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Development and evaluation of an immunomagnetic separation–ELISA for the detection of *Alicyclobacillus* spp. in apple juice

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ABSTRACT

The immunomagnetic separation (IMS) technique was used in combination with an enzyme-linked immunosorbent assay (ELISA) procedure to shorten the total analysis time and improve the sensitivity for the detection of *Alicyclobacillus* spp. in apple juice samples. The specificity of IMS–ELISA for twenty strains of *Alicyclobacillus* spp. and eighteen strains of non-*Alicyclobacillus* spp. was determined and there was little cross-reaction with non-*Alicyclobacillus* strains. Artificially contaminated apple juice with different concentrations of *Alicyclobacillus acidoterrestris* was detected by IMS–ELISA, and the detection limit of the assay in apple juice was 10^3 CFU/mL. Furthermore, the sample inoculated with 1 CFU/mL of *A. acidoterrestris* could be detected as positive after incubation for 24 h. The IMS–ELISA described, allows for the identification of suspect positive samples within 3 h of testing versus 3–5 days required by standard culture methods while significantly reducing the materials and labor required for the detection of *Alicyclobacillus* spp. in apple juice samples. As compared with the standard culture method performed concurrently on the same set of samples, the sensitivity, specificity and accuracy of IMS–ELISA for 102 naturally contaminated apple juice samples were 91.3%, 96.02% and 95.09%, respectively. These results demonstrated that the newly proposed IMS–ELISA procedure can be a potentially useful analytical method for the detection of *Alicyclobacillus* spp. in apple juice.

1. Introduction

Alicyclobacillus spp. are gram-positive, rod-shaped, nonpathogenic, spore-forming organisms and can grow in highly acidic environments of 2.5–6.0 at temperatures of 25–60 °C (Lee et al., 2004; Wang et al., 2012). For the spores are able to germinate and multiply in acidic beverages, *Alicyclobacillus* spp. can cause spoilage in pasteurized and heat-treated apple juice (Lee et al., 2002; Li et al., 2013). The formation of guaiacol and halophenols in the final product after bottling result in a decline in the quality of juices (Walker and Phillips, 2007). Therefore, *Alicyclobacillus* spp. has attracted much attention of governments, juice manufacturers and researchers. The development of rapid and accurate detection methods to detect and identify *Alicyclobacillus* spp. in acidic food products has become a focus of investigation (Smit et al., 2011; Steyn et al., 2011).

Currently, the existing methods for detection of *Alicyclobacillus* spp. have some limitations. The conventional detection methods based on physiological and nutritional characteristics are laborious and time-consuming, which often take up to 3–5 days to identify the target bacteria (Chang and Kang, 2005). The approaches such as polymerase chain reaction (PCR), Fourier transform infrared (FT-IR), electronic nose or 16S rDNA gene sequence analysis are still not widely applied for the detection of *Alicyclobacillus* spp. in practical samples due to the complicatedness of sample preparation, high equipment costs and staff skill requirements (Al-Qadiri et al., 2006; Concina et al., 2010; Connor et al., 2005; Wang et al., 2011). Furthermore, the target organisms are generally present in very low numbers. The complex interference of the background food components and the non-target flora make it a very difficult task to separate and concentrate the desired organisms.

At present, there has been a lot of interest in the development of separation and concentration techniques prior to detection. Several techniques such as centrifugation and membrane filtration have been utilized for this purpose (Bahceci et al., 2003; Grasso et al., 2009; Lee et al., 2007; Luo et al., 2004). However, with the advantages of reducing the total analysis time and improving the sensitivity of detection, immunomagnetic separation (IMS) technique has been evaluated as the most successful approach for the isolation and enrichment of target organisms (Španová et al., 2003; Wadud et al., 2010). In this process, bacterial cells bound to magnetic beads by specific antibodies can be separated from the background microflora in a magnetic field. If required, the isolated bacteria-beads complexes can be re-suspended in an enrichment broth so that cell numbers could increase rapidly to improve the sensitivity of detection assays (Jeníková et al., 2010; Malkova et al., 1998). The combination of IMS with detection methods such as direct plating, PCR and enzyme-linked immunosorbent assay (ELISA) has been widely applied in the

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enrichment and detection of *Salmonella*, *Escherichia coli*, *Staphylococcus aureus* and other pathogenic bacteria in food (Bennett et al., 2008; Malkova et al., 1998; Payne et al., 1992; Yazdankhah et al., 1999).

In this study, a proposed IMS–ELISA procedure was established for the detection of *Alicyclobacillus* spp. in apple juice. The detection limit and specificity of this method were evaluated. The sensitivity and accuracy of IMS–ELISA were compared with that of the standard culture method (K medium). This work is believed to be of both academic interest and practical importance for monitoring *Alicyclobacillus* spp. in apple juice and other beverage industry.

2. Materials and methods

2.1. Materials

The source and the enrichment medium of each strain were listed in Table 1. Twenty strains including *Alicyclobacillus* spp. and *Bacillus* spp. were purchased from German Resource Centre for Biological Material (DSMZ). Eight wild type isolates were obtained from microbiology laboratory of the College of Food Science and Engineering, Northwest A&F University (NWSUAF) (Wang et al., 2010). Three strains of *Alicyclobacillus acidoterrestris* (*A. acidoterrestris*) were generously provided by Keiichi Goto, Food Research Laboratories, Mitsui Norin Co., Ltd., Tokyo, Japan (AAT). Six strains including *Saccharomyces cerevisiae*, *Bacillus subtilis*, *Bifidobacterium bifidum*, *Enterococcus faecium*, and *Lactobacillus rhamnosus* were purchased from China Center of Industrial Culture Collection (CICC). *S. cerevisiae* was purchased from Institute of Microbiology, Chinese Academy of Sciences (IMCAS). All of the strains were preserved at −40 °C in 30% glycerol or on the slants at 4 °C for routine use.

Bovine serum albumin (BSA), Goat anti-rabbit IgG-horseradish peroxidase conjugate (GAR-HRP) and 3,3′5,5′-Tetramethyl benzidine (TMB) were purchased from Sigma-Aldrich Company (St. Louis, MO, USA). Tween-20, hydrogen peroxide and commonly used reagents were purchased from local suppliers at high purity. Immunomagnetic nanoparticles (IMPs) were prepared by the "oriented" immobilization of specific polyclonal anti-*Alicyclobacillus* IgG antibody onto the surface of silica-coated magnetic Fe_3O_4 nanoparticles (Fig. 1).

2.2. IMS–ELISA procedure

2.2.1. Immunomagnetic separation of bacteria

IMPs, pre-coated with specific polyclonal anti-*Alicyclobacillus* IgG antibody, were used for separation and enrichment of target bacteria. Five milligrams of IMPs and 2 mL of each sample were added into different tubes followed by cultivation at 37 °C for 30 min under stirring (150 rpm). IMPs were separated by magnetic decantation, washed three times with 0.01 mol/L sodium phosphate buffer (PBS, pH 7.4) and resuspended in 200 μL PBS for ELISA detection.

2.2.2. ELISA procedure

ELISA detection procedure was carried out in 96-well polystyrene micro-plates (Nunc, Roskilde, Denmark). ELISA plates were coated at 37 °C for 1 h with the specific anti-*Alicyclobacillus* IgG antibody diluted to 1 μg/mL in PBS plus 0.1% BSA (v/v). After incubation at 37 °C for 1 h, the solution was removed and the plates were washed three times with PBS plus 0.05% Tween-20 (PBS-T). The unbound active sites were saturated by PBS plus 0.5% BSA (v/v) and incubated at 37 °C for 2 h, and then the plates were washed three times. After removing residual buffer by tapping the plates on absorbent paper, the prepared plates were stored at −20 °C.

The prepared cell samples (either cell suspensions of bacteria to determine ELISA response, or cell suspensions following IMS), diluted as desired in PBS, were incubated in the plates (100 μL per well) at 37 °C for 1 h. After washing, the amount of immunocomplexes was quantified by the addition (150 μL per well) of GAR-HRP (diluted 1/2000, in PBS plus 0.1% BSA), and the plates were incubated at 37 °C for 1 h. The presence of enzyme-labeled antibody was detected by the use of substrates that contains TMB as chromogen. After color development at 37 °C for 15 min, the enzyme reaction was stopped by the addition (50 μL per well) of the halting solution (2 mol/L H_2SO_4). Optical density (OD) was read at 450 nm and 630 nm using a multi-scan iMark micro-plate spectrophotometer (Bio-RAD, USA).

All experiments were performed repeatedly and the data were presented as means ($n = 8$) ± Standard Deviation (S.D.). Data were subjected to One-way analysis of variance (ANOVA) using the Statistical Analysis System (SAS version 9.1, SAS Institute Inc., Cary,

Table 1
List of strains and the specificity testing of them using IMS–ELISA.

Strain	Species	Medium	Temperature	OD value	Strain	Species	Medium	Temperature	OD value
446	*A. acidocaldariuss*[a]	402[A]	60 °C	1.822	18134	*B. ginsengihumi*[a]	830[D]	30 °C	0.654
448	*A. acidocaldarius*[a]	402	60 °C	1.961	13	*A. acidoterrestris*[b]	402	45 °C	1.942
449	*A. acidocaldarius*[a]	402	60 °C	1.893	95	*A. acidoterrestris*[b]	402	45 °C	2.286
451	*A. acidocaldarius*[a]	402	60 °C	1.654	96	*A.acidoterrestris*[b]	402	45 °C	2.176
2498	*A. acidoterrestris*[a]	402	45 °C	2.191	YL-5	*A. contaminans*[c]	402	45 °C	2.136
3922	*A. acidoterrestris*[a]	402	45 °C	1.972	BS-1	*B. subtilis*[c]	402	45 °C	0.616
3923	*A. acidoterrestris*[a]	402	45 °C	2.102	YL-3	*B. subtilis*[c]	402	45 °C	0.586
3924	3924 *A. acidoterrestris*[a]	402	45 °C	2.369	BS-5	*B. subtilis*[c]	402	45 °C	0.742
4006	*A. cycloheptanicus*[a]	402	45 °C	1.859	LC-3	*B. subtilis*[c]	402	45 °C	0.549
12489	*A. hesperidum*[a]	402	50 °C	2.087	LC-4	*B. subtilis*[c]	402	45 °C	0.618
13609	13609 *A. herbarius*[a]	13[B]	55 °C	1.756	LC-8	*B. ginsengihumi*[c]	402	45 °C	0.577
14558	*A. acidiphilus*[a]	402	45 °C	1.869	BS-2	*B. ginsengihumi*[c]	402	45 °C	0.628
14955	*A. pomorum*[a]	402	45 °C	1.817	1027	*S. cerevisiae*[d]	402	30 °C	0.672
17614	*A. sendaiensis*[a]	402	50 °C	1.926	10034	*B. subtilis*[d]	402	30 °C	0.489
17975	*A. contaminans*[a]	13	55 °C	2.136	6169	*B. bifidum*[d]	MRS[E]	37	0.517
17978	*A. fastidiosus*[a]	13	45 °C	1.987	20420	*E. faecium*[d]	MRS	37	0.708
10	*B. subtilis*[a]	1[C]	30 °C	0.686	20975	*L. rhamnosus*[d]	MRS	37	0.612
30	30 *B. brevis*[a]	1	30 °C	0.713	23139	*L. rhamnosus*[d]	MRS	37	0.598
32	*B. megaterium* [a]	1	30 °C	0.598	1605	*S. cerevisiae*[e]	402	30	0.705

Various superscripts in lowercase indicate the sources of strains. [a]DSMZ: German Resource Centre for Biological Material. [b]AAT: Food Research Laboratories, Mitsui Norin Co., Ltd., Tokyo, Japan. [c]NWSUAF: College of Food Science and Engineering, Northwest A&F University. [d]CICC: China Center of Industrial Culture Collection. [e]IMCAS: Institute of Microbiology, Chinese Academy of Sciences.

Various superscripts in uppercase indicate the enrichment medium of strains. [A]402 medium: 0.2 g ammonia sulfate, 0.25 g calcium chloride, 0.5 g magnesium sulfate, 2.0 g yeast extract, 5.0 g glucose, and 3.0 g monopotassium phosphate per liter of deionized water (pH 4.0). [B]13 medium: 2.0 g yeast extract, 0.2 g ammonia sulfate, 0.5 g magnesium sulfate, 0.25 g calcium chloride, 0.6 g monopotassium phosphate, 1.0 g glucose, 0.01 g manganese sulfate per liter of deionized water (pH 3.0–4.0). [C]1 medium: 5.0 g peptone, 3.0 g meat extract per liter of deionized water (pH 7.0). [D]0.50 g yeast extract, 0.50 g proteose peptone, 0.50 g casamino acids, 0.50 g glucose, 0.50 g soluble starch, 0.30 g Na-pyruvate, 0.05 g magnesium sulfate, 0.30 g monopotassium phosphate per liter of deionized water (pH 7.2). [E]MRS: De Man–Rogosa–Sharpe broth, which were obtained from Oxoid (Basingstoke, England).