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Detection and identification of *Brettanomyces/Dekkera* sp. yeasts with a loop-mediated isothermal amplification method

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Abstract

Primer sets for a loop-mediated isothermal amplification (LAMP) method were developed to specifically identify each of the four *Brettanomyces/Dekkera* species, *Dekkera anomala*, *Dekkera bruxellensis*, *Dekkera custersiana* and *Brettanomyces naardenensis*. Each primer set was designed with target sequences in the ITS region of the four species and could specifically amplify the target DNA of isolates from beer, wine and soft drinks. Furthermore, the primer sets differentiated strains of the target species from strains belonging to other species, even within the genus *Brettanomyces/Dekkera*. Moreover, the LAMP method with these primer sets could detect about 1×10^1 cfu/ml of *Brettanomyces/Dekkera* yeasts from suspensions in distilled water, wine and beer. This LAMP method with primer sets for the identification of *Brettanomyces/Dekkera* yeasts is advantageous in terms of specificity, sensitivity and ease of operation compared with standard PCR methods.

Keywords: LAMP; Brettanomyces; Dekkera; Primer; ITS region; Beer; Wine; Soft drink; Identification; Quality control; Spoilage yeast

1. Introduction

Spoilage of foods and beverages from growth of contaminating yeasts results in major economic losses worldwide. Species of *Brettanomyces* (sexual state *Dekkera*) may cause turbidity and off-flavors in wines, beer and soft drinks (Demain et al., 1998). In the production of special beers (ale, lambic, porter, Berliner Weisse, etc.), *Brettanomyces/Dekkera* yeasts produce a characteristic flavor, but are usually beer-spoilage organisms. They compete poorly with brewer's yeast, but grow very well in filtered beer, developing an unpleasant characteristic acetic flavor. Acetic acid is the dominant off-flavor produced (Back, 1994; European Brewery Convention, 2005). *Brettanomyces/Dekkera* yeasts are also important in the wine industry, where they have been shown to produce a wide range of metabolites, including volatile phenols such as 4-ethylphenol and 4-ethylguaiacol (Chatonnet et al., 1995; Fugelsang and Zoecklein, 2003). Wines infected with *Brettanomyces/Dekkera* yeasts develop off-flavors that are described as 'phenolic', 'animal', 'horse sweat' and 'stable' (Chatonnet et al., 1992, 1995). In soft drinks, *Brettanomyces/Dekkera* yeasts produce large amounts of acetic acid from glucose and other C-sources and cause a characteristic flavor due to acetate-ester production (Back, 1999).

Traditional methods for identifying spoilage yeasts in wine and beer rely on culturing. In the case of *Brettanomyces/Dekkera* yeasts, culturing usually involves selective media containing cycloheximide and typically takes 1–2 weeks to perform (Kurtzman et al., 2003; European Brewery Convention, 2005). Identification with traditional methods takes 3–4 weeks and the results are often ambiguous (Yarrow, 1998). Some researchers have reported newer techniques for the rapid detection and identification of *Brettanomyces/Dekkera* yeasts. Ibeas et al. (1996) developed a two-step PCR that could detect as few as 10 intact *Dekkera* cells in contaminated sherry. Cocolin et al. (2000) developed direct methods to characterize yeast diversity in wine fermentations using

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denaturing gradient gel electrophoresis (DGGE) of PCR-amplified rRNA genes. Phister and Mills (2003) and Delaherche et al. (2004) developed a real-time PCR method for the detection and quantification of *Dekkera bruxellensis* in wine. Cocolin et al. (2004) developed a PCR-restriction enzyme analysis protocol to directly detect and identify *D. bruxellensis* and *Dekkera anomala* in wine samples.

Molecular techniques for the identification of *Brettanomyces/Dekkera* yeasts have also been developed to reduce the time needed for identification. Stender et al. (2001) developed species-specific peptide nucleic acid probes that were complementary to a unique target sequence on the 26S rRNA of *D. bruxellensis*, and a fluorescence in situ hybridization method for the identification of *D. bruxellensis*. Mitrakul et al. (1999) reported that RAPD-PCR could be successfully applied to discriminate *Brettanomyces/Dekkera* yeasts at the species and strain level. Egli and Henick-Kling (2001) used a PCR assay based on the rRNA internal transcribed spacer (ITS) region to differentiate *Brettanomyces* strains of four species. Morrissey et al. (2004) used a PCR restriction fragment length polymorphism analysis of the ITS region for the identification of indigenous yeasts at the species level in a traditional Irish cider fermentation.

Recently, Notomi et al. (2000) reported a novel nucleic acid amplification method, termed loop-mediated isothermal amplification (LAMP), that amplifies DNA with high specificity, efficiency and rapidity under isothermal conditions. This method employs a DNA polymerase with strand displacement activity and a set of four specially designed primers that recognize a total of six distinct sequences on the target DNA. Tsuchiya et al. (2005) developed sets of LAMP primers to detect yeasts of the genera *Brettanomyces* and *Dekkera* using sequences of the D2 region of the rRNA gene. However, these primer sets could not discriminate among species of *Brettanomyces/Dekkera*.

In this study, we developed LAMP primer sets, which amplify target sequences in the ITS region, for the specific identification of each of the four *Brettanomyces/Dekkera* species: *D. anomala*, *D. bruxellensis*, *Dekkera custersiana* and *Brettanomyces naardenensis*. These primer sets could detect *Brettanomyces/Dekkera* strains originating from beer, wine and soft drinks and differentiate the *Brettanomyces/Dekkera* strains from other genera of yeasts. Moreover, the LAMP method with these primer sets could detect about 1×10^1 cfu/ml of *Brettanomyces/Dekkera* yeasts from suspensions in distilled water, wine and beer. We discuss the advantages of the LAMP method in comparison with PCR-related methods.

2. Material and methods

2.1. Strains and culture conditions

The strains used in this study are listed in Table 1. The strains were grown on malt agar medium (3% malt extract, 0.3% peptone, 1.5% agar) at 20 °C.

Table 1
Yeast strains used in this study

Strain designation	Source and geographic origin
Dekkera anomala DSM70727 (T)	Stout beer, UK
Dekkera anomala ATCC56868	Cider
Dekkera anomala ATCC58984	Spoiled soft drink, Netherlands
Dekkera bruxellensis DSM70001 (T)	Lambic beer, Belgium
Dekkera bruxellensis ATCC64276	Tainted red wine, Australia
Dekkera custersiana DSM70736 (T)	Brewery, South Africa
Brettanomyces naardenensis NBRC1588 (T)	Lemonade, Netherlands
Brettanomyces naardenensis ATCC56870	Carbonated water
Saccharomyces cerevisiae NBRC10217 (T)	Brewer's top yeast, Netherlands
Saccharomyces bayanus NBRC11022 (T)	Turbid beer
Saccharomyces pastorianus NBRC11024 (T)	Unknown
Saccharomyces pastorianus NBRC11023	Brewer's yeast
Saccharomyces pastorianus NBRC10610	Unknown
Saccharomyces cerevisiae var. *diastaticus* DSM70487	Super-attenuated beer
Saccharomyces paradoxus NBRC10609 (T)	Soil, South Africa
Saccharomyces cariocanus NBRC10947 (T)	Fruit fly, Brazil
Saccharomyces mikatae NBRC1815 (T)	Soil, Japan
Saccharomyces kudriavzevii NBRC1802 (T)	Partially decayed leaf, Japan
Saccharomyces exiguus NBRC1128 (T)	Brewing, Japan
Saccharomyces servazzii NBRC1838 (T)	Soil, Finland
Saccharomyces unisporus NBRC0316 (T)	Unknown
Saccharomyces dairenensis NBRC0211 (T)	Dried persimmon
Saccharomyces kluyveri NBRC1685 (T)	Fruit fly, USA
Pichia anomala NBRC0127	Mash of Chinese wine
Williopsis saturnus NBRC0941	Soil, South Africa
Kluyveromyces lactis NBRC1090 (T)	Gassy cheese, UK
Candida utilis NBRC0988	Unknown
Candida boidinii ATCC48180	Unknown
Zygosaccharomyces bailii NBRC1137	Sour red wine, USA
Brewer's yeast and wild yeasts isolated from breweries	
Bottom fermenting yeast BFY61	
Bottom fermenting yeast BFY70	
Bottom fermenting yeast BFY84	
Top fermenting yeast TFY3	
Top fermenting yeast TFY23	
Trichosporon cutaneum WY54	
Candida intermedia WY55-1	
Debaryomyces hansenii WY69	
Pichia membranifaciens WY75	
Rhodotorula graminis WY93	
Dekkera bruxellensis WY96	
Dekkera bruxellensis WY97	
Dekkera bruxellensis WY98	
Saccharomyces cerevisiae WY101	
Saccharomyces cerevisiae var. *diastaticus* WY126	
Isolates from wines	
Zygosaccharomyces bailii WLY9	
Saccharomyces cerevisiae WLY10	
Pichia membranifaciens WLY13	
Lodderomyces elongisporus WLY14	
Aureobasidium pullulans WLY15	
Rhodosporidium fluviale WLY16	
Pichia anomala WLY17	
Pichia guilliermondii WLY18	

(T): type strain.