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Effects of culture conditions on the biofilm formation of *Cronobacter sakazakii* strains and distribution of genes involved in biofilm formation

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ABSTRACT

Biofilm of *Cronobacter sakazakii* on the surfaces of equipment and processing environments is the important source of persistent contamination in food samples. Based on 828 tests (23 × 4 × 3 × 3), the biofilm-forming abilities of 23 *C. sakazakii* isolates were assessed under different pH values, temperatures, and culture time. Biofilm formed by *C. sakazakii* was evaluated in tryptone soy broth in 96-well plates using crystal violet staining and its quantification was counted using the optical density measurements (OD570 nm). Among the evaluated conditions, *C. sakazakii* formed highest amount of biofilm at pH 5.0 (9 strains, 39.1%), at 28 °C (23 strains, 100%) and for 48 h (14 strains, 60.9%). In addition, the biofilm under pH7-24 h-28 °C, pH5-24 h-28 °C, and pH7-48 h-28 °C was detected by confocal laser scanning microscopy for detailed analysis of biofilm. Finally, PCR methods were newly developed for detection of the genes involved in biofilm formation. The bcsC, bcsG, flgJ, bcsA, fliD, and flhE were present in 100%, 100%, 91.3%, 95.7%, 100%, and 34.8% of tested strains respectively. Results indicated that the properties of polysaccharides in biofilm formation of *C. sakazakii* were affected by microbial environments and the biofilm-forming ability was strain-specific. The findings presented here provide useful information for development of efficient procedures against biofilm formation.

1. Introduction

Biofilms formation of foodborne pathogens on food contact surfaces led to serious public health hazards and economic losses (Bai & Rai, 2011). The pathogenic cells in biofilm are difficult to be completely eliminated, which is serving as the important source of persistent contamination in food products (Stepanovic, Cirkovic, Mijac, & Svabic-Vlahovi, 2003).

Cronobacter sakazakii is an important foodborne pathogen associated with outbreaks of a rare form of infant meningitis, necrotizing enterocolitis (NEC), and bacteremia through consumption of contaminated powdered infant formula (Biering et al., 1989; Gurtler, Kornacki, & Beuchat, 2005; Van Acker et al., 2001). Capsular biofilm formation of *Cronobacter* spp. was firstly reported to increase the resistance to dry stress in powdered milk (Iversen & Forsythe, 2003). Subsequently, *Cronobacter* spp. have been found to form biofilms on a wide range of surfaces including silicon, glass, stainless steel, latex, and polycarbonate (Iversen, Lane, & Forsythe, 2004) and biofilms formation were affected by hydrophilic and hydrophobic surfaces (Iversen et al., 2004), nutrients of medium and temperatures (Dancer, Mah, & Kang, 2009; Kim, Ryu, & Beuchat, 2006), extracellular matrix (Lehner et al., 2005).

To date, *Cronobacter* is a recently described genus including 10 species *C. sakazakii, Cronobacter malonaticus, Cronobacter turicensis, Cronobacter muytjensii, Cronobacter dublinensis, Cronobacter condiment, Cronobacter universalis, Cronobacter zurichensis, Cronobacter helveticus*, and *Cronobacter pulveris* (Brady, Cleenwerck, Venter, Coutinho, & De Vos, 2013; Iversen et al. 2008).

However, little knowledge about effects of culture conditions on biofilm formation of *C. sakazakki* was known. In this study, effects of different temperatures, time, and pH values on biofilm formation were evaluated. The detailed properties of biofilm under different conditions (pH7-24 h-28 °C, pH5-24 h-28 °C, pH7-48 h-28 °C) were

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further analyzed by confocal laser scanning microscopy. Finally, PCR methods were newly developed for successfully detecting genes (*bcsC*, *bcsG*, *bcsA*, *flgJ*, *fliD*, and *flhE*) involved in biofilm formation.

2. Materials and methods

2.1. Cronobacter sakazakii strains used in this study

C. sakazakii strains (n = 23) come from Guangdong Provincial Key Laboratory of Microbiology Culture Collection and Application, Guangdong Institute of Microbiology. All *C. sakazakii* strains isolated from powdered milk by FDA method, further confirmed by PCR assay targeting *rpoB* gene described previously (Stoop, Lehner, Iversen, Fanning, & Stephan, 2009) and *fusA* gene sequencing (Xu et al., 2014).

2.2. Biofilm formation of C. sakazakii under different conditions

The 828 tests about culture temperatures, incubation time, and pH values on biofilm formation were performed separately and the initial bacterial population was from different tubes. For determining the effects of temperatures on biofilm formation, the 30 μl overnight culture (OD600 nm = 0.5) of *C. sakazakii* strains (n = 23) was transferred into 96-cell plates (Cyagen, Co., Ltd, U.K) and 100 μl sterile tryptic soy broth (TSB, Qingdao, Haibo) was then added for incubation at 4 °C, 28 °C, 37 °C, and 44 °C for 24 h. To determine the effects of pH values, the 30 μl overnight culture (OD600 nm = 0.35) was transferred into 96-cell plates and 100 μl sterile TSB (pH 3, 5, 7, and 9) was mixed at 28 °C for 24 h. Likewise, the 30 μl culture (OD600 nm = 0.4) was transferred into 96-cell plates and mixed with sterile TSB (100 μl, pH7) at 28 °C for 12 h, 24 h, 36 h, and 48 h of incubation.

2.3. Analysis of biofilm formation

After incubation at different pH values (3, 5, 7, and 9-28 °C-24 h) or culture temperatures (4 °C, 28 °C, 37 °C, and 4 4 °C-pH7-24 h) or culture time (12 h, 24 h, 36 h, and 48 h-pH7-28 °C), the 96-cell plates were rinsed 3 times with deionized water and the adherent bacteria cells were stained with 1.0% crystal violet for 1 h. Then, the crystal violet was liberated by acetic acid (33%, v/v) following 30 min incubation. The sterile TSB was used as negative control and the OD values of each well (negative control and tested strains wells) were measured at 570 nm as described by Du et al. (2012) using ChroMate® microplate reader (Awareness Tech, INC. Palm, USA). The average value of the optical density of the negative control wells was subtracted from the values of each test well, and this difference was referred as OD570 nm for the biofilm-forming ability of *C. sakazakii* strains.

To analyze the influences of different temperatures, pH values and culture time on variability of biofilm formation by *C. sakazakii* strains, the boxplots of mean OD570 nm values and coefficient of variation (CV = standard deviation/mean OD570 nm × 100) and mean-CV (mean OD570 nm × 100) at different conditions were made. Each *C. sakazakii* strain was done in triplicate and the amount of biofilm was analyzed using EXCEL software.

2.4. Determination of biofilm by CLSM in selected strain

C. sakazakii isolate 7 was used to determine the detailed structures of biofilms under pH5-28 °C-24 h, pH7-28 °C-24 h, and pH7-28 °C-48 h by confocal laser scanning microscopy (CLSM) using Fluorescein Isothiocyanate-Concanavalin A (FTIC-ConA, Sigma, USA) and propidium iodide (PI, Sigma, USA) to bind polysaccharides and damaged cells respectively for revealing the changes of properties of extracellular matrix in biofilms, which was performed as described previously by Xiang, Sun, Xia, Song, and Huan (2010).

2.5. Detection of genes involved in biofilm formation by new PCR methods

The genes involved in biofilm formation were previously described through transposon mutagenesis (Hartmann et al., 2010). The primers and PCR parameters for detection of genes (*bcsC*, *bcsG*, *bscA*, *fliG*, *fliD*, and *flhE*) involved in biofilm formation were listed in Table 1. The DNA was extracted using genomic Extract Kit (DSBIO, Guangzhou). The PCR mixture (25 μl) consists of 0.5 μl primers (10 μM) for each, 200 μM dNTP, 10 × buffer 2.5 μl, *Taq* DNA polymerase 2.5U, DNA template 1.0 μl, and double distilled water. The products were detected by 1.5% agarose gel electrophoresis with Goldview staining (0.008%, v/v). The successful amplification of targeted fragments of different genes in Table 1 by PCR is considered as the positive for above genes, while the failure to amplify the targeted fragments means absence of genes. Genomic DNA from each strain was performed by PCR in triplicate.

3. Results and discussion

Pathogenic foodborne bacteria attaching food contact surfaces increase their resistance or tolerance to environmental stress, antibiotics, and detergents, which is a potential hazard to food safety and public health (Bai & Rai, 2011). This study aims to determine the influences of culture conditions on the biofilm formation by *C. sakazakii* strains. Under different incubation temperatures,

Table 1
Amplification of genes involved in biofilm formation.

Gene names	Primer (5'-3')	Amplicon size(bp)	PCR parameters (30 cycles)		
flgJ	F: TCAGGTGCCGATGAAGTTTG 3'	312		58.7°C, 40s	
	R: GCCCTTTCCAGGACGATGT 3'				
flhE	F: CATTACTGACGCTGCCTGTCC	256		58.2°C, 30s	
	R: GTAGTGCCCGTCTGGTCTTCC				
bcsA	F: AAGAAGAGTACGTGGACTGGGTGA	171	94°C, 2min;	59.5°C, 30s;	72°C, 45s
	R: CGCCGAGGATAATCAGGTTGTAG				
bcsG	F:GACGGGCTATCTGAATTTCCAC	146		58.7°C, 30s	
	R:GCCAGGTATCATGCCAGAACA				
filD	F:ATCGAGATCGAGCGTTCCAC	211		57.5°C, 30s	
	R:CGCCCTTATCAACTTTGACGTATT				
bcsC	F: AGATTTGAGCGGTTATTCTTTAGGC	156		58.7°C, 30s	
'	R:TCGGTCTTCGTGCGGGAGTG				

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