ELSEVIER

Food Microbiology 25 (2008) 22–28

FOOD MICROBIOLOGY

www.elsevier.com/locate/fm

A comparative study between overlay method and selective-differential media for recovery of stressed *Enterobacter sakazakii* cells from infant formula

Murad A. Al-Holy[a,*], Mengshi Lin[b], Hamzah M. Al-Qadiri[c], Barbara A. Rasco[d]

[a]*Department of Clinical Nutrition and Dietetics, Faculty of Allied Health Sciences, Hashemite University, P.O. Box 150459, Zarqa-Jordan, Jordan*
[b]*Food Science Program, 256 William Stringer Wing, Eckles Hall, University of Missouri, Columbia, MO 65211, USA*
[c]*Department of Nutrition and Food Technology, Faculty of Agriculture, The University of Jordan, Amman-Jordan, Jordan*
[d]*Department of Food Science and Human Nutrition, Box 646376, Washington State University, Pullman, WA 99164, USA*

Received 19 April 2007; received in revised form 15 August 2007; accepted 5 September 2007
Available online 11 September 2007

Abstract

This study compares the performance of different selective-differential media with the overlay method for recovery of stressed cells of *Enterobacter sakazakii* from infant formula milk (IFM). Five different selective-differential media were used in this study: OK medium, violet red bile agar (VRBA), Druggan–Forsythe–Iversen agar (DFI), *Enterobacteriaceae* enrichment (EE) agar, and fecal coliform agar (FCA). Tryptic soy agar supplemented with 0.1% sodium pyruvate (TSAP) was used as a control. The overlay method involved applying a thin layer (8 ml) of each of the selective media onto TSAP after spreading a sample onto TSAP. Reconstituted IFM was inoculated by ca 1×10^7 CFU/ml of a mixture of four strains of *E. sakazakii* and subjected to different stress conditions: heat (55 °C for 10 min), a freeze–thaw cycle (−20 °C for 24 h, thawed at room temperature, frozen again at −20 °C, and thawed), acidic pH (pH 3.56 for 15 min), alkaline pH (pH 11.04 for 15 min), and desiccation (*E. sakazakii* was inoculated onto powdered IFM at a level of ca 1×10^6 CFU/g, held at 21 °C, water activity of the inoculated product was 0.29 and examined at 0, 15, and 30 d). No major differences were noticed between the control (TSAP) and the overlay methods. However, the overlay method recovered significantly higher numbers of stressed *E. sakazakii* cells compared to selective-differential media. Also, the selective-differential media exhibited some variability in terms of their capabilities to recover stressed cells of *E. sakazakii*. Among all the examined selective-differential media, DFI performed better for recovering stressed *E. sakazakii* cells. This study suggests that the overlay method may serve as a potential alternative to direct selective plating for best recovery of *E. sakazakii* from IFM.

Keywords: *Enterobacter sakazakii*; Overlay method; Selective-differential media; Infant formula

1. Introduction

Enterobacter sakazakii, a Gram-negative, rod-shaped, motile bacterium that belongs to the family *Enterobacteriaceae*, has recently been involved in several cases of fatal neonatal meningitis (Bar-Oz et al., 2001; Gurtler et al., 2005; Bowen and Braden, 2006). *E. sakazakii* is distributed widely in the environment (Kandhai et al., 2004; Farber, 2004; Arts, 2005). Reconstituted powdered infant formula milk (IFM) has been implicated as a vehicle of transmission in several outbreaks and sporadic cases of *E. sakazakii* infection (Nazarowec and Farber, 1997; Bar-Oz et al., 2001). *E. sakazakii* can be recovered occasionally from IFM, however, mostly at a very low level (<1 CFU/g) (Iversen and Forsythe, 2007). Yet, if poor hygienic practices are used in preparing and handling IFM, the number of cells may rise quickly, mainly because this microorganism has a relatively short doubling time at room temperature (~40 min) (Richards et al., 2005). This becomes very critical since *E. sakazakii* has a relatively low infectious dose of 1000 CFU/ml in reconstituted IFM (Iversen and Forsythe, 2003). The reported fatality rate associated with *E. sakazakii* infections is 40–80% (Bowen

*Corresponding author. Tel.: +962 590 3333; fax: +962 390 3350.
E-mail address: murad@hu.edu.jo (M.A. Al-Holy).

0740-0020/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fm.2007.09.002

and Braden, 2006). *E. sakazakii* infection associated with powdered IFM prompted a recall of a commercial product in the United States in March 2002 (Center for Disease Control and Prevention, 2002).

The lack of an appropriate rapid method to test for the presence of *E. sakazakii* might have led to underestimating the prevalence of *E. sakazakii* in the environment and foods and the number of reported cases affected by *E. sakazakii*. *E. sakazakii* has a remarkable capability to survive in a dry environment such as powdered IFM for long time periods (~2 years) that gives it a competitive advantage of prevailing in dry IFM (Edelson-Mammel et al., 2005). Therefore, end product testing of powdered IFM is necessary. Also, having a rapid method would make it easier to trace isolates from clinical cases back to their source.

The US Food and Drug Administration (FDA) uses a time-consuming method to test for the presence of *E. sakazakii* in dry IFM. This method is based on reconstituting a milk sample and incubating it overnight followed by selective enrichment in *Enterobacteriaceae* enrichment (EE) broth and by streaking on violet red bile glucose agar (VRBG). Colonies from violet red bile glucose agar are streaked on tryptic soy agar (TSA) to check for the presence of yellow-pigmented colonies after 48–72 h incubation at 25 °C and finally by biochemical testing using API 20E system (US Food and Drug Administration, 2002). To date, several methods have been developed to detect *E. sakazakii* in food and environmental samples such as a fluorogenic selective medium (OK medium) which is based on the capability of *E. sakazakii* strain to produce α-glucosidase that reacts with 4-methylumbelliferyl-α-D-glucoside to give distinct fluorescent colonies of *E. sakazakii* (Oh and Kang, 2004) or by using 5-bromo-4-chloro-3-indolyl-α-D-glucopyranoside as a substrate for α-glucosidase in the Druggan–Forsythe–Iversen agar (DFI). Distinct blue-green colonies are produced on DFI medium (Iversen et al., 2004a). Another method involves a selective enrichment in lauryl sulfate broth supplemented with NaCl and vancomysin followed by streaking on tryptone soy agar with the resulting yellow-pigmented colonies are identified by API 20E system (Guillaume-Gentil et al., 2005). However, these methods are either not sufficiently selective or result in underestimating the presence of the organism in the tested powdered IFM and food samples because of the presence of selective and differential ingredients in those media that prohibit the resuscitation of injured cells. For example, some *E. sakazakii* strains, especially stressed cells are sensitive to sodium deoxycholate in DFI agar and to crystal violet and bile salts in violet red bile glucose agar (Gurtler and Beuchat, 2005; Iversen and Forsythe, 2007), precluding their detection in powdered IFM and other foods. Therefore, developing a rapid and accurate method for detection of *E. sakazakii* is crucial. The objective of this study was to investigate the capability of overlay methods coupled with selective-differential media for recovering *E. sakazakii* cells exposed to different stress conditions (heat, freeze, acid, alkaline, and desiccation).

2. Materials and methods

2.1. Bacterial cultures

Four *E. sakazakii* strains (ATCC 12868, ATCC 29004, FSM 292, and FSM 287) were exposed to different stress conditions and examined in terms of their recovery on a general (non-selective) medium which served as a control and on five other selective-differential media and by the overlay method, which combine an under general medium layer with upper selective-differential medium layer. The four strains of *E. sakazakii* were transferred individually from stock aqueous glycerol (15%) solutions stored at −20 °C to slants of TSA (Difco, Becton Dickinson, Spark, MD) and kept refrigerated. Prior to the experiment, the cultures were transferred from TSA slants to Brain Heart Infusion (BHI) broth (Difco) and grown for 24 h at 37 °C; thereafter, the four strains were inoculated in the reconstituted IFM. The initial level of *E. sakazakii* was determined by spread plating on TSA and incubation at 37 °C for 24 h.

2.2. Stress conditions

Stationary phase cells of four strains of *E. sakazakii* were exposed to heat, a freeze–thaw treatment, acid, alkaline, and desiccation stresses as described below. Unstressed cells served as a control. Ten milliliters samples of reconstituted IFM were sterilized at 121 °C for 15 min to be ready for further use. After applying each stress condition *E. sakazakii* count was determined by 10-fold serially diluting in 0.1% peptone water and spread plating on a general medium, selective-differential media or by the overlay method.

2.3. Heat stress

A commercial powdered IFM was purchased from a local grocery store before the experiment. Ten milliliters of the sterilized reconstituted IFM was inoculated with a mixture of four strains of *E. sakazakii* at an initial level of ca 1×10^7 CFU/ml. The tubes were heated at 55 °C for 10 min (equivalent to an average of one *D*-value, this time is enough to elicit about one log reduction in the initial *E. sakazakii* load). Heat treatment was conducted in a water bath (Iso temp 215, Fisher Scientific, Pittsburgh, PA). The tubes were submerged completely in the water bath where the temperature was controlled at the target temperature ±0.5 °C. The temperature of the tubes was monitored by a type T thermocouple (Barnat Co., Barrington, IL) connected with a portable thermometer. After completion of heating, the tubes were immersed promptly in a mashed ice bath at 0.0 ± 0.2 °C.