International Journal of Food Microbiology 146 (2011) 63–68

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Isolation of *Alicyclobacillus* and the influence of different growth parameters

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ARTICLE INFO

Article history:
Received 26 August 2010
Received in revised form 25 January 2011
Accepted 3 February 2011

Keywords:
Alicyclobacillus
Endospores
Isolation
Temperature
pH

ABSTRACT

Alicyclobacillus species are thermo-acidophilic, endospore-forming bacteria that are able to survive pasteurisation and have been implicated in a number of spoilage incidents involving acidic foods and beverages. The aim of this study was to compare three isolation methods used for the detection of *Alicyclobacillus acidoterrestris* and to investigate the influence of incubation temperature on the growth of *A. acidoterrestris* and *A. acidocaldarius*. Peach juice samples inoculated with *A. acidoterrestris* K47 were analysed using either the International Federation of Fruit Juice Producers (IFU) Method No. 12 (Method A), which involved spread plating onto *Bacillus acidoterrestris* (BAT) agar at pH 4.0; Method B, which involved pour plating using potato dextrose agar (PDA) at pH 3.7; or Method C, which made use of membrane filtration followed by incubation on K agar at pH 3.7. The performance of the three methods differed significantly, with the IFU Method No. 12 recovering the highest percentage of cells at 75.97%, followed by Method B at 66.79% and Method C at 3.43%. These findings strengthen the proposal of the IFU for the use of the IFU Method No. 12 as a standard international method for the detection of *Alicyclobacillus*. To investigate the effect on growth of different incubation temperatures *A. acidoterrestris* (three strains) and *A. acidocaldarius* (two strains) were incubated at either 45 °C or 25 °C. Growth at 25 °C was slower and maximum cell concentrations were lower (1×10^5–10^6 cfu/mL compared to 1×10^7–10^8 cfu/mL) than at 45 °C for *A. acidoterrestris*. *A. acidocaldarius* was unable to grow at 25 °C and cell concentrations decreased by 1–2 logs. Since a growth temperature of 25 °C could not inhibit growth of *A. acidoterrestris*, cooling to room temperature (20°–25 °C) is not an effective control measure for *A. acidoterrestris* inhibition.

1. Introduction

Species of *Alicyclobacillus* are thermo-acidophilic, endospore-forming, non-pathogenic bacteria that pose a problem to the food industry as they are able to survive pasteurisation temperatures (Eiroa et al., 1999; Vieira et al., 2002). These bacteria can multiply in acidic products such as fruit juice to cell concentrations high enough to produce off-flavour and odour taints, leading to product spoilage (Gocmen et al., 2005; Pettipher et al., 1997).

Alicyclobacillus has been isolated from a variety of environments, including thermal acid environments such as hot springs (Wisotzkey et al., 1992), various soils (Goto et al., 2007; Groenewald et al., 2008) and acidic food- and beverage products such as fruit concentrate and fruit juice (Matsubara et al., 2002; Splittstoesser et al., 1994), iced tea (Duong and Jensen, 2000) and canned diced tomatoes (Walls and Chuyate, 1998). A number of agar media and growth conditions for the isolation of species of *Alicyclobacillus* have been proposed, developed and compared (Murray et al., 2007), but no standard method has thus far been accepted.

The IFU Method No. 12 for the isolation of *Alicyclobacillus* was developed in 2004, revised in 2007 and proposed as a standard method for the isolation of *Alicyclobacillus* (IFU, 2007). However, the PDA method (McIntyre et al., 1995; Splittstoesser et al., 1994; Walls and Chuyate, 1998; Witthuhn et al., 2007) and K agar method (Walls and Chuyate, 1998) have also been used successfully.

The growth temperature range of *Alicyclobacillus* is between 35° and 65 °C (Karavaiko et al., 2005; Wisotzkey et al., 1992), and the optimum pH range 1.50–5.50 (Goto et al., 2007; Walls and Chuyate, 1998). Most researchers use incubation temperatures between 40 °C and 50 °C and growth media or agar is normally acidified to pH 3.5–4.0 (Chang and Kang, 2005; Goto et al., 2002a). Some studies have found that *A. acidoterrestris* is not able to grow at 25 °C (Bahçeci et al., 2005), while others found that growth is only inhibited at temperatures below 20 °C (Jensen and Whitfield, 2003). This has led to suggestions that cooling of products below 20 °C, although it represents an additional cost factor, could be used as a control measure against spoilage (Jensen and Whitfield, 2003).

The aim of this study was to compare three *Alicyclobacillus* isolation methods and to characterise the growth patterns of

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doi:10.1016/j.ijfoodmicro.2011.02.002

Alicyclobacillus isolated from the South African fruit processing environment under different conditions.

2. Materials and methods

2.1. Bacterial strains

Alicyclobacillus acidoterrestris K47 (Department of Food Science Culture Collection, Stellenbosch University) was isolated from white grape juice concentrate obtained from a manufacturer in South Africa (Witthuhn et al., 2007). *Alicyclobacillus acidoterrestris* FB2 and FB38, as well as *A. acidocaldarius* FB19, were isolated from fruit concentrate, wash water, condensate water, flume water and vinegar flies, respectively, at a fruit processing plant in the Western Cape province of South Africa (Groenewald et al., 2009). The type strains *A. acidoterrestris* DSM 3922^T and *A. acidocaldarius* DSM 446^T were also included in this study.

2.2. Comparison of three isolation methods

Three isolation methods primarily used by South African fruit processors were compared with regards to their efficacy in promoting only the growth of *A. acidoterrestris* from diluted peach juice concentrate (1:10).

2.2.1. Bacterial inoculation culture

An isolate of *A. acidoterrestris* K47 was inoculated into yeast starch glucose (YSG) (Goto et al., 2002b) broth (2 g/L yeast extract, 1 g/L glucose and 2 g/L soluble starch) acidified to pH 4.0 with 1 M H_2SO_4, and incubated at 45 °C for 5 days.

A volume of 100 μL of this 5 day old culture was spread onto BAT agar plates (IFU, 2007) and incubated at 45 °C for 4 days. BAT agar was prepared by mixing equal volumes of BAT broth and a 3–4% (m/v) agar solution after autoclaving. BAT broth consists of 250 mg/L $CaCl_2 \cdot H_2O$, 500 mg/L $MgSO_4 \cdot 7H_2O$, 200 mg/L $(NH_4)_2SO_4$, 3 g/L KH_2PO_4, 2 g/L yeast extract, 5 g/L glucose and 1 mL trace element solution, consisting of 660 mg/L $CaCl_2 \cdot H_2O$, 180 mg/L $ZnSO_4 \cdot 7H_2O$, 160 mg/L $CuSO_4 \cdot 5H_2O$, 150 mg/L $MnSO_4 \cdot H_2O$, 180 mg/L $CoCl_2 \cdot 5H_2O$, 100 mg/L H_3BO_3 and 300 mg/L $Na_2MoO_4 \cdot 2H_2O$. The trace element solution was prepared separately and sterilised by autoclaving. The broth was adjusted to pH 4.0 using 1 M H_2SO_4 and sterilised by autoclaving.

Colonies from the BAT agar plates were streaked onto fresh BAT agar plates, followed by incubation at 45 °C for 4 days, to ensure a pure culture. BAT broth at pH 4.0 was then inoculated with a single colony from one of the BAT agar plates and incubated at 45 °C for 5 days.

A volume of 30 mL of the inoculated broth was centrifuged in a Beckman Coulter TJ-25 centrifuge (Beckman Coulter Inc., Fullerton, California, USA) at 5 500 *g* for 6 min. The supernatant was discarded and the pellet resuspended in 30 mL saline (0.85% (m/v) NaCl). This process was repeated and after resuspension of the pellet in saline the optical density (OD) of the culture was measured at 540 nm using a Beckman Coulter DU 530 Life science UV/Vis spectrophotometer (Beckman Instruments Inc., Fullerton, California, USA). All samples were enumerated on PDA agar, pH 4.0, with incubation at 45 °C for 4 days. The approximate cell concentration was determined using a standard curve. All chemicals and media were supplied by Merck.

2.2.2. Method comparison

Volumes of 10 mL peach concentrate were diluted 1:10 in saline and placed in a water bath at 80 °C. Once the samples had reached a temperature of 80 °C they were inoculated with approximately 1×10^6 cfu/mL *A. acidoterrestris* K47 cells. The samples were heat shocked at 80 °C for 10 min, followed by cooling on ice. Subsequently, one of the following three procedures was carried out. All experiments were done in triplicate.

2.2.2.1. Method A — IFU Method No. 12 (IFU, 2007). The samples were serially diluted in saline and 100 μL of each sample was spread, in duplicate, onto BAT agar plates (pH 4.0), followed by incubation at 45 °C for 5 days.

2.2.2.2. Method B. Fifty millilitres of 1.5× PDA was prepared and aseptically acidified after autoclaving to pH 3.7 using 9.50% (m/v) tartaric acid. The heat shocked samples were serially diluted, in duplicate, in saline to final volumes of 100 mL and 50 mL PDA was added to each 100 mL dilution. The samples were mixed and divided into four to five petri-dishes using the pour plate technique. The plates were incubated at 46 °C for 4 days.

2.2.2.3. Method C. The samples were serially diluted in saline, in duplicate, to final volumes of 100 mL and each dilution was vacuum filtered through a 0.45 μm membrane filter (S-Pak, Millipore, USA). Each filter was placed onto a K agar plate (Walls and Chuyate, 1998) (2.5 g/L yeast extract, 5 g/L peptone, 15 g/L agar, 1 g/L glucose and 1 mL Tween 80, acidified to pH 3.7 using 10 mL 12.5% (m/v) filter sterilised malic acid). The filter was tapped several times to remove air bubbles so as to ensure contact with the medium. The plates were incubated at 40 °C for 5 days.

2.3. Effect of temperature on growth and endospore development

2.3.1. Bacterial cultures

Three hundred millilitres of BAT broth was inoculated with one of either *A. acidoterrestris* DSM 3922^T, FB2, FB38, *A. acidocaldarius* DSM 446^T or FB19 to a final cell concentration of 1×10^1–10^3 cfu/mL. Cultures of each strain were incubated at both 25 °C and 45 °C for 6 days and samples were analysed every 24 h. The vegetative cell concentration, endospore concentration and pH of every sample were measured as described. Uninoculated BAT broth incubated at both 25 °C and 45 °C served as controls. The experiment was performed in triplicate.

2.3.2. Growth parameters

2.3.2.1. Vegetative cell concentration and endospore concentration. The cell concentration was determined by serially diluting the sample in saline and using the pour plate technique with PDA adjusted to pH 4.0 after autoclaving with 1 M H_2SO_4. Duplicate plates were prepared for each sample and incubated at 45 °C for 4 days. The rest of the sample was subjected to a heat shock treatment at 80 °C for 10 min. The endospore concentration after heat shock was determined in the same way as the vegetative cell concentration before heat shock.

2.3.2.2. pH. Four millilitres of the sample was used to determine the pH using a Mettler Toledo 320 pH metre (Mettler–Toledo Ltd., Leicester, England). All chemicals were provided by Merck.

2.4. Statistical analysis

Differences in percentage recovery between the three different methods were tested using one-way analysis of variance (ANOVA). Effects of temperature on vegetative growth and endospore development were analysed using mixed model repeated measures ANOVA. All analyses were performed using Statistica 8. A 5% significance level was used as guideline for indicating significant results.