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Detection of *Penicillium expansum* by polymerase chain reaction

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Abstract

Penicillium expansum is a major causative agent of postharvest decay in a variety of fruits, including apples, peaches, nectarines, and cherries. It causes significant economic losses to the fruit industry and is also of potential public health significance, since it produces patulin, a mycotoxin known to cause harmful effects in animals. Rapid and specific detection of *P. expansum* is important for ensuring microbiological quality and safety of fruits and fruit juices. The traditional methods for identification of *P. expansum* are time-consuming and labor-intensive. In this study, we report a polymerase chain reaction utilizing primers based on the polygalacturonase gene of *P. expansum*. The PCR amplified a 404-bp DNA product from all the *P. expansum* isolates tested, but not in other common foodborne *Penicillium* species and *Escherichia coli*. Experiments to determine the sensitivity of the PCR indicated that it can detect the DNA equivalent from as low as 25 spores of *P. expansum*. The PCR could potentially be used as a rapid tool for screening fruits for the presence of *P. expansum*.
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Keywords: Penicillium expansum; Detection; PCR

1. Introduction

Postharvest spoilage of fruits results in abbreviated shelf-life and significant economic losses to the fruit industry. *Pencillium expansum*, a psychrotrophic mold and one of the most common fruit pathogens, causes a condition known as "blue mold rot" on a variety of fruits, including apples, cherries, nectarines, and peaches (Karabulut and Baykal, 2002; Karabulut et al., 2002; Vero et al., 2002; Venturini et al., 2002). Besides its economic impact, *P. expansum* is also of potential public health significance since it produces patulin, a mycotoxin known to cause immunological, neurological, and gastrointestinal toxic effects in animal models (Pitt, 1997). Further, exposure to high levels of patulin results in vomiting, salivation, anorexia, polypnea, weight loss, leukocytosis, erythropenia, and necropsy lesions of hemorrhagic enteritis in piglets (Krogh et al., 1984). Although the toxic effects of patulin in humans have not been proven conclusively, the presence of patulin has been demonstrated in apple juice (Scott et al., 1977) and grape juice (Moss, 1998). This a major concern since fruit juices, especially apple juice, are commonly consumed by infants and children. Use of moldy fruits contaminated with *P. expansum* greatly increases the risk of patulin contamination in fruit juices.

Rapid and specific detection of *P. expansum* is important for ensuring both microbiological quality and safety of fruits. The currently employed methods for identification of foodborne molds require culture isolation and application of morphological and phys-

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iological tests (Gourama and Bullerman, 1995; Zhou et al., 2000), which are time-consuming, labor-intensive, and often require mycological expertise (Shapira et al., 1996). Further, plate count techniques do not detect dead fungi, which could indicate past contamination of a product (Gourama and Bullerman, 1995). Nucleic acid-based methods, such as the polymerase chain reaction (PCR) and DNA probes, provide powerful and rapid tools for the detection of microorganisms (Hill, 1996; Kohne, 1983). Although PCR has been widely used for the detection of foodborne bacteria and viruses, its application for specific detection of foodborne molds is relatively limited. The objective of this study was to develop and optimize a PCR for rapid and specific detection of *P. expansum*.

2. Materials and methods

2.1. Fungal cultures

The fungal isolates used in this study are listed in Table 1. Each mold was grown on Malt Extract Agar (Difco, Detroit, MI, USA) for 4–6 days at 25 °C in the absence of light (Vero et al., 2002).

Table 1
Microorganisms tested by PCR

Cultures		Source
Penicillium expansum	FRR 1600	Dr. L. Bullerman,
Penicillium expansum	NRRL 2304	University of Nebraska
Penicillium expansum	2304	Ms. J. Byron, Cadbury
Penicillium expansum	1745	Schweppes, CT
Penicillium expansum	2220	
Penicillium expansum	1835	
Penicillium expansum	1801	
Penicillium expansum	C-1	Dr. W. Conway, USDA_ARS, MD
Penicillium expansum	M-8	Dr. W. Janisiewicz, USDA_ARS, WV
Penicillium expansum	46	Dr. D. Rosenberger,
Penicillium expansum	132	Cornell University
Penicillium roqueforti	127	
Penicillium solitum	115	
Penicillium eschinulatum	215	
Penicillium crustosum	902	
Penicillium commune	164	
Penicillium notatum	986	Presque Isle cultures, PA
Penicillium chrysogenum	981	
Escherichia coli	M1 (Biotype 1)	Ms. L. Hinckley, Diagnostic Testing Services, University of Connecticut

2.2. Isolation and purification of genomic DNA from *P. expansum*

The genomic DNA of *P. expansum* was isolated as per the method of Cenis (1992) with some modifications. Following growth of the fungal culture for 6 days at 25 °C, the mycelial mat was harvested from the surface of the agar with a sterile disposable loop and aseptically transferred into a 1.5 ml micro centrifugation tube containing 500 μl of TE buffer (10 mM Tris and 1 mM EDTA) (pH 8). The mycelial mat was pelleted by centrifugation for 5 min at 16,000 × *g* in a microcentrifuge (Fisher Scientific, Pittsburgh, PA), washed with 500 μl of TE buffer, and pelleted again. The supernatant was discarded and the fungal mat was resuspended in 500 μl of extraction buffer (Tris–HCl pH 7.5 200 mM, SDS 0.5% w/v, EDTA 25 mM, NaCl 250 mM) and 300 μl 3 M Na acetate. A volume of 100 μl of 0.1 mm Zirconia/Silica Beads (Biospec Products, Bartlesville, OK, USA) was added, and the mixture was vigorously agitated using a Fisher Vortex Genie 2 (Fisher Scientific) for 10 min. The resultant lysate solution was treated with 50 μl of proteinase K (2 mg/ml) (Sigma Aldrich, St. Louis, MO, USA), and incubated for 30 min at 37 °C in a circulating water bath. Following incubation, 100 μl of 1.5 M NaCl was added to the lysate and mixed. After incubation for 5 min at room temperature, the mixture was centrifuged at 16,000 × *g* to separate out insoluble cell debris. The supernatant was transferred into a new tube and was extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol (1:1:24) (Sigma Aldrich). The DNA was precipitated by adding 0.6 volume of isopropanol (Sigma Aldrich) and incubating at −20 °C for 1 h. The DNA was pelleted, washed with 70% ethanol, allowed to air dry, and finally resuspended in 100 μl of TE buffer.

2.3. Polymerase chain reaction

2.3.1. Primer selection

The primers were selected from an internal, conserved sequence of the polygalacturonase gene of *P.*

expansum published in the Genbank (Yao et al., 1998; Accession number, AF 047713). The forward (PEF, 5′ ATC GGC TGC GGA TTG AAA G 3′) and reverse (PER, 5′ AGT CAC GGG TTT GGA GGG A 3′) primers that amplify a 404-bp DNA fragment were selected on the basis of criteria described by Innis and Gelfand (1990). The primers were synthesized commercially (Integrated DNA Technologies, Coralville, IA, USA), and stored at − 20 °C.

2.3.2. Polymerase chain reaction

A PCR was carried out as described by Saiki et al. (1988) using reagents from a GeneAmp PCR kit (Perkin-Elmer Cetus, Norwalk, CT, USA). Amplification was carried out in 50 μl reaction mixtures containing 2.5 mM $MgCl_2$, 200 μM of each nucleotide, 1 μM of each primer, 1.25 U of Taq polymerase, and approximately 10 ng of template DNA from each of the mold cultures (Table 1). Following preliminary trials with different annealing temperatures and times, the thermal cycler (MJ Research, Watertown, MA, USA) was programmed for optimum PCR conditions. Initially, the reaction mixtures were heated at 92 °C for 5 min, then the PCR progressed for 30 cycles at a melting temperature of 92 °C for 1 min, annealing temperature of 55 °C for 45 s, and an extension temperature of 72 °C for 45 s. A final extension for 7 min at 72 °C was included at the end of the 30th cycle.

2.4. PCR with P. expansum FRR 1600 spores

In order to determine the sensitivity of the PCR, we performed PCR directly with spore suspensions of *P. expansum* using a rapid DNA extraction protocol (Zhou et al., 2000). *P. expansum* spores were harvested from a fungal mat grown on malt extract agar, and suspended in 10 ml of sterile phosphate buffered saline (PBS, pH 7.3). The spore suspension was serially diluted (1:10 in PBS) and surface plated on duplicate malt extract agar plates with a sterile glass spreader. The plates were incubated at 25 °C for 48 h before counting the total colony-forming units in the original spore suspension. The suspension was divided into 1.0 ml aliquots and stored at − 20 °C until needed. After determining the colony forming units in the spore suspension, it was diluted with PBS to obtain a population of approximately 7.0 log CFU/ml. A volume of 1 ml of *P. expansum* spore suspension (7.0 log CFU/ml) containing 100 μl of Zirconia/Silica Beads was vigorously agitated using a Vortex Genie for 10 min. The samples were subsequently incubated in a boiling water bath for 10 min to inactivate nucleases,

Fig. 1. Agarose gel electrophoresis of PCR products from *Penicillium* spp. and *E. coli*. Lane 1, 1 kb-plus ladder (Invitrogen Life Technologies, Carlsbad, CA, USA); Lane 2, *P. expansum* 132; Lane 3, *P. expansum* 46; Lane 4, *P. expansum* M-8; Lane 5, *P. expansum* C-1; Lane 6, *P. expansum* FRR 1600; Lane 7, *P. expansum* NRRL 2304; Lane 8, *P. expansum* 1745; Lane 9, *P. expansum* 1801; Lane 10, *P. expansum* 1835; Lane 11, *P. expansum* 2220; Lane 12, *P. expansum* 2350; Lane 13, *Penicillium roqueforti*; Lane 14, *Penicillium solitum*; Lane 15, *Penicillium eschinulatum*; Lane 16, *Penicillium crustosum*; Lane 17, *Penicillium commune*; Lane 18, *Penicillium notatum*; Lane 19; *Penicillium chrysogenum*; Lane 20, *E. coli*.

followed by centrifugation at $16,000 \times g$ to separate out insoluble cell debris. The resulting supernatant and its 10-fold serial dilutions were subjected to PCR directly as above, with the exception that an annealing temperature of 50 °C was used.

2.4.1. Detection of PCR product

Gel electrophoresis was used to detect the presence of the amplified PCR product from each mold isolate. A volume of 20 μl of PCR products was subjected to electrophoresis on a 1.2% agarose gel; the gel was stained with ethidium bromide, and viewed under ultraviolet light to detect the presence and size of the amplified DNA product.

3. Results

When the forward primer (PEF) and reverse primer (PER) were used in the PCR, they directed the amplification of a 404-bp DNA fragment from the chromosomal DNA of all the *P. expansum* isolates used (Fig. 1, lanes 2–12). However, no amplification was observed from the genomic DNA of other common foodborne *Penicillium* species (lanes 13 to 19) and *Escherichia coli* (lane 20). Similar to the results obtained with the genomic DNA, the PCR also generated a 404-bp DNA product from the crude spore extract of *P. expansum* (Fig. 2, lanes 2–6). The lowest limit of detection with the spore extracts was the DNA equivalent from 25 spores of *P. expansum* (Fig. 2, lane 6).

4. Discussion

A common approach employed for developing nucleic acid probes or PCR for detection of fungi is by targeting ribosomal RNA (rRNA) genes (Kappe et al., 1996; Vanittanakom et al., 2002; Zhou et al., 2000), which are highly conserved and present in multiple copies in molds. However, comparison of *P. expansum* 18S and 28S rRNA gene sequences with other published fungal rRNA genes in the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/Entrez) using the Blast program (Altschul et al., 1990) failed to yield any suitable *P. expansum*-specific sequences for its detection by PCR. Hence, we selected the primers based on the polygalacturonase gene (*Pepg1*) of *P. expansum*, which encodes the enzyme responsible for decay of fruit tissue (Yao et al., 1996, 1998). The primers were selected from an internal, conserved

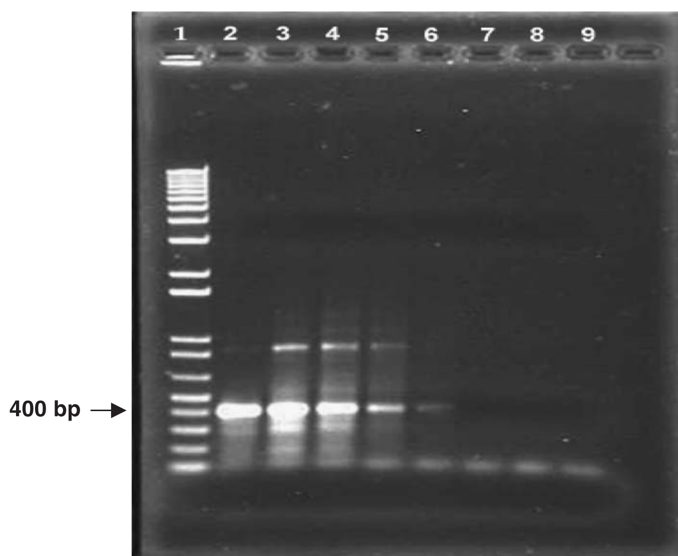

Fig. 2. Agarose gel electrophoresis of PCR products from *P. expansum* spore suspensions. Lane 1, 1 kb-plus ladder (Invitrogen Life Technologies, Carlsbad, CA, USA); Lanes 2–8, amplified products from spore concentrations of 2.5×10^5, 2.5×10^4, 2.5×10^3, 2.5×10^2, 2.5×10^1, and 2.5×10^0 CFU, 2.5×10^{-1}, respectively; Lane 9, sterile phosphate buffered saline (negative control).

fragment of *Pepg1* and were examined for specificity by comparing with other published prokaryotic and eukaryotic gene sequences available in GenBank. The results of the PCR confirmed that the amplified DNA sequence is specific for *P. expansum*, since the 404-bp amplicon was observed from the DNA of all the *P. expansum* isolates tested, but not in other foodborne *Penicillium* species and *E. coli*.

Since extraction of DNA from molds by conventional methods is time consuming, PCR was performed directly with *P. expansum* spores after a 10-min bead-agitation method (Zhou et al., 2000) to make the assay more practical and rapid. Fungal spores have tough cell walls and ordinary cell lysis treatments, such as chemical digestion and freeze/thaw cycles, cannot effectively disrupt them to release DNA (Zhou et al., 2000). The bead-agitation method used in this study has been previously reported as a rapid and effective method for obtaining high yields of DNA from fungal spores (Haugland et al., 1999; Kim et al., 1999; Shin et al., 1997; Zhou et al., 2000). The results of this study confirmed that the bead-agitation method could be used as a rapid method for obtaining DNA from *P. expansum* spores for PCR.

The crude spore extract may potentially contain substances that could affect PCR amplification efficiency, thereby reducing the sensitivity of detection (Zhou et al., 2000). Preliminary PCR experiments conducted with crude spore extracts of *P. expansum* at an annealing temperature of 55 °C had a sensitivity of detection of 2.5×10^2 CFU (results not shown). When the PCR was repeated with a lower annealing temperature (50 °C), the sensitivity of detection was increased to 10-fold, yielding a visible amplicon for samples containing 25 CFU (Fig. 2). However, it was observed that use of crude spore extract in the PCR as a DNA template resulted in several nonspecific DNA fragments along with the expected 404-bp product (Fig. 2). This could be attributed to the lower annealing temperature, since no nonspecific amplification was observed in the spore extract samples heated to an annealing temperature of 55 °C (results not shown).

In summary, the primers PEF and PER when used in PCR with the optimized conditions, amplify a specific 404-bp DNA fragment from purified DNA as well as crude spore extracts of *P. expansum*. The PCR can detect *P. expansum* in a relatively rapid timeframe (4–5 h) compared to 48 h required by the cultural methods, which employ isolation of mold on malt extract agar or Czapek yeast autolysate agar (Pitt and Hocking, 1985). However, further work is needed to validate its application for detecting *P. expansum* on food systems such as fruits. The present work elucidating appropriate PCR primers for detecting *P. expansum* in pure culture is a logical first step in achieving this goal.

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