Vol. 20(5), pp.202-209, May, 2021
DOI: 10.5897/AJB2021.17333
Article Number: 4F2111066752
ISSN: 1684-5315

http://www.academicjournals.org/AJB

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African Journal of Biotechnology

Full Length Research Paper

A rapid detection method on-site for *Zygosaccharomyces* based on loop-mediated isothermal amplification (LAMP) combined with a lateral flow dipstick (LFD)

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Received 23 February, 2021; Accepted 22 April, 2021.

The *Zygosaccharomyces* is notorious for its remarkable spoilage characteristics. In the present study, the visual and rapid identification of the genus *Zygosaccharomyces* was performed by a loop-mediated isothermal amplification (LAMP) assay using specific primers in Mini Dry Bath within 30 min at 65°C followed by a lateral flow dipstick (LFD) detection. The sensitivity evaluation revealed LAMP-LFD assay with 1.0×10^1 copies/μL of *Zygosaccharomyces* DNA as its detection limit, which was the same as the methods of real-time quantitative PCR (qPCR) and conventional polymerase chain reaction (PCR). However, qPCR or PCR methods not only need to be performed in a specialist analytical laboratory with expensive equipment but also with risk of aerosol pollution. The LAMP-LFD assay had no cross-reactivity against 10 other yeast species and its specificity was 100%. A total of 25 Qiangli loquat Dew samples (17 bottles bulged and eight normal-appearing) were detected within 40 min with 100 and 92.0% accurately and specifically, when compared with the qPCR assay and the microbiology culture method, respectively. Therefore, the simple, fast, sensitive and low-cost LAMP-LFD assay is an effective and useful tool for the on-site identification of the genus *Zygosaccharomyces*.

Key words: *Zygosaccharomyces*, on-site detection, loop-mediated isothermal amplification (LAMP), lateral flow dipstick (LFD).

INTRODUCTION

Based on The Yeasts Fifth Edition (Fleet, 2011) and the latest studies, the genus *Zygosaccharomyces* comprises 14 species, which are *Zygosaccharomyces rouxii*, *Zygosaccharomyces bailii*, *Zygosaccharomyces pseudobailii*, *Zygosaccharomyces parabailii*, *Zygosaccharomyces bisporus*, *Zygosaccharomyces lentus*, *Zygosaccharomyces kombuchaensis*, *Zygosaccharomyces mellis*, *Zygosaccharomyces sapae*, *Zygosaccharomyces machadoi*, *Zygosaccharomyces siamensis*, *Zygosaccharomyces gambellarensis*, *Zygosaccharomyces favi*, and *Zygosaccharomyces seidelii* (Hulin and Wheals, 2014; Brysch-Herzberg et al., 2020). To our knowledge, the spoilage potential of the genus *Zygosaccharomyces* has been widely recognized

within the food industry (James and Stratford, 2011; Hulin and Wheals, 2014). As fermenting microbes, and although the *Zygosaccharomyces* species consequent on products spoilage vary with the characteristics and product type involved (Harrison et al., 2011), the ability to survive under various stresses, such as resistance to extreme osmotolerance and high-level weak acid preservatives, make these problematic in food or medicine industry (Fugelsang and Edwards, 2007; Čadež et al., 2015; Wang et al., 2015; Karaman and Sagdic, 2019). In addition, the ability to ferment sugars to ethanol and CO_2 might cause containers, cans and glass bottles to deform, and even explode (Tournas et al., 2006; Frisón et al., 2015; Wang et al., 2017). Thus, an effective method is needed to promptly detect the genus *Zygosaccharomyces* for its effective control, thereby reducing potentially large economic losses, and protecting consumer safety.

Various detecting approaches for *Zygosaccharomyces* spp. have been developed (Rawsthorne and Phister, 2006; Harrison et al., 2011; Hulin and Wheals, 2014; Wang et al., 2016; Wang and Sun, 2019). As a standard method, the culture-dependent technique has several shortcomings, for example, it was a time-consuming process with poor repeatability and qualitative inaccuracy (Duarte et al., 2004; National Food Safety Standard of the People's Republic of China, GB 4789.15-2016). Molecular methods, such as PCR and qPCR assays, have been widely used with high sensitivity, specificity and accuracy (Casey and Dobson, 2004; Rawsthorne and Phister, 2006; Andorrà et al., 2010), and industry standards related to *Zygosaccharomyces* spp. have been issued (General Administration of Quality Supervision, Inspection and Quarantine of the People's Republic of China (AQSIQ), SN/T 4675.30-2017; SN/T 4780-2017). However, these involve steps with temperature increments and decrements, the normal presence of inhibitors, and the requirement of specialized laboratories, expensive equipment and highly trained laboratory staff (Rawsthorne and Phister, 2006; Andorrà et al., 2010; Zuehlke et al., 2013; Hulin and Wheals, 2014). Therefore, the establishment of a cost-effective, simple, fast and sensitive assay at the field level has become increasingly important to realize the early and successful detection of *Zygosaccharomyces*.

Compared to PCR and qPCR, loop-mediated isothermal amplification (LAMP) assay is an efficient and novel amplification technology with great specific and highly sensitive based on isothermal conditions (Zhang et al., 2019). Furthermore, it is simpler to perform, and merely use constant-temperature equipment, such as a water bath or Mini Dry Bath within 60 min. This can be potentially used for tests in the field (Punati et al., 2019; Joon et al., 2019; Tumino et al., 2020). In addition, LAMP has been extensively applied in food-borne pathogens, allergens, and genetically modified organisms in food analysis (Hara-Kudo et al., 2010; Rostamkhani et al., 2011; Saharan et al., 2015; Shi et al., 2017; Yuan et al., 2018; Li et al., 2019; Zhuo et al., 2019; Tumino et al., 2020). Furthermore, LAMP products traditionally observed by gel electrophoresis and hydroxynaphthol blue (HNB) or SYBR are either difficult to distinguish by fluorescent dyes, which need special equipment and the process is time-consuming, or easily exposed to aerosol pollution due to the opening of the lid (Shi et al., 2017; Punati et al., 2019; Zhang et al., 2019). Moreover, the amplified products are visualized directly on an LFD with one or two bands, which is more suitable for on-site detection (Shi et al., 2017).

To our knowledge, the detection of the genus *Zygosaccharomyces* by LAMP assay has not been reported to date. For rapid qualitative test of the genus *Zygosaccharomyces*, an accurate, sensitive and visualized LAMP-LFD assay was developed in this study. All strains of DNA were analyzed using LAMP-LFD, PCR and qPCR for comparison of the sensitivity. To validate the effectiveness and robustness of the LAMP-LFD assay, the specificity and sensitivity were evaluated and the Qiangli loquat Dew samples were performed. That the LAMP-LFD assay is a simple, fast, sensitive and low-cost tool for the genus *Zygosaccharomyces* on-site detection.

MATERIALS AND METHODS

Strains, media and DNA extraction

Table 1 presents the yeast strains assessed in the present study. The strains were cultivated in Sabouraud Dextrose Agar (SDA) (Hope Bio-Technology Co., Ltd, Qingdao, China) at 28°C for 48 h. All DNAs of the strains or Qiangli loquat Dew samples were extracted using the DNeasy PowerSoil Kit (QIAGEN, Germany), according to the manufacturer's protocol. The DNA was eluted in 100 µL of elution buffe followed by storing at -20°C.

LAMP-LFD, PCR and qPCR assays

Primer is crucial for amplification efficiency of LAMP assay. Based on the the internal transcribed spacer (ITS) regions ITS1 and ITS2 flanking the 26S ribosomal genes of *Zygosaccharomyces* DNA sequences obtained from the NCBI GenBank, the primers (Table 2)

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#These authors contributed equally to the article.

Table 1. Yeast strains used in the present study.

Species	Culture collection[a]
Zygosaccharomyces parabailii	ATCC MYA-4549
Canidia albicans	CMCC(F) 98001
Candida parapsilosis	ATCC 22019
Rhodotorulamucilaginosa	ATCC 66034
Pichia guilliermondii	CGMCC 2.1862
Trichosporonasahii	CGMCC 2.319
Cryptococcus albida	CGMCC 2.5512
Pichia kudriavzevii	CICC 33192
Candida metapsilosis	CICC 31269
Saccharomyces cerevisiae	ATCC 9763
Candida glabrata	ATCC MYA-2950
Zygosaccharomyces bailii isolate[b]	-
Zygosaccharomyces mellis isolate[b]	-
Zygosaccharomyces parabailii isolate[b]	-

[a]ATCC: American type culture collection, America; CMCC: National center for medical culture collections, China; CGMCC: China General Microbiological Culture Collection Center; CICC: China Center of Industrial Culture Collection. [b]Isolates were isolated from bottle bulged Qiangli loquat Dew by Key Laboratory of Microbial Technology and Bioinformatics of Zhejiang Province, Hangzhou, China.

Table 2. Primer sequence information.

Type	Primer	Sequence (5'-3')
Outer primers	*Zyg*-F3	TGAAGCGGCAARAGCTCAA
	Zyg-B3	GAGCTGCATTCCCAAACAAC
Inner primers	*Zyg*-FIP	AAGSRCCAGCCCCAGARTCRAAATCTGGTACCTTCGGTGC
	Zyg-BIP	CCTTGGAACAGGACGTCATRGATCGACTCTTCGAARGCRCT
Loop primers	*Zyg*-LF	CYTTCTACAAATTACAACTCGG
	Zyg-LB	CCGTATGGCGAGGATCCC

were designed by using the Primer Explorer version 5 software and synthesized from Shanghai Generay Biotech Co., Ltd. LAMP-LFD assay was carried out in a 25 µL reaction mixture, containing 1×*Bst* DNA polymerase buffer (New England Biolabs Ltd., Ipswich, MA, USA), 1.6 µM of *Zyg*-FIP and *Zyg*-BIP, 4.0 µM of *Zyg*-LF and *Zyg*-LB, 2.0 µM of *Zyg*-F3 and *Zyg*-B3, 1.4 µM of dNTP (TaKaRa Biotechnology Co., Ltd., Dalian, China), 0.32 U/µL of *Bst* 2.0 DNA polymerase (New England Biolabs Ltd., Ipswich, MA, USA), 8 mM of $MgSO_4$, 1×SYBY Green I, 2.0 µL of template DNA and nuclease-free water. The whole amplification procedure was performed at 65°C for 30 min and 80°C for 5 min in the Mini Dry Bath (Hangzhou Yooning Instrument, China). The amplified products were tested using LFD devices (Ustar Biotech Co., Ltd., Hangzhou, China), with colored lines at both the "C" and "T" lines, which denote positive samples, while the ones that only presented a signal at "C" were negative samples.

The PCR assay was conducted in the ProFlex PCR System (Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 25 µL reaction system, which contained 2×Phanta® Max Master Mix (Vazyme biotech Co., Ltd., Nanjing, China), 0.2 µM of each primer: Forward primer (Zygogen F3) 5'-ACACACAGTGGAGTTTC-3', Reverse primer (Zygogen R1) 5'-CGGTAAAACCTAATACGAC-3' (Hulin and Wheals, 2014), 2.0 µL of template DNA and nuclease-free water. The reactions were run in triplicate for 5 min at 95°C, followed by 35 cycles for 30 s at 95, 55 and at 72°C, respectively. 3 µL of amplified products were analyzed by gel electrophoresis on 1.5% gel with 6×loading buffer and visualized on the Tanon-1600 Gel Image System (Tanon Science & Technology Co., Ltd., Shanghai, China). Furthermore, the qPCR reaction performed in the StepOne™ Plus Real-Time PCR System (Applied Biosystems, Inc., Carlsbad, CA, USA) containing 1×SYBR® Premix Ex*Taq*™ (Tli RNaseH Plus) (TaKaRa Biotechnology Co., Ltd., Dalian, China), 0.2 µM of Forward primer, 0.2 µM of Reverse primer (the same as the PCR assay previously), 2.0 µL of template DNA and nuclease-free water with running in triplicate for 30 s at 95°C, followed by 40 cycles at 95°C for 5 s, and at 60°C for 30 s as its amplification procedure.

Sensitivity and specificity evaluation of the LAMP-LFD assay

In order to determine the sensitivity of the LAMP-LFD assay, in terms of the detection limits compared with the PCR and qPCR assays, the amplification reactions were performed with the *Z. bailii* DNA. The DNA fragment was purified from the agarose gel using the AxyPre™ DNA Gel Extraction Kit (Axygen Scientific, USA), and cloned into the pMD™18-T vector using the *E.coli* DH5α competent

cell, followed by plasmid extraction. Each dry plasmid DNA was then suspended in nuclease-free water. The copies of gene were determined by multiplying the DNA concentration through Avogadro's number and divided by the product of the plasmid size (bp) and the average weight of the base pair (Ahmed et al., 2014). The 10-fold serial dilutions ranged from 10^6 to 10^0 gene copies per microliter of DNA were prepared with three replicates for the LAMP-LFD, PCR and qPCR assays. The limit of detection (LOD) was determined as the concentration of the lowest dilutions, in which all three replicates had positive signals.

In order to verify the LAMP-LFD specificity, the LAMP results were compared with the DNA of *Zygosaccharomyces* and 10 other yeasts: *Canidia albicans, Candida parapsilosis, Rhodotorula mucilaginosa, Pichia guilliermondii, Trichosporon asahii, Cryptococcus albida, Candida metapsilosis, Saccharomyces cerevisiae, Candida glabrata* and *Pichia kudriavzevii.* In particular, the LAMP assay was conducted at 65°C for 30 min, as previously described. All samples were determined in triplicate, and the experiment was performed three times.

Evaluation of the LAMP-LFD assay using Qiangli loquat Dew samples

In order to evaluate the efficiency and robustness of the LAMP-LFD assay, a total of 25 Qiangli loquat Dew samples were used in detecting *Zygosaccharomyces*, while 17 of the samples were bottle bulged Qiangli loquat Dew samples, and the other eight samples were normal-appearing Qiangli loquat Dew samples. The DNA extracted from the Qiangli loquat Dew samples were tested by the LAMP-LFD assay and compared with the qPCR with three replicates for each sample. Simultaneously, the microbiology culture method was also used in the trial. Strains collected from the 25 Qiangli loquat Dew samples were, respectively cultivated in SDA at 28°C for 48 h. Then, the DNAs of the strains were extracted and verified by sequencing the D1/D2 region of the 26S rRNA gene.

RESULTS AND DISCUSSION

Sensitivity of the LAMP-LFD, PCR and qPCR assays

To contrast between the LAMP, PCR and qPCR assays, the sensitivities of PCR and qPCR were tested with the same plasmid DNA (1.0×10^0 to 1.0×10^6 copies/μL). The LFD readouts with clear-colored line reaction at the control "C" and test "T" lines were positive (+), while those with only the control "C" were negative (-) (Figure 1a). In Table 3, the LFD readouts of the LAMP amplified products that corresponded to the different concentrations of the plasmid DNA are shown. The conventional PCR amplified products were detected by agarose gel electrophoresis with different diluents (Figure 1b). The cycle threshold (Ct) values were plotted against copies/μL to establish the sensitivity of the amplification in different diluents (Figure 1c). When the concentration of the plasmid DNA was 1.0×10^0 copies/μL, the LAMP-LFD assay displayed a signal only at "C". Furthermore, the PCR assay had no band, and qPCR assay (two in three parallel results) had no Ct values. These results revealed that the LAMP-LFD assay detection limits were as low as 1.0×10^1 copies/μL, which was consistent with both the PCR assay and qPCR assay (Table 3).

The low-copies result is not unexpected, because specific primers were the key players in these molecular methods. Although the detection of the genus *Zygosaccharomyces* by LAMP assay has not been reported to date, the sensitivity of the LAMP assay was in agreement with other microorganisms studies (Saharan et al., 2015; Yuan et al., 2018; Li et al., 2019; Wu et al., 2019; Zhang et al., 2019; Zhuo et al., 2019). Seveval PCR or qPCR methods have detected *Zygosaccharomyces* spp. in previous studies, while the limits of these molecular methods were not for comparison validly to the results in the present study because of the different unit (CFU/μL) (Rawsthorne and Phister, 2006; Jaramillo et al., 2015). Some studies have suggested that species or genus specific PCR primers can be used to identify the genus *Zygosaccharomyces* (Harrison et al., 2011; Hulin and Wheals, 2014), but missing LOD data. Moreover, there was very few paper detected *Z. rouxii* using the qPCR assay with LOD approximately 20 times more sensitive than the LAMP assay in the present study (Syromyatnikov et al., 2018). This may be attributed to the set of primers only applied to the specific *Zygosaccharomyces* spp.

However, the PCR assay and qPCR assay involved steps with temperature increments and decrements and required a specialized laboratory, an expensive PCR or qPCR system, UV illumination equipment, and highly trained laboratory staff (Rawsthorne and Phister, 2006; Andorrà et al., 2010; Zuehlke et al., 2013; Hulin and Wheals, 2014). In addition, the qPCR assay needs approximately 2 h to accomplish each DNA test, while the PCR assay needs approximately 3 h. Furthermore, the amplified products analyzed by gel electrophoresis require the opening of the lid of the tube, which could cause aerosols pollution (Shi et al., 2017; Zhang et al., 2019). Moreover, the LAMP-LFD assay for the detection of *Zygosaccharomyces* could be rapidly completed in the Mini Dry Bath within 30 min at 65°C and visualized on an LFD without opening the lid in the field level (Figure 1d).

The minimum equipment required to run the LAMP-LFD assay includes Eppendorf tubes with reagents, heat blocks, nuclease-free water, pipettes and tips and lateral flow dipsticks.

Specificity of the LAMP-LFD assay

A highly specific assay at the field level is important for the detection of the genus *Zygosaccharomyces*, which could avoid cross-reactions and false-positive data. The specificity of the LAMP-LFD assay was determined with the *Zygosaccharomyces* DNA and other yeasts DNA. Then, 10 ng each of the *Z. bailii, Z. parabailii*, *Z. mellis*, *C. albicans*, *C. parapsilosis, R. mucilaginosa*, *P. guilliermondii*, *T. asahii, C. albida*, *C. metapsilosis*, *S. cerevisiae*, *C. glabrata* and *P. kudriavzevii* DNA were used in the tests. The results revealed that the DNA from *Z. parabailii*, *Zygosaccharomyces mellis* and *Z. bailii*

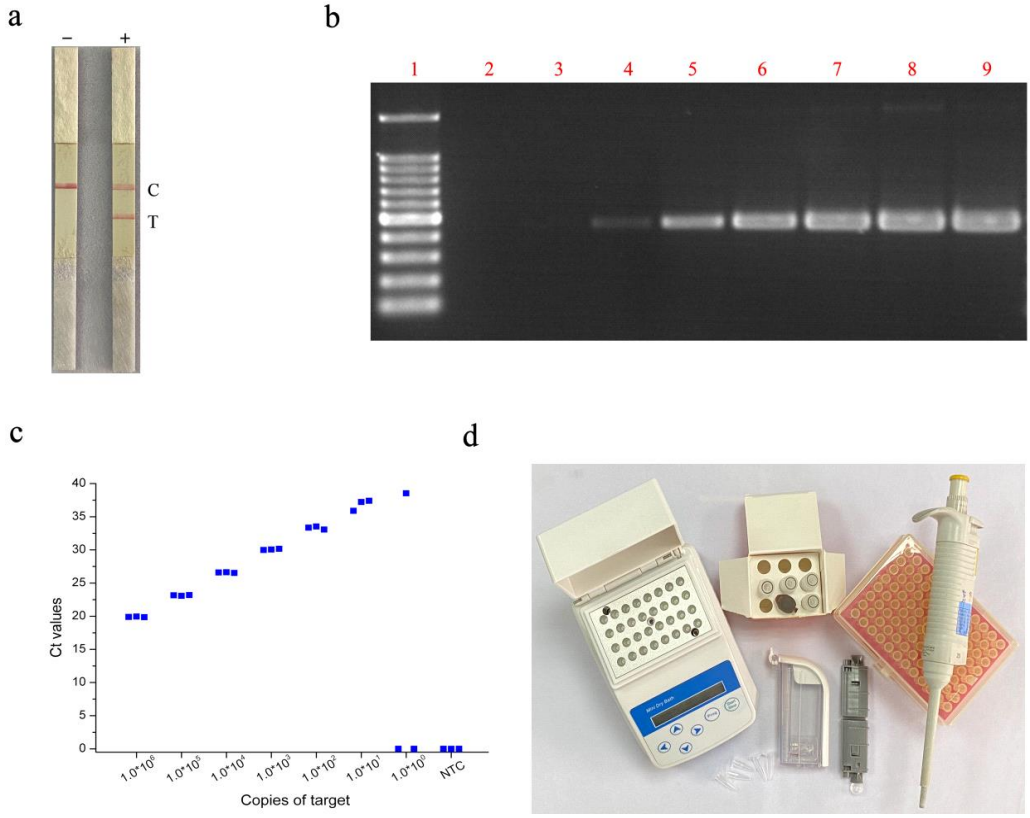

Figure 1. The sensitivity of the LAMP-LFD, PCR and qPCR assays for the detection of *Zygosaccharomyces*. a: Lateral flow dipstick readout. A positive result (+) with dual labels, both "C" and "T", negative result (-) with only "C". b: PCR assay; 1: 100 bp marker, 2: No template control, 3: 1.0×10^{0} copy/μL, 4: 1.0×10^{1} copies/μL, 5: 1.0×10^{2} copies/μL, 6: 1.0×10^{3} copies/μL, 7: 1.0×10^{4} copies/μL, 8: 1.0×10^{5} copies/μL, 9: 1.0×10^{6} copies/μL. c: The LOD of the qPCR assay (n=3). d: The minimum equipment needed for the LAMP-LFD assay.

Table 3. The LFD readouts of the LAMP amplified products in different concentrations.

Copies of target	LFD readouts
0	-
1.0×10^{0}	-
1.0×10^{1}	+
1.0×10^{2}	+
1.0×10^{3}	+
1.0×10^{4}	+
1.0×10^{5}	+
1.0×10^{6}	+

were detected as positive by the LAMP-LFD assay. The DNA samples that originated from the other 10 species of yeasts gave negative results. The data revealed no cross-reactions in the LAMP-LFD assay (Table 4). Therefore, the designed primers for the LAMP-LFD assay to detect *Zygosaccharomyces* had conservative property and specificity features. In other words, the specificity of the LAMP-LFD assay applied to the *Zygosaccharomyces* DNA was 100%. As expected, the results showed that the specificity of the primers play a crucial role in the LAMP-LFD assay, which was consistent with the specificity reported in other literatures (Wu et al., 2019; Zhuo et al., 2019).

The LAMP-LFD assay detection for Qiangli loquat Dew samples

In order to determine the efficacy and robustness of the LAMP-LFD assay for the detection of the genus *Zygosaccharomyces* in practical application, the DNA samples extracted from 25 Qiangli loquat Dew samples, respectively, were detected in contrast to the qPCR assay and microbiology culture method. As shown in Figure 2, the detected results for the 17 DNA samples (Nos. 1-4, 6, 8, 10-14, 17, 18, 20, 22, 23 and 25), which were obtained from the corresponding 17 bottles bulged Qiangli loquat Dew samples, were shown as positive (+) with blue color, and displayed two lines in the LFDs

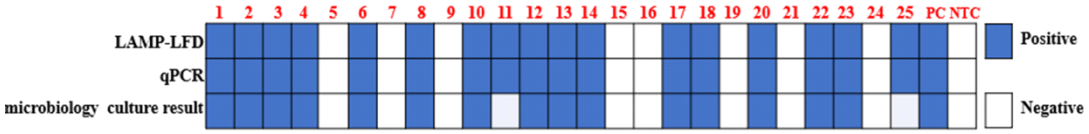

Figure 2. The LAMP-LFD, culture and qPCR assays for the detection of *Zygosaccharomyces* in the Qiangli loquat Dew samples. Nos. 1-4, 6, 8, 10~14, 17, 18, 20, 22, 23 and 25 were the bottles bulged Qiangli loquat Dew samples, while Nos. 5, 7, 9, 15, 16, 19, 21 and 24 were the normal-appearing Qiangli loquat Dew samples. PC: Positive control, NTC: No template control.

Table 4. Specificity test for the detection of *Zygosaccharomyces* by LAMP-LFD assay.

Species	LFD readout
Zygosaccharomyces parabailii ATCC MYA-4549(positive control)	+
Zygosaccharomyces parabailii isolate	+
Zygosaccharomyces mellis isolate	+
Zygosaccharomyces bailii isolate	+
Canidia albicans CMCC(F)98001	-
Candida parapsilosis ATCC 22019	-
Rhodotorula mucilaginosa ATCC 66034	-
Pichia guilliermondii CGMCC 2.1862	-
Trichosporon asahii CGMCC 2.319	-
Cryptococcus albida CGMCC 2.5512	-
Pichia kudriavzevii CICC 33192	-
Candida metapsilosis CICC 31269	-
Saccharomyces cerevisiae ATCC 9763	-
Candida glabrata ATCC MYA-2950	-
No template control	-

(Figure 1a). However, eight of these (Nos. 5, 7, 9, 15, 16, 19, 21 and 24) corresponded to the eight normal-appearing Qiangli loquat Dew samples, which displayed as negative (-), with the white color displaying only the "C" line (Figure 1a). It could also be observed that 17 qPCR-positive samples were positive and eight negative samples were negative in the LAMP-LFD assay (Figure 2). In another words, these LFD results were 100% consistent with the results of the qPCR assay. Nevertheless, all culture-positive samples (15 samples) were identified as *Z. parabailii* through the sequencing of the D1/D2 region of the 26S rRNA gene (data were not shown), and these were also positive in the LAMP-LFD and qPCR assays. In other words, compared to the microbiology culture result, the LAMP-LFD assay and qPCR for the detection of the genus *Zygosaccharomyces* in the Qiangli loquat Dew samples was 92.0% accurately and specifically, which is the gold standard method. Two samples (Nos. 11 and 25) with negative culture turned positive in the qPCR and LAMP-LFD assays. This was probably because the *Zygosaccharomyces* in these two samples were dead, and the LAMP-LFD and qPCR assays could not discriminate between live and dead cells (Cangelosi and Meschke, 2014).

Although there are no report related to direct identification of *Zygosaccharomyces* from Qiangli loquat Dew samples by using LAMP (Rawsthorne and Phister, 2006; Jaramillo et al., 2015; Wang et al., 2017), the LAMP-LFD was 100 and 92.0% accurately and specifically, when compared with the qPCR assay and the microbiology culture method, respectively in this study. But the qPCR assay takes approximately 2 h to accomplish each test, while the microbiology culture takes much more time. These indicate that the LAMP-LFD assay is an effective tool for the identification of the genus *Zygosaccharomyces* at the field level.

Conclusion

The present study is the first to develop the LAMP-LFD assay for the detection of the genus *Zygosaccharomyces*. The detection limit of the LAMP-LFD assay was the1.0×10^{1} copies/μL and 100% specificity to *Zygosaccharomyces* DNA. In addition, the LAMP-LFD assay can be performed under an isothermal condition without sophisticated equipment in only 40 min (in the present study), and the amplified products can be

directly and visually detected. Furthermore, the results could be easily understood by non-technical people. Based on the high specificity and sensitivity of the LAMP-LFD assay, the investigators detected 25 Qiangli loquat Dew samples with 100% and 92.0% positive agreement, respectively, and negative samples with no positive signal when compared with the qPCR assay and microbiology culture method. In conclusion, the LAMP-LFD assay is simple, fast, sensitive and low-cost, and is an effective tool for the identification of the genus *Zygosaccharomyces* at the field level.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

This work was supported by the Science and Technology Department of Zhejiang Province, China (LGN20C050001).

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