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Short Communication

PCR Detection of Heat-Resistant Fungi

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ABSTRACT

Heat-resistant fungi inhabit soil and can contaminate fruit and can also survive high-temperature treatments such as pasteurization. Fungal spoilage of heat-processed food has emerged as a major problem in the food industry. Moreover, heat-resistant fungi are capable of producing numerous mycotoxins; therefore, they pose a serious threat to human health. To ensure the safety of food production, Polymerase Chain Reaction (PCR) based methods for detection of heat-resistant fungi have been developed. Conventional PCR methods are highly specific for the detection of *Talaromyces macrosporus* and *Talaromyces trachyspermus*, which have high heat tolerance and the potential to produce mycotoxins. The applicability of conventional PCR methods to food analyses has been successfully tested on artificially contaminated blueberries. PCR detection of *T. macrosporus* and *T. trachyspermus* would be useful for reducing global food loss and ensuring a safe food supply.

Keywords: Heat-resistant fungi; PCR; *Talaromyces*

DESCRIPTION

Fungi are universally present in soil, water, and air and can contaminate and spoil agricultural products and foods. According to FAO's "The State of Food and Agriculture 2019," around 14% of all food produced in the world is discarded post-harvest on farms or during storage and transportation [1]. Microbial decay accounts for a large amount of these food losses. Molds and yeasts have greater tolerance for desiccation and acidity than most bacteria and can grow at lower temperatures [2]. Mold growth on foods not only results in the loss of good taste and nutritional value but may also produce harmful secondary metabolites. Some of these metabolites are subject to global regulation as mycotoxins and are a problem in international food distribution [3].

Several ascomycetes produce large numbers of ascospores with strong heat-resistance [4]. Occasionally, they contaminate foods during heat-processing by adhering to raw materials and containers. Some species of *Byssochlamys* and *Talaromyces* can survive heat-processing of bottled fruit juice and canned fruit, sometimes causing spoilage, because their ascospores can tolerate heating at 85°C [4]. Moreover, *Byssochlamys fulva* and *Byssochlamys nivea* are known to produce mycotoxins, such as patulin and byssochlamic acid [5]. *Talaromyces macrosporus* produces duclauxin, and *Talaromyces trachyspermus* produces spiculisporic acid [6].

We identified two heat-resistant fungal isolates (BFF228 and BFF75) from quick-frozen low-bush blueberries [7]. BFF228 and BFF75 were identified as *Devriesia thermodurans* and *Hamigera striata* by morphological characterizations and phylogenetic analyses of internal transcribed spacer regions, respectively. Seifert et al. [8] isolated four species of *Devriesia* described as heat-resistant, and BFF228 exhibited the morphological and growth characteristics of *Devriesia* **spp.** *Hamigera* **spp.** have previously been isolated from spoiled canned blueberries and semi-finished strawberry products [9]. Heat-resistant fungi belonging to the genera *Byssochlamys*, *Eupenicillium*, *Neosartorya*, *Talaromyces* and *Thermoascus* have also been detected in various fruits and fruit products, such as blueberries, lemon peels, orange juice, and sweetened beverages [7,9-11].

The detection and identification of fungi are performed mainly by phenotypic methods, including macroscopic analysis and

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microscopy. Morphological identification is often time-consuming and requires profound mycological knowledge and trained staff with considerable expertise to be performed properly. Moreover, it does not invariably provide adequate identification results because growth and sporulation depend on proper incubation conditions and media to prevent misidentifications. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and Fourier transform infrared spectroscopy have recently been applied to efficiently identify fungi [12,13]. However, both techniques have high set-up and consumable costs and require highly skilled personnel capable of operating the systems on a routine basis.

Polymerase Chain Reaction (PCR) methods have advantages over phenotypic methods for identifying fungi that cause food spoilage. Nakayama et al. [14] designed genus-specific primer sets based on the β-tubulin gene sequences of the *Byssochlamys* and *Hamigera* genera and identified *Byssochlamys* and *Hamigera* at the genus level by PCR. The detection limits of the genus-specific primer sets were 1 ng and 10 pg of template DNA by conventional and nested PCR methods, respectively [14]. Based on the β-tubulin gene sequences of the genus *Byssochlamys*, species-specific primer sets were designed for *B. fulva*, *B. nivea*, *Byssochlamys lagunculariae*, and *Byssochlamys zollerniae*, allowing for accurate species-level detection using conventional PCR methods. The detection limit for each species-specific primer set was 100 pg and 10 pg of template DNA by conventional and nested PCR, respectively [15]. Moreover, genus- and species-specific PCR methods proved useful for detecting heat-resistant fungi, including *Thermoascus* and *Neosartorya* species, respectively. Using conventional and nested PCR methods, the detection limits for each primer set were 100 pg and 10 pg of *Thermoascus* genus DNA and 40 pg and 4 pg of *Neosartorya* genus DNA, respectively [16,17]. The sensitivity of detection was improved 10-fold using nested PCR methods.

We developed species-specific PCR methods to detect the heat-resistant fungi *T. macrosporus*, and *T. trachyspermus* using primer sets targeting the isocitrate lyase gene, which codes for a unique enzyme in the glyoxylate cycle and hydrophobin gene sequences [18,19]. Hydrophobin genes are suitable targets for the species-specific detection of fungi because hydrophobins are unique to fungi and exhibit considerable variability in their overall sequences [20]. Other heat-resistant fungi that cause food spoilage, including *Byssochlamys*, *Hamigera*, *Neosartorya*, and *Thermoascus* species, were not detected using the *Talaromyces*-specific primer sets. The detection limit for each species-specific primer set was 50-100 pg of template DNA without using nested PCR. These methods also detected fungal DNA extracted from blueberries inoculated with *T. macrosporus* [18,19]. Furthermore, conventional PCR methods using crude DNA extract as the template DNA yielded amplicons specific to *T. macrosporus* and *T. trachyspermus* [19]. The crude genomic DNA templates were prepared using small colonies and a microwave irradiation protocol [21]. The microwave irradiation protocol is a simple, rapid method for extracting genomic DNA, requiring no specialized techniques. Therefore, this protocol shortens the time required for fungal genomic DNA extraction and provides crude genomic DNA that is a suitable PCR template.

Recently, quantitative PCR (qPCR) and Loop-Mediated Isothermal Amplification (LAMP) assays have been designed for specific detection of the *DNA* replication licensin factor gene of heat-resistant fungus *Talaromyces flavus* [22,23]. The specificity of these assays was confirmed by the use of 5 *T. flavus strains* and 35 other fungal isolates. The detection limits of the qPCR and LAMP assays were 200 fg and 1 fg of template DNA, respectively [22,23]. Both the qPCR and LAMP assays exhibited higher sensitivity than other PCR methods. However, there are several problems with these two assays. The apparatus and reagent kits for qPCR assays are expensive and the system optimization for LAMP assays makes it difficult to completely avoid false positives.

For the routine application of PCR methods on-site in the food and beverage industry, they must be cheap, simple, fast, and reliable compared with other available methods of fungal identification. Conventional PCR methods require only basic PCR reagents, thermocyclers, and electrophoresis apparatus. This equipment is relatively cheap, inexpensive to maintain, and simple to operate.

DISCUSSION AND CONCLUSION

The conventional PCR method using specific primer sets and crude genomic DNA extracts from mycelia cultivated for a short period was sufficient for specific detection of heat-resistant fungi. Thus, conventional PCR methods for detecting heat-resistant fungi possess numerous advantages for use in the food and beverage manufacturing environment. This method would be useful for quality control of raw materials and processed products.

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