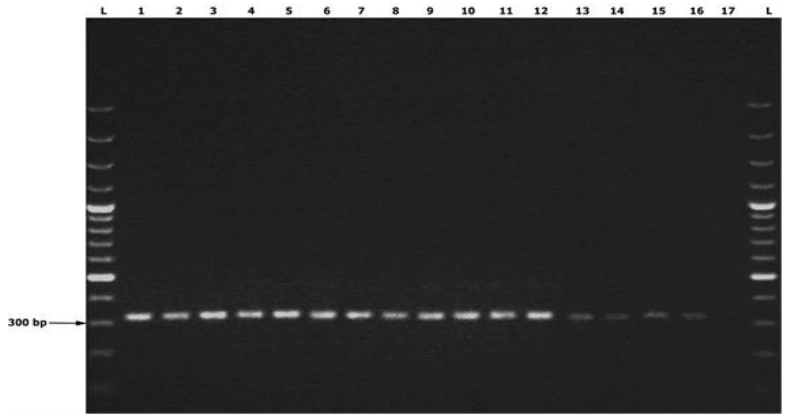

Figure 2. Gel electrophoresis of the PCR products of an aflR gene.
Lane L: 1500 bp low range DNA ladder; lane 1,2: PCR product of method #1a; lane 3,4: PCR product of method #1b; lane 5,6: PCR product of method #2a; lane 7,8: PCR product of method #2b; lane 9,10: PCR product of method #3a; lane 11,12: PCR product of method #3b; lane 13,14: PCR product of method #4a; lane 15,16: PCR product of method #4b; lane 17: PCR product of the control sample (H_2O)

When compared to the other methods, method #4 showed a lower PCR yield (Fig. 2). Possible reasons could be the co-extracted PCR inhibitors or degraded DNA. The amplification products obtained from PCR of DNA obtained from four described methods confirmed that all tested isolation procedures provide enough fungal genomic DNA for further PCR analysis, while the worst recovery and reproducibility is obtained when DNA is extracted using both liquid nitrogen and sonication. Such a method appears to be too aggressive resulting in more degraded genomic DNA and less efficient PCR amplification.

Conclusion

In conclusion, all tested methods were found to be effective for the isolation of *A. flavus* genomic DNA. Nevertheless, they differ according to obtained DNA purity, quantity, time consumption and costs. According to the obtained PCR yield, genomic DNA obtained by methods #1, #2, and #3 was successfully used for gene amplification. DNA extraction method #2 was found to be more suitable for those laboratories with low budgets, while method #1 is appropriate for those laboratories with no budgetary problems or with insufficient hands-on experience performing organic extractions. Also, both methods are less time consuming and allow the efficient isolation of *A. flavus* genomic DNA in less than 2 hours. RNase treatment can be omitted because genomic DNA obtained by all tested methods without the RNase treatment can be successfully amplified by PCR.

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