Rapid and Specific Detection of Live Spoilage Yeasts Using a Real-time NASBA-Molecular Beacon Detection System

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ABSTRACT

Rapid detection of spoilage and pathogenic organisms in foods is essential for food safety and quality control. The objective of this project is to develop a real-time, isothermal Nucleic Acid Sequence-Based Amplification (NASBA)-Molecular Beacon system to detect only the presence of live microbes in processed foods. Yeast, a common spoilage agent in food, was chosen as the target for system development. A set of universal primers specific for the 18S rRNA gene were used to amplify an approximately 1.5 kb PCR fragment from representative spoilage yeasts obtained from food industry. The DNA sequences of these fragments, as well as those in the GenBank database, were used to develop a pair of NASBA primers and a Molecular Beacon probe targeting the NASBA amplification product. Detection of the presence of approximately 100 yeast cells per sample is achieved without cross-activity to molds, bacteria and raw food materials by the NASBA-Molecular Beacon system, and the analysis is completed within 6 hours. This is a significant improvement comparing to the current industrial practices, which take from 48 hrs to a couple of weeks to identify and characterize these organisms. The system has great potential for food industry applications.

INTRODUCTION

Each year, approximately 10-25% of our food supplies are lost due to spoilage. The outgrowth of spoilage microorganisms can lead to changes in nutrition value, physiochemical and sensory properties of the products, and cause huge financial loss and resource waste to the community. Molds and yeasts can survive and grow at a wider range of pH and water activity than bacteria, therefore are more problematic. Current industrial procedures take from 2 days to a couple of weeks to detect the presence of these organisms, which are unsuitable for foods especially those with limited shelf life. Therefore rapid, specific and sensitive detection approaches are greatly in need for proper food quality control.

Polymerase chain reaction (PCR)-based detection methods are rapid but DNA from dead cells could serve as amplification template and potentially lead to false positive results. Reverse-transcriptase PCR using RNA as amplification template can minimize the problem, but the handling procedures are very complicated and not practical for food industry applications. The objective for this study is to develop a NASBA-Molecular Beacon real-time system to detect only live microorganisms. The system uses RNA as the template, however the amplification is conducted at 41°C by a set of enzymes distinctively different than DNA polymerase used in PCR, so the presence of DNA contamination will not interfere with the detection result (). Further, a fluorescent labeled Molecular Beacon probe, highly specific to distinguish the difference of even a single nucleotide (), will be included to enable the real-time, specific detection of the amplification product.

EXPERIMENTAL APPROACH

PCR and sequence determination of the 18S rRNA gene fragments from representative strains

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Sequence analysis of the 18S rRNA gene fragments

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NASBA Primers and Molecular Beacon probe development for real-time detection

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Specificity and sensitivity analysis using the NASBA-Molecular Beacon system

RESULTS

Primers-and-probe sets development

A pair of universal 18S rRNA gene specific primers for eukaryotes (Wan et al., 2006) were used to amplify the 1.5 kb 18S rRNA gene fragment from 4 spoilage yeast isolates from food industry. The DNA sequences of the PCR fragments were determined.

The above sequenced DNA fragments were further aligned with published 18S rRNA gene fragments from molds, yeasts, plants and animals. Three conserved sequences suitable for NASBA primers and Molecular Beacon probe development were identified. The NASBA primers franking fragment were synthesized by Sigma Genosys and the Molecular Beacon probe was fluorescence-labeled with 5' 6-FAM BHQ-1 3' and synthesized by Biosearch Technologies, Inc. (Novato, CA).

Specificity Analysis

Using the developed primer and probe sets, representative spoilage yeasts from a major beverage manufacturing facility were tested positive by the NASBA-Molecular Beacon real-time detection system. No cross reactivity was detected against representative spoilage mold, bacteria strains, and common food ingredients (Fig. 1).

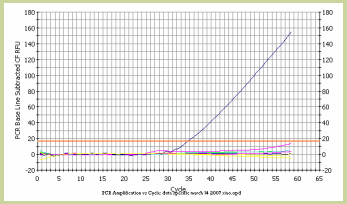

Fig.1. Real-time NASBA-Beacon specificity analysis. *Zygosaccharomyces bailii* AC1 (▼). Detection curves below the base line include *Lactococcus lactis* 2301 (▼), *E. coli* DH5α (▼), *Pseudomonas putida* 49451 (/); *Byssochlamys fulva* ATCC 24474 (▼, orange (▼), apple (▼).

Sensitivity analysis

Using the developed system, detection of the presence of approximately 100 cells in apple juice was achieved (Fig. 2).

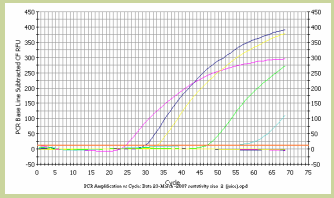

Fig 2. Real-time sensitivity analysis of *Zygosaccharomyces bailii* AC1 in apple juice using NASBA-Molecular Beacon targeting 18S rRNA gene. $8.0x10^5$ cells(▼), $8.0x10^4$ (▼), $8.0x10^3$ (/), $8.0x10^2$ (▼), $8.0x10^1$ (▼).

CONCLUSION & DISCUSSION

NASBA is an isothermal amplification of RNA template using the concurrent activity of AMV reverse transcriptase (RT), RNase H and T7 RNA polymerase, together with two primers to produce antisense, single-stranded RNA as the major end product. It has been mostly used to detect RNA viruses, and its most recent application include to detect live pathogenic bacteria cells. Adding the fluorescent-labeled Molecular Beacon probe to the system enabled real-time monitoring of the amplification process using an optical module.

In this study, a pair of NASBA primers and a Molecular Beacon probe targeting the 18S rRNA gene were developed for rapid detection of spoilage yeasts by real-time, isothermal amplification detection. The presence of spoilage yeasts can be detected without cross reactivity with spoilage bacteria, molds and food ingredients.

Although specificity analyses were conducted using limited number of microbial strains, the primer-and-probe sets were developed by comparing DNA sequence homology among a wider range of yeasts, molds, and other eukaryotes. Therefore the set of primer-and-probe derived could be used to detect other yeasts as well. The total detection procedures from microbial cell collection to PCR signal display can be completed within 5 to 7 hrs, which is a significant improvement compare to conventional methods for yeasts detection, normally taking from 2 days to a couple of weeks. As few as 100 cells per sample can be detected in apple juice, indicating the effectiveness of the system in real food sample analysis.

The availability of this rapid yeast detection system adds new value to the OSU CleanPlant® microbial detection package. Further studies will be conducted to expand the detection spectrum for molds, spoilage and pathogenic bacteria.

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