Journal of Microbiological Methods 92 (2013) 1–8

Contents lists available at SciVerse ScienceDirect

Journal of Microbiological Methods

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

Optimisation of a direct plating method for the detection and enumeration of *Alicyclobacillus acidoterrestris* spores

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ARTICLE INFO

Article history:
Received 9 August 2012
Received in revised form 16 October 2012
Accepted 16 October 2012
Available online 23 October 2012

Keywords:
Alicyclobacillus acidoterrestris
Detection method
Growth medium
Membrane filtration
Spores

ABSTRACT

A direct plating method for the detection and enumeration of *Alicyclobacillus acidoterrestris* spores has been optimised. The results of the application of four types of growth media (BAT agar, YSG agar, K agar and SK agar) regarding the recovery and enumeration of *A. acidoterrestris* spores were compared. The influence of the type of applied growth medium, heat shock conditions, incubation temperature, incubation time, plating technique and the presence of apple juice in the sample on the accuracy of the detection and enumeration of *A. acidoterrestris* spores was investigated. Among the investigated media, YSG agar was the most sensitive medium, and its application resulted in the highest recovery of *A. acidoterrestris* spores, while K agar and BAT agar were the least suitable media. The effect of the heat shock time on the recovery of spores was negligible. When there was a low concentration of spores in a sample, the membrane filtration method was superior to the spread plating method. The obtained results show that heat shock carried out at 80 °C for 10 min and plating samples in combination with membrane filtration on YSG agar, followed by incubation at 46 °C for 3 days provided the optimal conditions for the detection and enumeration of *A. acidoterrestris* spores. Application of the presented method allows highly efficient, fast and sensitive identification and enumeration of *A. acidoterrestris* spores in food products. This methodology will be useful for the fruit juice industry for identifying products contaminated with *A. acidoterrestris* spores, and its practical application may prevent economic losses for manufacturers.

1. Introduction

The presence of *Alicyclobacillus acidoterrestris* spores in fruit juices and fruit juice concentrates is a significant problem for the fruit industry. These Gram-positive, thermoacidophilic, spore-forming, non-pathogenic bacteria cause flat-sour type spoilage in commercially pasteurised fruit juices, which is attributed to the production of offensive-smelling metabolites. Spoilage caused by *A. acidoterrestris* is difficult to detect visually. The spoiled fruit juice appears normal, or might exhibit a light sediment, but no gas formation. The pasteurisation process can destroy vegetative forms of microorganisms, though it is practically impossible to eliminate thermally resistant spores. Undesirable changes in the quality of contaminated products make them deficient and undrinkable, which results in a decrease of their market value and, thus, substantial economic losses (Chang and Kang, 2004). Therefore, this microorganism is currently considered to represent a major challenge for the fruit juice industry. Recent studies have indicated that other *Alicyclobacillus* species are also able to cause spoilage in food products. For example, *Alicyclobacillus acidocaldarius*, *Alicyclobacillus pomorum*, *Alicyclobacillus herbarius*, and *Alicyclobacillus acidophilus* have been isolated from contaminated products and have been found to have the ability to produce off-flavours in fruit juices (Cerny et al., 1984; Goto et al., 2002, 2003; Matsubara et al., 2002). Nevertheless, *A. acidoterrestris* is the most important microorganism associated with spoilage within the *Alicyclobacillus* genus. It is commonly believed that *A. acidoterrestris* is the principle cause of most spoilage problems, so the majority of studies on *Alicyclobacillus* spp. related spoilage are focused on *A. acidoterrestris* (Chang and Kang, 2004; Spinelli et al., 2009). Thus, *A. acidoterrestris* is considered to represent important target microorganisms in the quality control of apple juice products. Therefore, the development of a sensitive, selective, repeatable and reproducible method for the detection and enumeration of *A. acidoterrestris* spores is of high importance. In the food industry, rapid, simple and cost-effective methods are necessary to take precautions against possible microbiological food safety risks. In practice, culture-based methods are mainly used for routine analysis of fruit juice products because these techniques are easy to perform and reliable. Alternative detection methods, such as Fourier transform infrared (FT-IR) absorbance spectroscopy, flow cytometry and polymerase chain reaction (PCR)-based methods are expensive and complicated, making them useless for industrial application. Moreover, the detection method should allow accurate evaluation of the methods used for the elimination of *A. acidoterrestris* spores in liquid media.

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http://dx.doi.org/10.1016/j.mimet.2012.10.007

Various isolation media and growth conditions for the isolation of *Alicyclobacillus* spp. have previously been proposed and compared. Nevertheless, a standard detection method has not yet been formulated. The recommendations regarding spore identification methods, such as the appropriate type of growth media, pH value, heat shock conditions, plating method and incubation temperature and time, are different among different countries and groups representing the fruit juice industry (Murray et al., 2007).

The International Federation of Fruit Juice Producers (IFU) proposed a standard method for the detection of *Alicyclobacillus* in fruit juices in 2004 and revised it in 2007 (IFU Method No. 12, entitled "Method on the Detection of Taint Producing *Alicyclobacillus* in Fruit Juices"). This method recommends the application of K agar (pH 3.7) in combination with BAT (*Bacillus acidoterrestris* thermophilic, pH 4.0) or YSG (yeast starch glucose, pH 3.7) medium. Under this method, the plates are incubated at temperature of 45 °C for 2–5 days (IFU, 2007; Steyn et al., 2011). The American Public Health Association proposed the use of K agar and incubation of plates at a temperature of 43 °C for 3 days (Evancho and Walls, 2001). The Japan Fruit Juice Association (JFJA) recommends the use of YSG agar and incubation at 30 °C for 5 days for the isolation of *Alicyclobacillus* (JFJA, 2003). The use of K agar medium has also been proposed and applied successfully for this purpose (Walls and Chuyate, 1998). Based on K agar, SK agar (pH 4.0) was developed as a novel *Alicyclobacillus* isolation medium for higher recovery of spores (Chang and Kang, 2005). Generally, BAT agar, YSG agar and K agar are currently the most frequently used growth media for the detection of *Alicyclobacillus* spp.

Although various plating methods for the isolation of *A. acidoterrestris* spores have been developed, the effectiveness of these methods differs. The type of growth medium, type of plating method and application of additional procedures, such as membrane filtration, may influence the efficacy of the procedures used for the detection and enumeration of *A. acidoterrestris* spores in food products. The aim of this work was to develop and optimise an effective method for the detection and enumeration of *A. acidoterrestris* spores, especially in fruit industry products. For this purpose, the three most frequently used types of growth media (BAT agar, YSG agar and K agar) and a novel SK agar medium applied for the recovery of *A. acidoterrestris* spores were investigated. Moreover, the influence of the type of isolation medium used, heat shock and incubation conditions, plating technique (spread plating and membrane filtration) and the presence of apple juice in the sample on the accuracy of the enumeration of *A. acidoterrestris* spores were determined. The effects of the isolation media and growth conditions on the morphology of *A. acidoterrestris* colonies were also investigated.

There is little systematic research reported in the literature addressing comparisons of the recovery of *A. acidoterrestris* spores using the three most commonly recommended types of growth media (i.e., BAT, YSG and K agar media) and SK agar medium. Murray et al. (2007) investigated ten types of agar media, including BAT agar, YSG agar and K agar, applied to enumerate *Alicyclobacillus* spores. Witthuhn et al. (2011) compared three methods for the isolation of *Alicyclobacillus* from diluted peach juice concentrate using three types of isolation media, i.e., BAT agar (method A), PDA (method B) and K agar (method C, in combination with membrane filtration). The highest recovery level, on the order of 76%, was obtained using method A, while method C was the least effective (3%). Witthuhn et al. (2007) found that the application of PDA and OSA media results in a higher recovery of *Alicyclobacillus* vegetative cells and spores compared to the application of K agar, YSG agar and BAM. The use of SK agar for the recovery of *Alicyclobacillus* has been reported only by Chang and Kang (2005) to date. Additionally, there are few published studies investigating the application of membrane filtration techniques for the enumeration of *Alicyclobacillus* spores (Lee et al., 2007; Pettipher and Osmundson, 2000; Goto et al., 2002).

2. Materials and methods

2.1. Bacterial strain

A culture of *Alicyclobacillus acidoterrestris* ATCC 49025 to be used in our experimental work as an artificial contaminant was obtained from Microbiologics, Inc. (Minnesota, USA) in the form of a lyophilised, standardised microorganism preparation (EZ-FPC™ product, pellets). The concentration of *A. acidoterrestris* vegetative cells in the standardised preparation was equal to $3.3{\cdot}10^3$ CFU per pellet. The microorganism preparation was stored at 4 °C.

2.2. Preparation of stock spore suspensions

Pellet of the EZ-FPC™ product was solubilised in sterile deionised water according to the manufacturer's instructions. A culture of *A. acidoterrestris* ATCC 49025 was spread on potato dextrose agar (PDA) at pH 4.0 (BTL, Poland) and incubated at 46 °C for up to 14 days, or until at least 80–90% of the cells had sporulated, as determined by microscopic examination. Spores of each strain were harvested by adding 1–2 ml of sterile deionised water to the PDA culture plate and gently rubbing the surface of the medium with a sterile cotton swab. The rubbing procedure was repeated three times for each plate. Pooled suspensions of strains collected from 15 plates were centrifuged at 4000 ×*g* for 10 min. The supernatant was discarded, and the spores were resuspended in sterile deionised water, followed by centrifugation at 4000 ×*g* for 10 min again. The washing-centrifugation procedure was repeated three times. After centrifugation, the supernatant was discarded, and the spores were resuspended with sterile deionised water. The spore suspension was mixed and placed in cryogenic tubes. Suspensions were stored at −20 °C until used in further experiments.

2.3. Recovery of A. acidoterrestris on different growth media

Four types of isolation media (BAT agar, YSG agar, K agar, SK agar) were applied for the recovery of *A. acidoterrestris* spores. The growth media used in the experimental work were prepared from individual components obtained from BTL (Poland). The components of the trace element solution for BAT agar were obtained from POCH (Poland). The compositions of the applied media are summarised in Table 1.

The mixture of growth medium components was autoclaved at 121 °C for 15 min. The mixture was then cooled down to 50 °C and adjusted to the desired pH value using 1 mol l^{-1} sulphuric acid

Table 1
Composition of the growth media used for the detection and enumeration of Alicyclobacillus acidoterrestris.

Component	BAT agar	YSG agar	K agar	SK agar
Yeast extract	2.00 g	2.00 g	2.50 g	2.50 g
Glucose	5.00 g	1.00 g	1.00 g	1.00 g
$CaCl_2{\cdot}2H_2O$	0.25 g			0.50 g
$MgSO_4{\cdot}7H_2O$	0.50 g			
$(NH_4)_2SO_4$	0.20 g			
KH_2PO_4	3.00 g			
Trace elements solution[a]	1.00 ml			
Peptone			5.00 g	5.00 g
Soluble starch		2.00 g		
Tween 80			1.00 ml	1.00 ml
Agar	18.00 g	18.00 g	15.00 g	15.00 g
Deionised water	1000 ml			
pH	4.0	3.7	3.7	4.0

[a] Trace elements solution: 0.66 g $CaCl_2{\cdot}2H_2O$, 0.18 g $ZnSO_4{\cdot}7H_2O$, 0.16 g $CuSO_4{\cdot}5H_2O$, 0.15 g $MnSO_4{\cdot}4H_2O$, 0.18 g $CoCl_2{\cdot}6H_2O$, 0.10 g H_3BO_3, 0.30 g $Na_2MoO_4{\cdot}2H_2O$ per litre distilled water.