Eur Food Res Technol (2009) 230:79–87
DOI 10.1007/s00217-009-1139-4

ORIGINAL PAPER

Evaluation of DNA extraction methods for PCR detection of fungal and bacterial contamination in cocoa extracts

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Received: 17 June 2009 / Revised: 11 August 2009 / Accepted: 25 August 2009 / Published online: 16 September 2009

Abstract Direct and sensitive PCR detection of contaminant microflora in cocoa extracts is affected by the quality of the template DNA. This study compares the efficacy of five different commercial DNA extraction methods, selective enrichment broths and use of glycolitic enzymes to obtain quality DNA for PCR detection of both fungi and bacteria in artificially inoculated cocoa extract samples. PCR-based methods were applied to detect contaminant microflora in cocoa extracts using as model organisms: *Aspergillus nidulans*, *Bacillus subtilis, Escherichia coli* and *Salmonella enterica*. The quality of the extracted DNA was assessed in terms of PCR inhibitor content with results indicating that the HighPure PCR template (Roche) kit was the best methodology under the conditions assayed. PCR protocols using this commercial kit and a combination of glycolitic enzymes and enrichment procedures gave a detection limit of 100 conidia/g and 100 cfu/g for filamentous fungi and bacteria, respectively. The selected extraction and PCR procedures were also tested to assess their suitability for detecting filamentous fungi and bacteria on an industrial scale. They were sensitive enough to detect fungal and bacterial contaminants within the legally required limits. The results obtained with the molecular approach were in agreement with those of standard microbiological tests but require a considerably shorter analysis time. Thus, the molecular approach provides a sensitive and rapid alternative to check for microbial contamination in cocoa extracts.

Keywords *Aspergillus* · *Bacillus* · Cocoa · DNA extraction · PCR · *Salmonella* · *E. coli*

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Introduction

Cocoa powder, extensively used as a food ingredient in cakes, biscuits, ice-creams and sweets, is produced from cocoa beans, i.e. seeds from fruit pods of the tree *Theobroma cacao*. Microbial contamination may occur at many critical points in the cocoa production chain [29], and its origin can fall into three categories: (1) fungal pathogens present in the raw materials, (2) natural microflora, mostly yeasts, lactic and acetic acid bacteria, which are responsible for fermentation of mucilagenous pulp [6, 15], and (3) environmental microorganisms, mainly belonging to filamentous fungal species.

Most of the contaminants originally present or produced within the intermediate stages of processing are eliminated during fermentation and roasting. However, sporulating

microorganisms can survive these treatments, producing a variety of chemical compounds that increase acidity and are detrimental to the chocolate flavour [7, 16, 29, 38]. Moreover, fungal contamination poses a major problem for the end-product retailer as levels can increase at the end of fermentation, during drying, or if the product becomes humid during storage and transport [4, 17, 23, 24, 26]. Furthermore, many fungal species, especially from the genera *Aspergillus* and *Penicillium*, produce mycotoxins causing acute or chronic intoxication [12, 19, 21]. In fact, the occurrence of ochratoxin A has been reported in cocoa powder and cocoa marketed products in various countries [2, 5, 20, 30, 33]. Also there have been recent reports on the presence of abundant mycotoxin-producing fungi during cocoa processing [22, 28].

Traditional microbiological detection methods are too time-consuming and arduous to be adapted to the high-throughput sampling and analysis required by industry for statistical and quality control, especially for slow-growing organisms like fungi. Nevertheless, microbial contaminant detection can be readily performed by DNA technologies and, particularly, the polymerase chain reaction (PCR). PCR is increasingly being used to detect and in some cases quantify microorganisms in food samples [18, 31]. However, a major limitation of PCR-based detection of microorganisms in food samples is the quantity and purity of the DNA template. Particularly, the polyphenols (flavanols) and polysaccharides in cocoa beans can reduce the yield and quality of extracted DNA [25], making it unsuitable for most molecular applications, including PCR amplification [1, 8, 14].

There are several studies comparing different DNA extraction procedures for cocoa products [10, 11]; however, none of them deals with PCR detection of microbial contamination. The aforementioned studies show that the most suitable methods use adsorptive columns that retain contaminants under conditions in which DNA is desorbed and vice versa; however, they do not provide an estimation of the sensitivity limit for any target organism. Thus, an extraction method must be developed for the rapid isolation of high-molecular-weight DNA to obtain quality template DNA from the microorganisms (bacteria, fungal mycelia and fungal conidia) present in cocoa products.

In this paper, five commercial DNA extraction methods have been compared concerning extraction efficiency, DNA purity and DNA suitability for PCR amplification. The comparison of extraction and purification procedures was performed in cocoa extracts artificially inoculated with fungal conidia. Presence of PCR inhibitors was evaluated using DNA extractions from uninoculated cocoa samples and known amounts of fungal DNA. After selecting a DNA extraction method, selective enrichment strategies and the use of glycolitic enzymes were compared for the sensitive and reliable detection of *Aspergillus nidulans*, *Bacillus subtilis*, *Escherichia coli* and *Salmonella enterica* in artificially inoculated cocoa extract samples. PCR detection methods employing the previously selected DNA extraction kits and procedures were finally evaluated on industrial samples of different types of cocoa powder. In parallel, samples were also tested using official conventional microbiology procedures to confirm the results and evaluate the performance of the PCR system.

Materials and methods

Samples and reference strains

Aspergillus nidulans SRF-200, *E. coli* DH5, *B. subtilis* 168, and *S. enterica* LT2 were used as reference strains to set up DNA extractions and PCR protocols. They are held in the Institute of Agrochemistry and Food Technology (IATA-CSIC).

Cocoa Bean Powder (CBP) is a labelled trademark registered by the Natraceutical Group and is obtained after physical extraction of fat in the form of a reduced fat cocoa powder (10–12% fat content). Intermediate cocoa powder is the product obtained after the first heating process of CBP. CocoanOx is a labelled trademark from Natraceutical Group and is a cocoa powder enriched with flavanoids [34]. Samples of 500 g of CBP (12% fat content, batch 00257), intermediate cocoa bean powder (12% fat content, batch 00551) and CocoanOx (12% polyphenols, batch 01698) were kindly provided by Natraceutical Group.

Sample preparation and artificial inoculation

Sterilised samples of 100 mg of cocoa bean powder were used to perform artificial inoculations, DNA extractions and PCR sensitivity assays. For artificial inoculation of cocoa powder samples, fungal conidia of *A. nidulans* were prepared by flooding three plates (6 days old) of MEA (malt extract 6.0 g/L, maltose 1.8 g/L, dextrose 6.0 g/L, yeast extract 1.2 g/L) with 5 mL of sterile nanopure water containing 0.005% Tween 80 (Fluka Biochemika, Steinheim, Germany), and rubbing the surface with a glass rod. The conidial suspension was filtered through Whatman paper No 1, diluted in sterile nanopure water, as necessary, and quantified both by plate count on MEA and microscopy, using a Neubauer counting chamber. Artificial inoculations were prepared in 25 mL Erlenmeyer flasks containing 9 mL of MEA medium and 100 mg of the different sterilised cocoa powder samples. A flask was inoculated with 1 mL of 10^7 conidia mL^{-1} to assess the different extraction methodologies as described below. Afterwards, flasks were inoculated with 1 mL of 10, 10^2, 10^3, 10^4, and

10^5 conidia mL^{-1} to determine the sensitivity of the selected methodology. One non-inoculated flask was included in all cases as negative control. The flasks were incubated overnight with 150 rpm shaking at 30 °C to promote germination of conidia.

For artificial inoculation of cocoa powder, samples with bacterial cell suspensions of *B. subtilis, E. coli* and *S. enterica* cultures were grown in Luria–Bertani medium (LB) either at 30 or 37 °C on a rotary shaker at 200 rpm overnight, washed and concentrated by suspension in physiological solution (NaCl 0.09%). Artificial inoculations were performed identically, but using LB medium for enrichment and inoculating different amounts of bacteria (1 mL of 10, 10^2, 10^3, 10^4, and 10^5 conidia mL^{-1}).

DNA extraction

From each flask of fungi-inoculated culture and for each extraction method assayed, 1 mL was sampled in duplicate and its solid residue was pelleted by centrifugation at 10,000*g* for 5 min. Pellets were washed with 0.5 mL of TE (10 mmol L^{-1} Tris–HCl; 1 mmol L^{-1} EDTA, pH 8), centrifuged at 13,000*g* for 3 min and resuspended in 50 µL of sterile nanopure water. An amount of 1 mL of pure conidia of *A. nidulans* at a known concentration (10^2 conidia/mL) was used as positive control. The solid residue from an uninoculated flask was used as negative control.

DNA was isolated following the manufacturer's protocol for each of the five different commercial methodologies: RealPure Extraction Genomic DNA Kit (Ref RBMEG02, REAL™, Durviz, Valencia, Spain); UltraClean DNA Isolation Kit (Ref 12224-50, Mo Bio, USA), Nucleospin Plant Kit (Ref 635979, Macherey-Nagel), Chelex 100 Resin (Ref 143-2832, BioRad Laboratories, USA), and the High Pure PCR Template Preparation Kit (Ref 11796828001, Roche Diagnostics, Valencia, Spain). In the final step, DNA was eluted in 100 µL of sterile TE buffer (10 mM Tris–HCl, 1 mM EDTA pH 8.0) and kept at −20 °C until used as template for PCR amplification.

For sensitivity evaluation and improvement, 1 mL was sampled in duplicate from each flask of both fungi- and bacteria-inoculated cultures and solid residue was processed as described above. An amount of 1 mL of pure conidia of *A. nidulans* (10^6 conidia/mL) was also used as positive control. For fungal DNA extractions, 30 µL of the lytic enzymatic preparation Glucanex from *Trichoderma harzianum* at 1 mg/mL (Novozymes for ref L1412 from Sigma–Aldrich) were added and the samples were incubated at 37 °C for 1 h under mild stirring conditions. Bacterial DNA extractions were incubated for 1 h at 37 °C with the enzyme Achromopeptidase (EC 3.4.21.50 from *Lysobacter sp*, ref A3547 from Sigma–Aldrich). After enzyme digestion, pellets were washed in 0.5 mL of TE (10 mmol L^{-1} Tris–HCl; 1 mmol L^{-1} EDTA, pH 8), centrifuged at 13,000*g* for 3 min and resuspended in 50 µL of sterile nanopure water.

PCR analysis

For fungal DNA detection, the 5.8S-ITS region of the ribosomal DNA was amplified by PCR using universal primers ITS4 and ITS5 [36]. PCR reactions were performed in 50 µl of final volume, containing 50 mM KCl, 10 mM Tris–HCl, 80 µM of each dNTP, 1 µM of each primer, 2 mM $MgCl_2$ and 1 U of DNA polymerase (Netzyme, Molecular Netline Bioproducts, N.E.E.D, SL, Valencia, Spain). 5 µL of DNA extract was used in all cases as a template. Serial logarithmic dilutions were performed to reduce the concentration of possible contaminants and DNA polymerase inhibitors. The reaction mixtures were performed in a thermal-cycler Techne (TC-512) starting with 3 min of denaturation at 95 °C followed by 35 cycles consisting of 1 min at 95 °C, 1 min at 52 °C and 1 min at 72 °C. A final extension step of 10 min at 72 °C was also included.

Generic PCR detection of bacterial species was performed using the Bacterial Screening Test of Takara, following the manufacturer's instructions. Two commercial-specific PCR primer pairs were used to detect both Gram-negative and Gram-positive bacteria, respectively (noted ENT and BS, respectively, by the manufacturer).

Salmonella was detected by PCR according to Malornya and coworkers [18]. Thus, DNA was amplified by PCR using primers 139 (5′-GTG AAA TTA TCG CCA CGT TCG GGC AA-3′) and 141 (5′-TCA-TCG CAC CGR CAA AGG AAC C-3′). PCR reactions were performed in 50 µL of a reaction mixture containing 2.5 µL of reaction buffer (10 mM Tris–HCl pH 9.0, 50 mM KCl, 2 mM $MgCl_2$, 0.1% Triton X-100, 0.2% BSA), 80 µM of each dNTP, 1 µM of each primer, 2 mM $MgCl_2$ and 1 U of Taq-DNA polymerase (Netzyme), 5 µL of DNA extract or its corresponding dilution as a template. The reaction mixtures were incubated in a thermalcycler (Techne TC-512) for 35 cycles of 1 min at 95 °C, 1 min at 55 °C and 1 min at 72 °C.

PCR products were separated on 1% agarose gels with TAE buffer (40 mM Tris–Acetate, 1 mM EDTA pH 8.0). After electrophoresis, gels were stained with ethidium bromide (0.5 mg mL^{-1}) and the DNA bands were visualised under UV light. Sizes were estimated by comparison with a DNA standard length (GeneRuler ™ 100pb DNA ladder, MBI Fermentans, Vilnius, Lithuania).

Industrial sample evaluation

The abovementioned industrial cocoa samples from different origins were analysed for the presence of microbial contaminants by classical microbiological detection and

Table 1 General characteristics of each DNA extraction method used in this study

Method	Lysis procedure	DNA recovery	Processing time	Additional reagents Spetial equipment required
RealPure Extraction genomic DNA Kit (DURVIZ)	Chemical	Solvent precipitation	1-2 h	Isopropanol, microcentrifuge tubes, ethanol
UltraClean DNA Isolation Kit (MOBIO)	Chemical and mechanical	Column adsorption	0.5-1 h	High speed stirrer
Nucleospin Food Kit (Mackerey Nagel)	Chemical and enzymatic	Column adsorption	1-2 h	None
Chelex Extraction Kit (Biorad)	Thermal	None	0.5 h	None
High Pure PCR Template Preparation Kit (Roche)	Chemical and enzymatic	Column adsorption	1-2 h	None

Table 2 Yield and quality of DNA from cocoa extracts obtained by the different DNA extraction methods used in this study

Sample	Extraction methods	Elution volume	DNA concentration Pygogreen (μg/g)	A_{260}/A_{280} range	PCR amplification
100 mg CBP + 10^7 cfu *A. nidulans*	RealPure Extraction Genomic DNA Kit (DURVIZ)	100 μL	21.4	1.17	Inhibited
100 mg CBP + 10^7 cfu *A. nidulans*	UltraClean DNA Isolation Kit (MOBIO)	100 μL	12.3	1.09	Inhibited
100 mg CBP + 10^7 cfu *A. nidulans*	Nucleospin Plant Kit (Mackerey Nagel)	100 μL	6.7	1.26	Sporadically inhibited
100 mg CBP + 10^7 cfu *A. nidulans*	Chelex Extraction Kit (Biorad)	1 mL	531.8	1.33	Inhibited
100 mg CBP + 10^7 cfu *A. nidulans*	High Pure PCR Tem plate Preparation Kit (Roche)	100 μL	26.5	1.52	Non-inhibited

PCR detection. Samples of 100 mg of cocoa-based products were resuspended in 25 mL of MEA and LB media and incubated at 30 °C overnight for fungal and bacterial detection, respectively. Solid pellet was recovered and processed, and PCR reactions were carried out as described above. Fungal and bacterial detection was performed in DNA extracted from both MEA and LB-medium incubations in order to estimate whether a single enriched culture extract could be used in both molecular detection methods. In parallel, microbial counts were performed on Dichloran Rose Bengal Chloramphenicol Agar (DRBC, Oxoid, Basingstoke, Hampshire, UK) and LB plates, and incubated at 30 and 37 °C for fungi and bacteria, respectively.

Results

Comparison of DNA extraction methods in cocoa samples: determining DNA concentration and purity

Five extraction protocols (see "Materials and methods") were selected to represent various cell-lysing methods and DNA purification protocols (see Table 1). A 100 mg starting sample was used for all DNA extraction protocols to ensure uniformity and three replicates of each sample were analysed. The efficacy of the DNA extraction and purification procedures was assessed by measuring final DNA concentration which was most accurately evaluated by the PycoGreen Reagent method (GE Healthcare) (results are summarised in Table 2). DNA quality was evaluated using the absorbance ratio 260/280. As can be seen in Table 2, the ratio A_{260}/A_{280} was poor in all cases, probably due to high polyphenol and polysaccharide content. The Chelex extraction method produced the highest yields with 531.8 μg/g and a A_{260}/A_{280} ratio of 1.33; however, DNA quality was extremely poor, giving rise to smeared, short fragments (data not shown). This probably accounts for the unusually high DNA concentration estimated by fluorescence. Furthermore, no PCR amplification was obtained using this DNA extraction method (see below). The HighPure PCR template kit (Roche) produced a yield of 26.49 μg/g and a ratio A_{260}/A_{280} of 1.52 and enabled PCR amplification (see below). The Nucleospin Plant Kit (Macherey–Nagel) produced a ratio A_{260}/A_{280} close to 1.3 which, although far lower than the expected 1.8, also enabled PCR amplification. The remaining methods gave even lower yields and ratios A_{260}/A_{280}.

Comparison of DNA extraction methods in cocoa samples: detection of PCR inhibitors by spiked *A. nidulans* DNA in cocoa extract

The following assays were performed to determine whether the presence of PCR inhibitors was responsible for PCR failure in some of the kits. An uninoculated cocoa sample was extracted and purified by each method and the purified extract was spiked with known amounts of *A. nidulans* DNA extracted from mycelia. PCR was performed on tenfold and one 100-fold dilutions of the DNA-spiked cocoa samples extracted by each extraction method. Additionally, PCR reactions using universal primers ITS4 and ITS5 were performed in triplicate for each sample and dilution, respectively. Samples were classified as "inhibited" if amplification failed for all reactions from a replicate, and "sporadically inhibited" if at least one reaction succeeded (Table 2). The PCR assays indicated that DNA extracts from cocoa samples obtained by Nucleospin Plant Kit and Roche Diagnostics kit were suitable for PCR amplification (Table 2). However, suitability of the Nucleospin Plant kit amplification depended on the dilution rate, whereas with the HighPure Roche kit direct PCR amplification of DNA extracts was possible. Therefore, although the spectrophotometric analysis of DNA purity was lower (1.52) than the expected (1.8), this method was suitable for PCR amplification. No PCR amplification signal was obtained using DNA extracts and their dilutions from the RealPure kit, UltraClean Isolation Kit and Chelex Extraction kit. These results suggest PCR was inhibited in all samples obtained with the aforementioned DNA extraction kits.

Apart from DNA extract yield and purity, other factors such as operating time, auxiliary materials and equipment required were taken into account to choose the optimal extraction kit (Table 1). According to these considerations and due to the fact that the Roche High Pure PCR Template kit yielded better detection limits than the other methods, this was the only method used in the following steps of this study.

Evaluation of the DNA extraction procedure for fungal DNA detection in inoculated cocoa samples

Given the well-known resistance of conidia walls to lysis, it was necessary to assess the effectiveness of different procedures to extract DNA from cocoa extracts artificially contaminated with *A. nidulans* conidia (see "Materials and methods"). DNA extracted from artificially inoculated cocoa extracts was analysed to evaluate whether the DNA extraction method selected (Roche High Pure PCR Template kit) was suitable for fungal PCR detection in cocoa extracts.

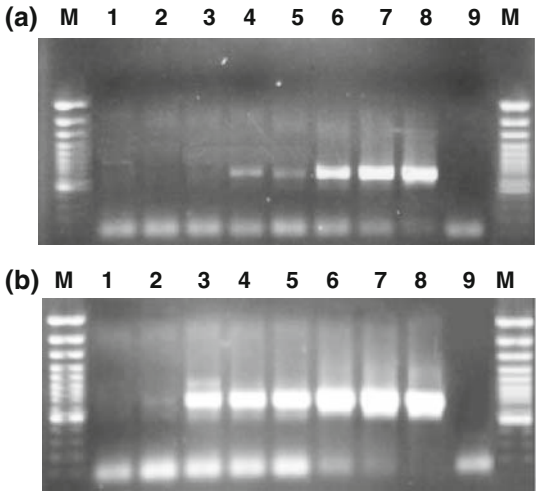

Fig. 1 PCR sensitivity for fungal contamination without pre-incubation (**a**) and with pre-incubation and lytic enzymes in the extraction protocol (**b**). *Lanes M* correspond to the 100-bp molecular weight marker. *Lanes 1–9* represent PCR products of approximately 600 bp from extracts obtained from samples that were spiked with 10–10^8 conidia/g, respectively. *Lane 9* negative control

PCR amplification yield varied depending on the extraction procedure (Fig. 1). As can be seen in Fig. 1b, better amplification was obtained when DNA was processed using both the pre-incubation procedure and lytic enzymes. Results obtained with this DNA extraction procedure demonstrate that at least 100 conidia/g should be present for a positive PCR reaction. Sensitivity was lower without the use of lytic enzymes and without the pre-incubation procedure, showing a detection limit of 10^4 conidia/g of cocoa sample (Fig. 1a). The influence of different cocoa matrixes enriched with polyphenol fraction on DNA extraction and PCR amplification was also examined (Fig. 2). PCR amplification was also possible at a range from 10^2 to 10^4 conidia/g of cocoa sample, as demonstrated by analogous PCR experiments with DNA extracted using artificially inoculated cocoa extracts, enriched with the polyphenol-rich extract CocoanOx with *A. nidulans* conidia, processed by the pre-incubation procedure and using lytic enzymes (Fig. 2).

Evaluation of the DNA extraction procedure for detection of bacterial DNA in inoculated cocoa samples

To determine whether the kit is suitable to detect bacterial contamination in cocoa extracts, PCR analyses were performed with DNA extracted from cocoa extracts that had been artificially inoculated with *B. subtilis* (Gram-positive) and *E. coli* (Gram-negative) cells (Fig. 3a, b). For *B. subtilis*, the PCR assay was evaluated using pre-incubation procedures with and without the enzyme Achromopeptidase prior to DNA extraction. When enzyme-digested DNA

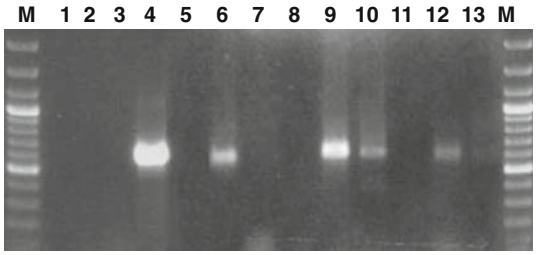

Fig. 2 PCR detection in extracts from cocoa samples enriched with polyphenols. *Lanes M* correspond to the 100-bp molecular weight marker. *Lane 1* extraction negative control, *lane 2* PCR negative control, *lane 3* void lane, *lane 4* positive control (*A. nidulans*, 10^5 conidia/g); *lanes 5–13* represent PCR products from extracts obtained from samples spiked with 10^4–10^2 cfu/g and their dilutions: *5* 10^4 cfu/g 1/1, *6* 10^4 cfu/g 1/10, *7* 10^4 cfu/g 1/100, *8* 10^3 cfu/g 1/1, *9* 10^3 cfu/g 1/10, *10* 10^3 cfu/g 1/100, *11* 10^2 cfu/g 1/1, *12* 10^2 cfu/g 1/10, *13* 10^2 cfu/g 1/100

extract was used as a template for PCR amplification, at least 10 cfu of *B. subtilis* had to be added to 100 mg of cocoa extract (10^2 cfu/g) to obtain a positive PCR result (Fig. 3b). Sensitivity was lower when the enzyme Achromopeptidase was not used (data not shown). Identical detection limits were obtained when the DNA used as a template for PCR amplification was extracted from cocoa extracts artificially inoculated with *E. coli* cells. Again 10^2 cfu/g of cocoa extract was necessary to obtain the PCR product (Fig. 3a). It is worth noting that no enzyme digestion prior to DNA extraction was used to extract DNA from cocoa extracts artificially inoculated with *E. coli.* Also evaluated was the suitability of the HighPure Roche Kit to detect *Salmonella* spp contamination by PCR amplification in cocoa extracts. Although a different primer pair was used to amplify DNA from *Salmonella* spp, identical sensitivity values were obtained and 10^2 cfu/g of cocoa extract were needed to obtain the PCR product (Fig. 3c).

Application of PCR analysis to industrial cocoa samples

To determine whether the methodology is suitable to detect fungal and bacterial contamination in industrial cocoa extracts, an assay was performed using DNA extracted from samples corresponding to different stages of product preparation (intermediate and final products). Additionally, to determine the applicability of using only one common enrichment procedure, DNA was extracted using both fungal and bacterial enrichment procedures. PCR amplification was performed with primers ITS4 and ITS5 and the Takara kit for detection of fungi and bacteria, respectively. When DNA samples were examined using the universal primer pair ITS4/ITS5, a fungi-specific PCR product was obtained only for the intermediate product enrichment in MEA medium at 30 °C (Fig. 4). The remaining samples failed to yield ITS-PCR products independent of the enrichment

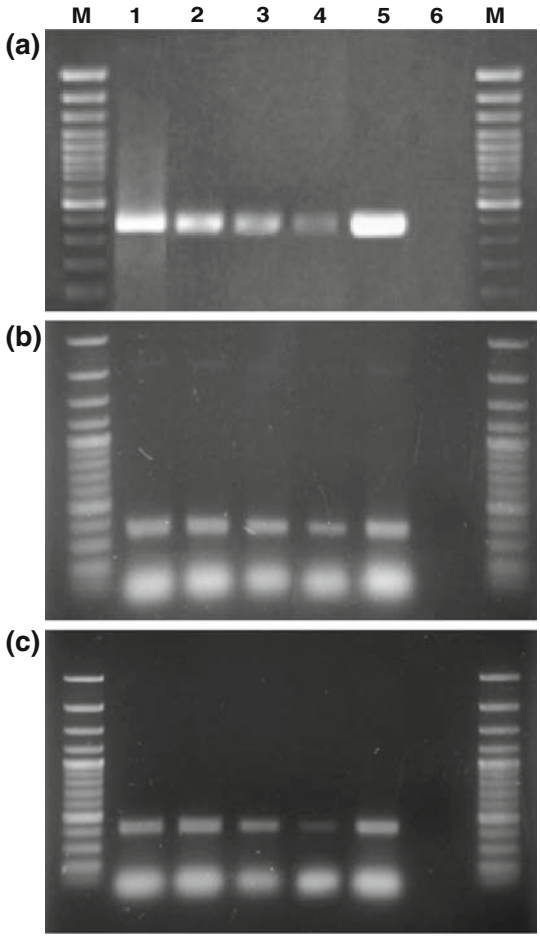

Fig. 3 Sensitivity of the PCR technique developed for bacterial contamination. **a** Gram-negative bacterial detection using primers provided by the Takara Kit. *Lanes 1–4* represent PCR products that were spiked with 10^5–10^2 cfu/g. *Lane 5* positive control; *lane 6* negative control. **b** Gram-positive bacterial detection using the primers provided by the Takara kit. *Lanes 1–4* represent PCR products that were spiked with 10^4–10^1 cfu/g. *Lane 5* positive control, *lane 6* negative control. **c** *Salmonella* detection by PCR and using primers 139 and 141. *Lanes M* correspond to the 100 bp molecular weight marker. *Lanes 1–4* represent PCR products that were spiked with 10^4–10^1 cfu/g. *Lane 5* positive control, *lane 6* negative control

procedure. When DNA samples were examined using the Takara Bacterial Detection Kit, the PCR product was obtained for Gram-positive and Gram-negative bacteria in both the final product and the intermediate product (Fig. 5). Although both types of enrichment were assessed for fungal and bacterial detection, in order to determine whether a common procedure could be used, only the specific enrichment method (LB, 37 °C for bacteria and MEA, 30 °C for fungi) ensured proper detection of contaminating flora in the samples analysed.

To compare the PCR-based detection methods with the classical isolation procedures, samples were also plated and enumerated after incubation on DRBC and LB media at 30 and 37 °C for fungi and bacteria, respectively. Fungi could

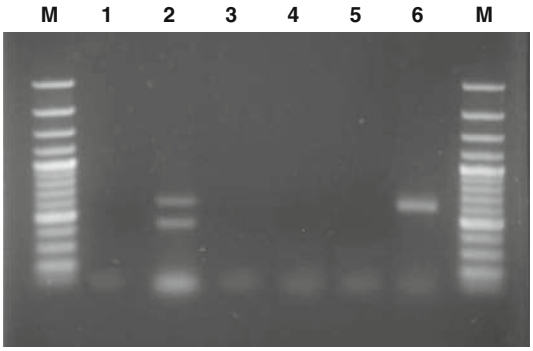

Fig. 4 PCR detection with primers ITS-4 and ITS-5 in industrial cocoa samples. *Lanes M* correspond to the 100 bp molecular weight marker. *1* final product CBP preincubated in MEA at 30 °C, *2* intermediate product CBP preincubated in MEA at 30 °C, *3* final product preincubated in LB at 30 °C, *4* intermediate product preincubated in LB at 30 °C, *5* negative control, *6* positive control (*A. nidulans*, 10^5 conidia/g)

only be detected in the intermediate product at a very low level (100 conidia/g). By contrast, a higher level of bacterial contamination was found for intermediate and final product samples and 5.7×10^6 cfu/g and 334 cfu/g were isolated, respectively. Consequently, plate counts were in agreement with the results of the PCR method and its expected sensitivity.

Discussion

Until now, few papers have compared DNA extraction methods in terms of their extraction efficiency in cocoa products [9–11]. Although DNA extraction methods were compared for detection of genetically modified soybean DNA in chocolate, they were not applied to PCR detection of microbial contamination. The present study compares five commercial DNA extraction methods with respect to DNA extraction efficiency and quality. It also assesses their ability to obtain amplifiable DNA from contaminated fungi and bacteria in cocoa products. The study is based on the premise that regular culture methods to detect microorganisms are laborious and time-consuming. By contrast, DNA amplification technology has proven beneficial when working with food-borne pathogens, due to its high specificity, sensitivity and speed. The main limitation of applying PCR to food-contaminating microorganisms concerns the presence of inhibitory substrates. Such compounds are co-extracted with the DNA and may be present in the sample, causing a PCR reaction failure which leads to a false negative result. This particular problem occurs with cocoa products, which are rich in polyphenols and polysaccharides, well-known Taq polymerase inhibitors [25].

Yield and purity of the extracted DNA were compared in terms of spectrophotometric properties of the sample and its suitability for PCR amplification. Due to the presence of inhibitors, evaluation of the extracted DNA through spectrophotometric analysis and agarose gel electrophoresis were not fully reliable in predicting PCR results. Neither can the absorbance ratio 260/280, reflecting DNA purity, be relied upon. Different substances of food origin, such as calcium ions [3], polyphenols and polysaccharides or fats [27], which commonly cause inhibition of PCR amplification, are undetectable in the A_{280} reading. Therefore, yield and purity alone cannot be used to predict the success of a subsequent PCR. The presence of potential PCR inhibitors

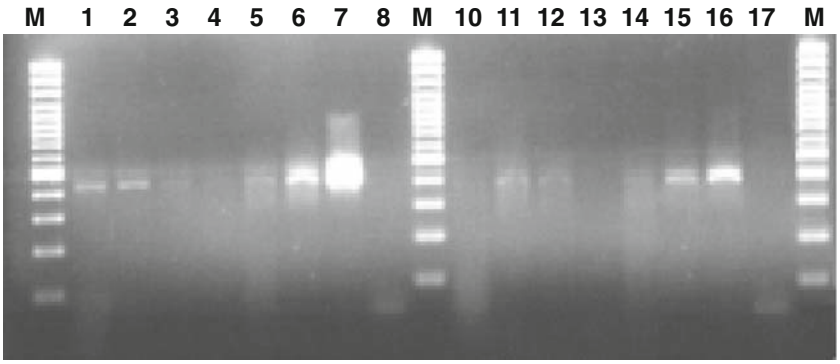

Fig. 5 PCR detection with the Takara Kit in industrial cocoa samples. *Lanes M* correspond to the 100 bp molecular weight marker. *Lanes 1–8* represent PCR products of approximately 450 bp from industrial samples using specific primers for enterobacteria. *Lane 1* final product CBP preincubated in LB at 37 °C, primers ENT; *lane 2* intermediate product preincubated in LB at 37 °C, primers ENT; *lane 3* final product CBP preincubated in MEA at 30 °C primers ENT; *lane 4* intermediate product preincubated in MEA at 30 °C primers ENT; *lane 5* final product preincubated in LB at 37 °C (Acr) primers ENT; *lane 6* intermediate product preincubated in LB at 37 °C (Acr) primers ENT; *lane 7* positive control primers ENT; *lane 8* negative control. *Lanes 10–17* represent PCR products of approximately 400 bp from industrial samples using specific primers for *B subtilis*. *Lane 10* final product CBP preincubated in LB at 37 °C primers BS; *lane 11* intermediate product CBP preincubated in LB at 37 °C primers BS; *lane 12* final product preincubated in MEA at 30 °C primers BS; *lane 13* intermediate product preincubated in MEA at 30 °C primers BS; *lane 14* final product preincubated in LB at 37 °C (Acr) primers BS; *lane 15* intermediate product preincubated in LB (Acr) at 37 °C primers BS; *lane 16* positive control BS; *lane 17* negative control primers BS

was tested by subjecting cocoa products to each of the five extraction/purification methods and spiking the extract with known concentrations of purified DNA from *A. nidulans* followed up by PCR. A similar approach has previously been used for coffee extracts [32]. Extracts from 100 mg of cocoa powder were tested for their PCR inhibitory effects. This experiment took into account the ability of the methods to remove Taq DNA polymerase inhibitors from the DNA sample. Although the Chelex Extraction Kit produced the highest yields of DNA, no PCR amplification was obtained using this DNA extraction method. PCR amplification using the Nucleospin Plant Kit was only achieved depending on the dilution rate. By contrast, results indicate that the Roche High Pure PCR Template Kit was the DNA extraction method to remove inhibitors most effectively, as PCR amplification was achieved directly from DNA extracts. Additionally, the detection limit was comparable to that obtained when PCR was performed without the addition of cocoa extract. Given these results the Roche High Pure PCR Template Kit was selected as potentially the best method for cocoa extracts.

The selected DNA extraction procedure was evaluated using cocoa extracts inoculated with different concentrations of conidia from *A. nidulans*, and cells from *B. subtilis* and *E. coli*, respectively, and its detection limit was determined. Enrichment of the cocoa extract, inoculated with *A. nidulans* conidia in a nutrient-rich medium (MEA), together with the use of lytic enzymes prior to DNA extraction/purification significantly improved detection limits, being able to detect an initial sample inocula of 100 conidia of *A. nidulans*. The 16–18 h incubation of samples guarantees differentiation between live contaminant flora and non-viable fungal debris and enables young and labile mycelium to form as conidia germination is initiated. Direct PCR amplification leads to lower limits of sensitivity. By contrast, the detection limit without the use of either lytic enzymes or an enrichment step was about 1 and 2 log orders less sensitive, respectively. Similar to previous studies, direct amplification of PCR products without an enrichment step was difficult. It is not possible to compare our results with previous studies on PCR detection in cocoa extracts, as previous studies tested sensitivity using only DNA from the lecithin gene used in the chocolate [9, 10]. However, our results range within the detection limits reported for other fungi in other substrates such as soil [37]. PCR amplification in artificially inoculated cocoa extracts enriched in the polyphenol fraction with *A. nidulans* conidia was only possible when tenfold DNA dilutions were used. Thus, the assay also proved suitable for PCR amplification in extracts with high levels of polyphenols, suggesting that the Roche High Pure PCR Template Kit is sensitive enough to extract/purify amplifiable DNA from fungi contaminating any kind of cocoa extract product.

Following optimization of fungal DNA detection methods, DNA extraction procedures (enrichment and enzyme digestion prior to DNA extraction) and determination of detection limits were also evaluated using cocoa extracts inoculated with different concentrations of cells from *B. subtilis* and *E. coli* as a model for Gram-positive and Gram-negative bacteria, respectively. PCR results were in the same range of the detection limits previously reported for the fungal PCR detection (100 cfu/g as initial sample inocula). Both DNA extraction procedures included an enrichment step. Enzyme digestion by Achromopeptidase was required prior to DNA extraction for *B. subtilis* detection, whereas no enzyme digestion was used for *E. coli* detection. Previous studies examined the performance of a direct PCR assay on *E. coli* spiked in other substrates, such as bovine faeces, without the enrichment step [13, 35], and their results were similar to those recorded in this study, which found that direct amplification of PCR products from cocoa products without an enrichment step is difficult. Prior enrichment of the culture affords certain advantages, by increasing the amount of target sequences, diluting non-target DNA and compounds interfering with PCR, and ensuring a positive result from viable cells. Additionally, the same kit was assayed for *Salmonella* spp in order to cover the range of bacteria whose detection is legally required in the food industry. The PCR analysis also successfully detected 100 cfu/g as initial sample inocula.

The previously established procedures were also applied to different samples provided by an industrial cocoa dealer. Testing of industrial cocoa samples revealed that reliable and sensitive PCR detection of fungal and bacterial contamination was achieved after enrichment in MEA medium at 30 °C for 20 h and LB medium at 37 °C for 20, respectively. The samples that tested positive by classical microbiological methods could also be detected by PCR after one-step enrichment. Furthermore, results obtained by classical microbiological methods were in agreement with the detection limit of the PCR procedure (above 100 cfu/g).

In conclusion, the Roche High Pure PCR Template Kit provides efficient DNA extraction by removing inhibitors from cocoa extracts. The inclusion of enrichment and enzyme digestion prior to DNA extraction and amplification provides a more sensitive way to evaluate fungal and bacterial contamination in cocoa extracts. The method is applicable to high-throughput analysis and routine diagnosis of industrial cocoa samples. Finally, the molecular approach described in this study could be used to develop a real-time PCR procedure which might provide a more accurate quantification of the microflora contaminant in industrial cocoa extracts.

Acknowledgments The authors thank Leila Moulay (Natraceutical S.A) for selecting and providing cocoa samples for evaluation.

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