DEGRADATION OF CELLULOSE AND CURLI, INACTIVATION OF SHIGA TOXIN PRODUCING *ESCHERICHIA COLI* CELLS, AND CONTROL OF BIOFILMS USING TREATMENTS WITH SELECTED ENZYMES, ORGANIC ACIDS OR COMMERCIAL DETERGENTS

by

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(Under the direction of JINRU CHEN)

ABSTRACT

This study was undertaken to determine the efficacies of different concentrations of cellulase or protease, 2% acetic and lactic acid and a manufacturer-recommended concentration of an acidic and alkaline detergent to reduce cellulose or curli, inactivate the cells of Shiga toxin producing *Escherichia coli* (STEC), and control the biofilms formed by STEC producing different amounts of cellulose and/or curli on polystyrene and stainless steel surfaces. It was found that treatments with 2% acetic and lactic acid significantly reduced the cell populations of STEC and the amounts of curli, and treatments with 2% lactic acid also significantly decreased the amounts of cellulose produced by STEC cells ($P < 0.05$). The residual amounts of cellulose and curli after the treatments with the two organic acids positively correlated to the surviving cell populations of individual STEC strains used in the study. Treatments with cellulase and protease degraded cellulose and curli, respectively, but the treatments had no influence on the fate of STEC cells. Treatments with the two commercial detergents not only degraded cellulose and curli, but also

reduced the cell populations of STEC to undetectable levels. Thus, no positive correlations between the residual amounts of cellulose or curli and the surviving cell populations of STEC from the treatments with the two detergents was observed. Cells expressing cellulose and/or curli formed the greater amounts of biofilm. More dense of biofilms were formed on polystyrene than on stainless steel surface. Acidic and alkaline detergents were more effective than the organic acids in removing biofilms.

INDEX WORDS: Shiga toxin producing *Escherichia coli*, Bacterial cellulose, Curli, Biofilm, Cellulase, Protease, Acetic acid, Lactic acid, Acidicdetergent, Alkaline detergent, Polystyrene, Stainless steel

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CHAPTER 1

INTRODUCTION

Shiga toxin producing *Escherichia coli* (STEC) is a group of foodborne pathogens causing serious clinical manifestations, ranging from mild and severe diarrhea to severe complications such as hemorrhagic colitis and hymolytic-uremic syndrome (Karmali 2003; O'Brien *et al*. 1982; O'Brien *et al*. 1987; Riley *et al.* 1983). Although more than 200 serotypes of STEC have been reported (Eklund *et al*. 2001), the dominant serotypes were O157, O26, O111, O103, O121, O45 and O145. They have been linked to outbreaks and sporadic cases of STEC infections (Boerlin *et al.* 1999; Brooks *et al.* 2005; Goldwater and Bettelheim 1994). Cattle are the major reservoirs of STEC, however, *E. coli* O157 and non-O157 strains have been isolated from retail beef, pork, poultry and lamb meat (Doyle and Schoeni 1987; Samadpour *et al.* 1994). STEC infections are usually transmitted *via* the consumption of food contaminated with STEC during food production, transport and preparation (Mainil and Daube 2005; Fairbrother and Nadeau 2006; Vanselow *et al.* 2005).

To sustain their stability in the environment, cells of *E. coli* adhere to surfaces and form biofilms. Cell surface appendages such as curli and cellulose contribute to the biofilm development process (Cookson *et al.* 2002; Zogaj *et al.* 2001; Zogaj *et al.* 2003). Curli expressed by *E. coli* enhance the ability of the cells to contact surfaces (Kikuchi *et al.* 2005; Pawar *et al*. 2005; Ryu and Beuchat 2005). They are characterized as β-sheet rich, thin, wiry, coiled fibers with various lengths (Barnhart *et al*. 2006; Olsén *et al*. 1989). Cellulose is a long chain polymer of glucose with β-1, 4 glycosidic bonds. It is highly insoluble, inelastic and has extreme strength (Ross *et al.* 1991). Cellulose maintains its structural stability (Zogaj *et al*. 2001) through a high

degree of polymerization and crystallinity and the ability to form hydrogen bonding networks (Ross *et al.* 1991; Steinbüchel *et al.* 2005; Williams and Cannon 1989). Co-expression of cellulose and curli confers hydrophobic properties to *E. coli* cell surface and mediates the interaction between bacterial cells and their contact surfaces (Zogaj *et al*. 2003). Previous studies have demonstrated that the cellulose and curli play a critical role in protecting *E. coli* cells from environmental stress (White *et al.* 2003; Zogaj *et al.* 2003). However, their influence on biofilm control has not been determined. The objectives of this study were to

1. Investigate the efficacies of the selected enzymes, organic acids and commercial detergents in degrading cellulose and curli, inactivate the cells of Shiga toxin producing *Escherichia coli* (STEC), and control the biofilms formed by STEC producing different amounts of cellulose and/or curli on polystyrene and stainless steel surfaces.
2. Determine the correlations between the residual amounts of cellulose or curli and the surviving cell populations of STEC after the aforementioned treatments

CHAPTER 2

LITERATURE RIVEW

I. Shiga toxin producing *Escherichia coli* (STEC)

1. Introduction on *E. coli*

Escherichia coli belong to the family of *Enterobacteriaceae* and are one of microflora living in the lower intestine of mammals. They are gram negative and facultative anaerobic rods. Under anaerobic condition, they have the ability to ferment to gain energy and to produce mixed acids/gas as end products (Jurtshuk 1991). Kauffman serotyping is widely used as a tool for classification of *E. coli*, and this classification is based on *E. coli* cell surface antigens O (somatic), H (flagella) and K (capsular) (Lior 1996; Nataro and Kaper 1998). There are 174 O serotypes, 53 H serotypes and 103 K serotypes (Meng *et al*. 2007; Nataro and Kaper 1998).

E. coli causing diarrheal diseases in human are categorized into six groups based on pathogenic mechanisms, virulence properties and clinical manifestations (Levine 1987; Meng *et al*. 2007; Nataro and Kaper 1998) including enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), diffuse-adhering *E. coli* (DAEC), enteroaggregative *E. coli* (EAEC), and enterohemorrhagic *E. coli* (EHEC). Among diarrheagenic *E. coli*, EHEC is a subset of STEC (Nataro and Kaper 1998) and is a distinct pathogenic group of *E. coli*. EHEC is associated with bloody diarrhea, hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS). Predominant strains of EHEC belong to serogroups O157, O111, O26, O113 and O103 (Goldwater and Bettelheim 1994).

2. STEC

STEC are enteropathogens producing one or more toxins which is related to the exotoxin of *Shigella dysenteriae* serotype 1 (Konowalchuk *et al.* 1977; O'Brien *et al*. 1987; O'Brien *et al*. 1984). Shiga-like toxin produced by *E. coli* O157:H7, which's associated with bloody diarrhea and hemorrhagic colitis (HC), were recognized by O'Brien in United States (1984, 1987) since the finding of verotoxin producing *E. coli* (VTEC) by Konowalchuk (1977). Then it has been referred as Shiga-like toxin producing *E. coli* (STEC). Now, both VTEC and STEC have been referred to *E. coli* producing one or more types of Stx like Stx1, Stx2, Stx2c, Stx2d, Stx2e (Cleary 2004; Nataro and Kaper 1998). As the strong association with HUS outbreaks, STEC O157:H7 have been a concern worldwide, while non-157 has commonly reported in Australia, Germany and Austria (Elliott *et al.* 2001, Gerber *et al.* 2002).

3. Shiga toxins (Stx)

The major virulence factor of STEC is Shiga toxin (Stx), which leads to death and other symptoms in infected human. The Stx of *E. coli* consist of two groups including Stx1, close to the Stx toxin of *Shigella* dysenteriae type 1, and Stx2 having several variants such as Stx2, Stx2c, Stx2d, Stx2e, and Stx2f (Duffy *et al*. 2001; Melton-Celsa and O'Brien 1998; O'Brien and Holmes 1987). Stx2 may be more related to severe disease and HUS than Stx1 alone or Stx1 and Stx2 (Ostroff 1989). Stx consists of two subunits structurally: a subunit A (33 kDa) that is a biologically active part and a pentameric subunit B (7.5 kDa) that binds to the specific cell receptor. The subunit A is nicked by protease yielding peptide A_1 (28 kDa) and peptide A_2 (4 kDa). The subunit A binds to a hollow ring of the subunit B which mediates toxin binding on

receptors like globotriaosylceramide (Gb3) or Gb4 on the surface of eukaryotic cells. After binding, the subunit A releases peptide A1, which has RNA N-glycosidase activity and cleaves a specific adenine in the 28S rRNA in the cytoplasm. Afterwards, the subunit A of Stx is transferred to the Golgi apparatus and then to the endoplasmic reticulum. The cleavage interrupts the binding of amino acyl-tRNA with 60s ribosomal units, and finally inhibits the protein synthesis. The disruption of protein synthesis causes cell death (Sandvig and van Deurs 1996). The role of Stx in the intestinal disease has been reported as causing lightly or bloody diarrhea (Boerlin *et al.* 1999; Fontaine *et al.*1988; Sjogren *et al*. 1994). Another role of Stx is that it is a major virulence factor for HC and HUS when STEC or EHEC is associated with those diseases (Nataro and Kaper 1998). The damage of endothelial cell by Stx has been recognized as the main cause of HUS and damages occur in the renal cells, the gastrointestinal tract and other organs (Karmali 2003).

4. Other virulence factors

STEC are able to survive in acidic foods and in the high acidic environment such as human stomach. Several mechanisms may be related to acid tolerance of *E. coli* including the arginine dependent system, the glutamate dependent system, and acid- induced oxidative system depending on the sigma factor *rpoS* (Lin *et al.* 1996).

In addition to Shiga toxins, a whole cluster of virulence factors encoded by a chromosomal region, called the locus of enterocyte effacement (LEE), is present in many STEC isolates. Those factors are responsible for causing the attaching and effacing lesions typical for many strains of STEC and EPEC. Cell surface adhesive structures of *E. coli* confer cell the

ability to adhere to the bowel mucosa and mediates the first step of the infection process. The ability to produce attaching and effacing (A/E) lesion on epithelial cells is the important properties of STEC O157 and some other STEC (Kaper *et al*. 1998). These lesions are characterized by localized destruction of intestinal epithelial cell microvilli, intimate attachment of bacteria to the epithelial cells, and assembly of highly organized cytoskeletal structures in the cells beneath intimately attached bacteria (Kaper *et al*. 1998).

Plasmid pO157 is commonly present in *E. coli* O157:H7 isolated from patients having hemorrhagic colitis. The plasmid may facilitate bacterial attachment to Henle407 cells, but not HEp-2 cells (Karch *et al.* 1987). However, the relation of pO157 with adhesion of *E. coli* O157 is controversial (Law 2000). They have been reported that the loss of pO157 decreased adhesion, enhanced adhesion or had no effect on adhesion by other investigators (Law 2000). Intimin was shown to be involved in adhesion of *E. coli* O157 for attachment to HEp-2 cells (McKee and O'Brien 1996). Introduction of the intimin gene, *eae* into enterohemorrhagic *E. coli* O157 *eae* mutant restored full virulence of the pathogen (Donnenberg *et al.* 1993). The *eae* gene is frequently found in non-O157 STEC commonly isolated from humans, and 37% of 35 non-O157 STEC had the *eae* gene (Willshaw *et al*. 1992).

Another virulence factor of EHEC is enterohemolysin (Ehx). The toxin produced small turbid zones of haemolysis after 18-24 h incubation on blood agar containing washed erythrocytes (Beutin *et al*. 1989). The ehx operon is important for survival of STEC, and Ehx shows strong association with intimin (Law 2000). Ehx was more associated with O111 strains causing HUS (16 of 18) than those causing diarrhea (4 of 18) (Schmidt and Karch 1996).

5. Public health significance

STEC especially *E. coli* O157:H7 has been a public health concern because of their involvement in large outbreaks of severe disease such as HUS and HC (Karmali 2003). Since the first report of HC outbreak by *E. coli* O157:H7 infection in 1982 (Riley *et al.* 1983), *E. coli* O157:H7 has also been linked to HC, HUS and thrombotic thrombocytopaenic purpura (TTP) (Mainil and Daube 2005). The incidence of *E. coli* O157:H7 infection increased in the 1980s and 1990s, and the pathogen is responsible for approximately 73,000 illnesses and 61 deaths annually in the United States (Mead 1999). In 2009, 459 cases of O157 and 264 cases of non-O157 STEC were identified by the Foodborne Disease Active Surveillance Network (FoodNet) of CDC's Emerging Infections Program. FoodNet collects data from 10 states regarding diseases caused by enteric pathogens transmitted through food, and they monitors the incidence of these infections by conduction active, population-based surveillance for laboratory confirmed cases (CDC 2010). The highest incidence of STEC in O157 infections was among children aged <4 years in Colorado. Table 2.1 shows the 2009 incidence of laboratory-confirmed STEC infections and post-diarrheal HUS reported by FoodNet (CDC 2010). The incidences of laboratory-confimed infections of STEC O157 and other foodborne pathogens from 1996-2009 reported by Fiid Net are shown in Figure 2.1. The incidence of STEC O157 infections declined during the period of 1996-2004, but the incidence has increased from 2004-2006 (CDC 2010).

6. Incidence of O157 and non-O157

There are more than 200 *E. coli* serotypes which produce Shiga toxin (Griffin *et al*. 2003), and more than 100 of them are linked with human illness (Eklund *et al*. 2001). Although the most

frequently isolated and studied serotype of STEC is *E. coli* O157:H7 (Griffin and Tauxe 1991; Pradel *et al*. 2000), other strains have been also related with disease outbreaks, mainly O26, O111, O103, O121, O45 and O145 in the United States (Boerlin *et al.* 1999; Brooks *et al.* 2005). In the United State, the incidence of O157:H7 were 38% in 82 STEC isolates collected from stool of patients, followed by O26:H11, O121:H19, and O103:H2 (Jelacic *et al*. 2003). Hedican *et al.* (2009) identified 206 STEC isolates from human stool samples collected by health maintenance organization laboratory and a hospital laboratory in Minnesota, the United States during 2000 to 2006. Moreover, 48% of STEC isolates were O157 and 52% were non-O157 serotypes. Serotype O157 was more involved in bloody diarrhea (78%), hospitalization and HUS (7%) than non-O157 serotypes.

Table 2.1. Incidence[*] of laboratory-confirmed bacterial and parasitic infections in 2009 and post-diarrheal hemolytic uremic syndrome (HUS) in 2008, by site and pathogen, compared with national health objectives[†], Source: MMWR 2010.

Pathogen	California[§]	Colorado[§]	Connecticut	Georgia	Maryland	Minnesota	New Mexico	New York[§]	Oregon	Tennessee	Total 2009	National health objective[¶]
Bacteria												
Campylobacter	29.37	14.09	15.25	7.58	8.34	17.24	16.88	11.33	18.63	8.22	13.02	12.30
Listeria	0.45	0.25	0.74	0.31	0.25	0.06	0.15	0.61	0.50	0.24	0.34	0.24
Salmonella	17.87	11.76	12.34	24.57	13.56	11.05	16.88	9.97	10.95	12.81	15.19	6.80
Shigella	5.49	2.37	1.23	6.71	4.90	1.51	4.64	1.08	1.13	6.00	3.99	N/A[**]
STEC[††] O157	1.15	2.29	1.26	0.20	0.43	2.49	0.45	0.66	1.74	0.61	0.99	1.00
STEC non-O157	0.12	1.46	0.63	0.31	0.57	1.21	1.21	0.38	0.29	0.35	0.57	N/A
Vibrio	0.60	0.33	0.77	0.28	0.53	0.17	0.05	0.26	0.47	0.13	0.35	N/A
Yersinia	0.33	0.15	0.63	0.36	0.18	0.25	0.10	0.30	0.45	0.37	0.32	N/A
Parasites												
Cryptosporidium	1.66	1.64	1.11	3.26	0.71	6.65	7.36	1.62	5.01	1.26	2.86	N/A
Cyclospora	0.00	0.00	0.51	0.06	0.04	0.02	0.05	0.02	0.00	0.03	0.07	N/A
HUS[§§]	0.47	0.48	0.95	0.95	0.00	1.39	--	1.31	3.29	3.60	1.40	0.90
Surveillance population (millions)	3.13	2.75	3.50	9.69	5.63	5.22	1.98	4.26	3.79	6.21	46.35	

[*] Per 100,000 population

[†] Data are preliminary.

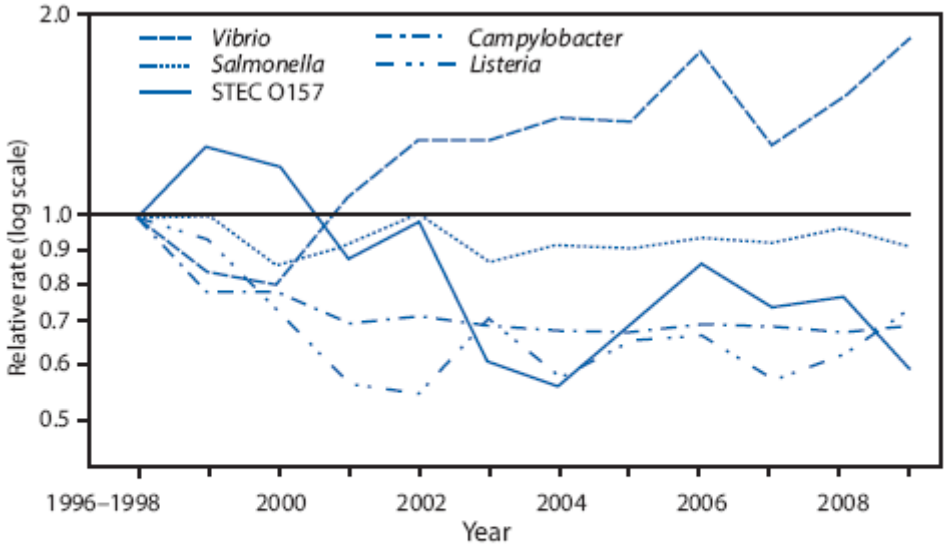
[§] This FoodNet site includes only selected counties. California: Alameda, San Francisco, and Contra Costa; Colorado: Adams, Arapahoe, Denver, Douglas, Jefferson, Boulder, and Broomfield; New York: Albany, Allegany, Cattaraugus, Chautauqua, Chemung, Clinton, Columbia, Delaware, Erie, Essex, Franklin, Fulton, Genesee, Greene, Hamilton, Livingston, Monroe, Montgomery, Niagara, Ontario, Orleans, Otsego, Rensselaer, Saratoga, Schenectady, Schoharie, Schuyler, Seneca, Steuben, Warren, Washington, Wayne, Wyoming, Yates

[¶] Current *Healthy People 2010* objective 10-1 targets for incidence of *Campylobacter, Salmonella*, Shiga toxin-producing *Escherichia coli* O157, and *Listeria* infections, and HUS.

[**] No national health objective exists for these pathogens.

[††] Shiga toxin-producing *Escherichia coli*.

[§§] Incidence of postdiarrheal HUS in children aged <5 years; denominator is surveillance population aged <5 years in sites that conduct hospital discharge data review (New Mexico excluded).



* Shiga toxin-producing *Escherichia coli*.
† The position of each line indicates the relative change in the incidence of that pathogen compared with 1996–1998. The absolute incidences of these infections cannot be determined from this graph. Data from 2009 are preliminary.

Figure 2.1. Relative rates of laboratory-confirmed infections with *Campylobacter*, STEC*O157, *Listeria*, *Salmonella*, and *Vibrio* compared with 1996-1998 rates, by year. Foodborne Diseases Active Surveillance Netwerk, United States, 1996-2009†. Source: MMWR 2010.

7. STEC in Foods

The human infection of STEC are mostly caused by eating food contaminated with STEC during food production, transport, and slaughter (Fairbrother and Nadeau 2006; Vanselow *et al.* 2005). In the United States, the major vehicles of STEC outbreaks are foods, which are associated with 52% of outbreaks of *E. coli* O157:H7 infection during 1982 to 2002 (Rangel *et al*. 2005). For example, animal carcasses can be contaminated with STEC from animal hide or gastrointestinal contents, which subsequently affect the prevalence of the pathogen in animal products (Elder *et al.* 2000; Hussein, 2007).

Hussein and Bollinger (2005) reported that the prevalence of E. coli O157:H7 for the past three decades was 0.1-54.22% in ground beef, 0.1-4.4% in sausage, 1.1-36.0 % in unspecified retail cuts, and 0.01-43.4% in whole carcasses. In retail meat, prevalence of *E. coli* O157:H7 was 3.7% in beef, 1.5% in pork, 1.5% in poultry and 2.0% in lamb (Doyle and Schoeni 1987). As for non-O157: H7, prevalence was 23% in beef, 4% in pork, 48% in lamb, 12% in chicken, and 7% in turkey (Samadpour *et al.* 1994). Food products originated from cattle such as raw milk, cheese curds, butter, and ice cream bars, have served as the source of STEC infection (Fairbrother and Nadeau 2006). STEC was also found in fresh produce including mushrooms, sprouts, lettuce and spinach in retail outlets of the United States (Samadpour *et al.* 2006).

8. STEC contamination during food processing

The contamination of foods is also caused through the exposure to environment such as ruminant feces. Survival of STEC in feces influences subsequent distribution of the pathogen or the contamination of fresh produce via irrigation or fertilization water (Erickson and Doyle 2007). In addition, food contact surfaces such as stainless steel and plastic can be transitional sources of pathogens during food processing. Cross-contamination of *E. coli* O157:H7 were observed in pre-slaughter and abattoirs (Avery *et al.* 2004), and the transfer of *E. coli* O157:H7 was evaluated from beef tissue to polyethylene board surfaces (Flores *et al*. 2006).

Generally, the transmission of pathogens occurs during hide removal and dressing defects such like evisceration during the slaughtering and leads to the contamination of the carcasses. Elder *et al*. (2000) showed a significant correlation between the prevalence of *E. coli* O157 in the fecal or hide and animal carcasses. In addition, cross-contamination of *E. coli* O157:H7 has

occurred in food preparing operation such as grinding or cutting (Flores 2004; Flores and Stewart 2004; Flores *et al*. 2006). Flores (2004) reported the distribution of *E. coli* O157:H7 inoculated on beef to intact beef during beef trims in the bowl cutter.

II. Biofilm

1. Definitions and mechanisms

A biofilm is a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface (Carpentier *et al.* 1993; Costerton *et al.* 1987). The bacterial cells including both viable and nonviable cells primarily constitute the structure of biofilm as well as extracellular polymeric substances. In addition, extracellular polymeric substances contain polysaccharides, proteins, phospholipids, teichoic and nucleic acids with 85-95 % of water (Carpentier 1993; Chmielewski and Frank 2003).

Development of biofilms consists of five steps (Houdt and Michiels, 2005): (1) reversible attachment of planktonic bacteria cell to surface, (2) irreversible attachment by production of extracellular polymers by the bacteria and/or by adhesions interacting with the surface, (3) development of biofilm structure, (4) development of microcolonies, and (5) dispersion of cells from the biofilm. The steps of biofilm formation reported by Kumar and Anand (1998) was slightly different, including (1) formation of conditioning film, (2) attachment of bacterial cells, (3) development of microcolonies, (4) biofilm formation, and (5) dispersion of biofilmss.

Prior to biofilm formation, conditioning films consisting of proteins and polysaccharides changes the properties of the surface by absorbing organic and inorganic molecules to the surface (Davey and O'Toole 2000; Kumar and Anand 1998). The reversible attachment of bacteria cells

to the surface occurs by interaction forces including van der Waals, hydrophobic, electrostatic and London dispersion forces (Chmielewski and Frank 2003; Nikolaev and Plakunov 2007). Then, the cell is irreversibly attached to the surface by hydrogen bonding, dipole-dipole interaction, and ionic covalent bonding (Bower *et al*. 1996). The irreversibly attached cells use the nutrients from the surrounding environment to grow and divide, and then form microcolonies (Kumar and Anand 1998). Extracellular polymeric substances are also produced by the attached cells during this stage, which assist cells to firmly attach to the surface and to trap nutrients from environment (Characklis and Mashall 1990; Houdt and Michiels 2005). In addition to the continuous attachment of cells, extracellular polymeric substances form the complex biofilm architecture (Characklis and Mashall 1990). When the biofilm is mature, the bacterial cells growing in the biofilm are detached, and the released cells move to another surface (Characklis and Mashall 1990; Marshall 1992).

Environmental factors and the properties of microbial cells affect the process of biofilm formation and its characteristics. Important environmental factors in the biofilm formation are pH, salinity, osmolarity, oxygen partial pressure, and accessible nutrient sources, hydrophobicity of the phase interface, and the force and type of liquid motion around surface (Nikolaev and Plakunov 2007).

2. Bacterial attachment and biofilm formation in food industry

Biofilm have been observed in various food processing environments including dairy, meat, poultry and seafood (Gudbjörnsdóttir *et al*. 2004; Lindsay *et al*. 1996; Sharma and Anand 2002). In addition, biofilm have been observed on common materials of food contact-surfaces

such as stainless steel, glass, polypropylene and rubber (Herald and Zottola 1988; Mafu *et al*. 1990). *Listeria monocytogenes* have been shown to form biofilms on stainless steel, and plastic surfaces (Frank and Koffi 1990; Jeong and Frank 1994; Mafu *at al*. 1990). *Pseudomonas spp.* was reported to form biofilm on stainless steel surface accompanying with extracellular polymeric substances production (Barnes *et al*. 1999). Various studies also showed that *Salmonella* attached to, and formed biofilms on surfaces of plastic, cement and stainless steel (Helke and Wong 1994; Jones and Bradshaw 1997; Joseph *et al.* 2001). Similarly, *E. coli* O157:H7 has capability to form biofilm on various surface materials such as stainless steel, glass, teflon and polystyrene (Ryu and Beuchat 2005; Sharma *et al*. 2005; Uhlich *et al*. 2006).

The formation of biofilms following bacterial attachment may be undesirable in food systems (Kumar and Anandm 1998). The biofilm on food contact surfaces may result in cross-contamination of products (Kumar and Anand 1998; Zottola and Sashara 1994). Anand *et al*. (1989) reported that the cross-contamination of chicken carcass is associated with the presence of microorganisms during slaughtering process. Kusumaningrum et al. (2003) observed the transfer of *Salmonella* enteritidis, *Staphylococcous*, and *Campylobacter jejuni* from kitchen sponges to stainless steel, and subsequently to foods such as the slices of cucumber or roasted chicken fillet.

Biofilms are persistent because the attached cells in biofilms have physical adaptation (Hood and Zottola 1997) with resistance to cleaning and sanitizing regimes (Lundén *et al*. 2002), thus leading to endemic populations. Lundén et al. (2002) showed that persistent strains of *Listeria* have enhanced ability of adhesion within shorter period of time comparing to those of

non-persistent strains. During the biofilm formation, exopolysaccharide in biofilm produced by the attached cell plays an important role to combat environmental stress (Sutherland 2001).

Numerous studies have observed the attachment of STEC cells to surfaces and formation of biofilms (Cookson *et al*. 2002; Rivas *et al.* 2007). Individual STEC strains had different ability to attach to stainless steel surface and microtitre plates (Rivas *et al*. 2007). Some non-O157 STEC strains isolated from the feces of a healthy cow expressed type 1 fimbriae and curli fimbriae that are associated with adhesion to polystyrene microtitre plates. However, the adherence of STEC O157:H7 to the same surface was poor and did not elaborate any fibmtriae (Cookson *et al*. 2002). Similarly, Uhlich *et al.* (2008) reported that STEC O157:H7 isolates from the spinach- and lettuce- related outbreaks in 2006 did not produce biofilm on glass.

3. Significance of cell surface structures in the biofilm formation

During the process of biofilm formation, extracellular polymers produced by bacterial cells trap nutrients and may develop complex architecture with pedestal-like structures, water channels and pores. As a result, the growth patterns, and physiology and metabolism of bacteria cell are changed (Houdt and Michiels 2005). Cell surface structures play a role in development of biofilm formation (Houdt and Michiels 2005). In biofilm of *E. coli*, the cell surface polymers such as fimbriae, other proteins, exopolysaccharides and flagella are essential in the attachment, thus leading to biofilm formation (Donlan 2002). Fimbriae and other proteins make cell surface hydrophobic and contribute to attachment to hydrophobic substrata, while exopolysacchride and lipopolysaccharides contribute to attachment to hydrophilic surfaces (Corpe 1980; Donlan 2002). The contribution of cell surface structures such as flagella, curli and polysaccharide have been

recognized in certain steps of biofilm formation (Table 2.2), particularly exopolysaccharide including PGA polysaccharide and colanic acid as well as curli are involved in microcolony formation, development of biofilm architecture, and maturation of biofilm in *E. coli* (Houdt and Michiels 2005).

Table 2.2. The influence of cell surface structures on the development of biofilm.

Development of biofilm	Involving surface structures
Surface contact and reversible attachment	Flagella and motility
Irreversible attachment	Fimbriae (Type 1 fimbriae) Curli Exopolysaccharide (β-1,6-*N*-acetyl-D-glucosamine (PGA) polysaccharide)
Microcolony formation and early development of biofilm architecture	Motility Curli Autotransporter protein (Antigen 43) Exopolysaccharide (colanic acid, PGA polysaccharide)
Maturation	Exopolysaccharide (colanic acid) Curli Conjugative pili
Dispersal	Flagella and motility

Modified from Houdt and Michiels (2005)

a. Curli

Curli are heteropolymeric proteinaceous filamentous appendages and associated with the adherence of biofilm forming *E. coli* strains (Olsén *et al*. 1989), particularly STEC (Cookson *et al.* 2002). The expression of curli has been observed in many pathogenic isolates of *Escherichia coli* (Gophna *et al*. 2001; Olsén *et al*. 1989; Uhlich *et al*. 2001), and similar structures known as thin, aggregative fimbriae have also been observed on *Salmonella enteric* serovar Enteritidis and *Salmonella enteric* serovar Typhimurium (Collinson *et al*. 1996; Collinson *et al.* 1999; Römling

et al. 1998). Curli are capable of binding to a number of substrates including fibronectin, laminin, and Congo-red dye (Olsén *et al.* 1989). Bacterial aggregation and biofilm formation have been observed in curli expressing bacteria cells (Cookson *et al*. 2002; Olsén *et al*. 1989; Pawar *et al*. 2005; Ryu and Beuchat 2005).

In *E. coli*, *csgBAC* and *csgDEFG*, are required for the expression of curli (Houdt and Michiels 2005). The curli structural subunit, CsgA, is secreted from *E. coli* cells in a soluble form and is polymerized on the bacterial surface by the nucleator protein CsgB (Bian and Normark 1997**;** Hammar *et al*. 1995). The absence of the CsgB causes the secretion of a soluble, less adhesive CsgA (Bian and Normark 1997). CsgD, which is transcriptional regulator, is required for the transcription of *csgBAC* (Hammar *et al*. 1995; Römling *et al*. 1998). CsgG is the outer membrane lipoprotein, and required for the stability of the CsgB and CsgA proteins and transportation of curli proteins across the outer membrane (Loferer *et al*. 1997). Csg F is also a periplasmic protein, the mutation of *csgF* gene inhibits curli formation by secreting unpolymerized CsgA. Also, CsgE is related to bind between fibronectin and Congo-red dye (Hammar *et al*. 1995; Stapthopoulos *et al*. 2000).

Physically, curli observed on the cell of *E. coli* is thin, wiry, coiled surface fimbriae of various lengths (Olsén *et al*. 1989). The diameter of curli is ~ 2 to ~ 12 nm under higher magnification of transmission electronic microscope (Chapman *et al*. 2002; Olsén *et al*. 1989), while the cross-sectional diameter of the aggregated curli is as large as 60 nm (Prigent-Combaret *et al*. 2000). The major subunits of curli, CsgA and CsgB, are composed of five-repeating units and each consists of 19-24 amino acids. These repeating units also contain serine, asparagines and glutamine (Collinson *et al.* 1999; Wang *et al.* 2007). The structural and biochemical

properties of curli are similar to amyloid fibers (Barnhart *et al*. 2006), which are composed of aggregated *β*-sheets in an ordered fibrillar structure with straight, rigid and unbranched fibrils (Kayed *et al*. 2003). The *β*-sheets are composed of two or more *β*-strands, which are in parallel or anti-parallel alignments *via* inter-strand hydrogen bonds (Gillespie *et al.* 1997). The *β*-sheets structure is related to stability of protein architecture and protein-protein interactions (Gillespie *et al.* 1997).

Curli are generally expressed under low temperature (< 30ºC), low osmolarity and during stationary phase growth (Olsén *et al.* 1989; Olsén *et al.* 1993; Maurer *et al.* 1998). Increasing NaCl content or elevating temperature will cause recovering phenotype of curli producing strains to white phenotype (Olsén *et al.* 1993; Römling *et al.* 1998). NaCl inhibits the expression of curli by causing the repression of the *csg*A transcription (Olsén *et al.* 1993). In media containing Congo-red dye incubated at 26-28 ºC, curli producing colonies display a red, dry and rough morhpotype, while curli deficient colonies are smooth, colorless and moist (Hammar *et al.* 1995; Olsén *et al.* 1993; Römling *et al.* 1998).

The expression of curli is regulated by several systems, and one of them is the stationary-phase specific sigma factors RpoS. The RpoS stimulates the transcription of *csg*A in the stationary growth phase, which assists the survival of cells in the environment with limited nutrients (Olsén *et al.* 1993). A two component system, OmpR/EnvZ, positively regulates curli expression. OmpR is a cytoplasmic protein and EnvZ is a sensor kinase that senses external osmolarity, mediating phosphorylation of OmpR. OmpR positively regulates the expression of *csg*D, and the absent of ompR represses the *csg*D transcription (Römling *et al.*1998). An integration host factor (IHF) and histone like protein (HN-S) are also involved in curli expression.

Absence of IHF and deletion of HN-S both decrease curli production, subsequently resulting in a decreased transcription of *csg*D in *Salmonella* (Gerstel and Römling 2003). In addition, in *E. coli* K-12 strains hns mutants promotes csgBA transcription, finally facilitate curli expression (Arnqvist *et al.* 1994; Gestel and Römling 2003).

Curli bind to and interact with many host proteins including fibronectin, laminin, plasminogen, MHC (major histocompatibility complex) class I and Toll-like receptor (TLR)-2 and this binding may facilitate bacterial dissemination through the host (Barnhart and Chapman 2006; Bian *et al.* 2000). For the binding to external host proteins, curli have two binding sites, one has 24 amino acids and is located on the N-terminal of the curli protein and the other one has 26 amino acids and is located on the C-terminal end of the curli protein (Olsén *et al.* 2002). In infected patients, the binding of curli to host proteins results in the release of inflammatory molecules, which cause the onset of pain, fever, oedema and hypotension (Ben Nasr *et al.* 1996).

In *E. coli*, the expression of curli enhances the adherence of cells to various eukaryotic cell lines. Specifically, curli expression promots adherence of *E. coli* K-12 to uropithelial cells (Kikuchi *et al.* 2005). *E. coli* could invade human cervical epithelial (HeLa) cells (Gophna *et al.* 2001) or human laryngeal epithelial (Hep-2) cells (Uhlich *et al.* 2002) through curli expression. In *Salmonella* and *E. coli*, the expression of curli is sometimes accompanied with the expression of another cell surface component, cellulose. The expression of both curli and cellulose leads to the development of typical colony morphotypes of cells (White *et al.* 2003; Zogaj *et al.* 2003) and biofilms. Additionally, CsgD indirectly regulates the production of cellulose by activating *adr*A (Hammar *et al.* 1995; Zogaj *et al.* 2001). Curli promots *Salmonella* enteritidis adherence to Teflon and stainless steel surfaces (Austin *et al.* 1998). Curli expressing *E. coli* have better

ability to form biofilm on polystyrene and rubber, surfaces leading to long-term survival of the cells (Cookson *et al.* 2002; Pawar *et al.* 2005).

b. Exopolysaccharides (EPS)

EPS is polysaccharides synthesized by bacterial cells and is located outside the cell walls (Sutherland 1990; Sutherland 2001). Bacterial EPS is synthesized in two forms, including capsular and slime polysaccharides (Kumar *et al.* 2007). EPS has different chemical and physical properties ranging from neutral to polyanionic macromolecules. Neutral macromolecules are comprised of sugar and non-sugar components. Sugars in EPS include pentoses, hexoses, amino sugars and uronic acids, whereas non-sugar components include acetic acid, succinic acid, pyruvic acid, phosphoric acid and sulfuric acid (Kenne and Lindberg 1983; Sutherland 2001). Polyanionic macromolecules such as D-glucuronic acid, D-galacturonic acid, D-mannuronic acid or ketal linked pyruvate are predominant in bacterial EPS (Sutherland 1990; Sutherland 2001). The EPS present in biofilms has similarity with the polymers synthesized by planktonic cells, and it is produced as part of a stress response by biofilm cells (Sutherland 2001). The amounts of EPS within biofilm depend on the available carbon substrates and nutrients (Sutherland 2001). Excessive available carbon substrates and other nutrients such as nitrogen, potassium or phosphate promote the synthesis of EPS (Sutherland 2001). EPS has the backbone of 1,4-β- or 1,3-β- linkages consequently conferring biofilm considerable rigidity and mechanical stability against shear force (Mayer *et al.* 1999).

The exopolysaccharides produced by *E. coli* include cellulose, colanic acid and *β*-1,6-*N*-acetyl-D-glucosamine (PGA) polysaccharide (Danese 2000; Prigent-Combaret *et al.* 2000; Wang

et al. 2004; Solano *et al.* 2002). Cellulose is recognized in *E. coli* and *Salmonella* typhimurium as a major component of EPS, and contributes to colony morphotype (Solano *et al.* 2002; Zogaj *et al.* 2001); (2) Colanic acid produced by *E. coli* K-12 contribute to the development of the three dimensional structure and depth of biofilms, but is not associated with the initial attachment to an abiotic surface (Danese 2000); and (3) PGA polysaccharide works for the *E. coli* attachment to abiotic surfaces, intercellular adhesion and biofilm formation (Wang *et al.* 2004).

c. Cellulose

Cellulose is a long chain polymer of glucose with β-1,4 glycosidic bonds. It is insoluble, inelastic, and has a tensile strength (Ross *et al.* 1991; Yamanaka *et al.* 1989). The polymer is the major component of plant cell walls, but is also synthesized by a variety of bacterial cells as an extracellular matrix (Da Re *et al.* 2006; Grimm *et al.* 2008). Cellulose contains 300 to over 15,000 glucose residues, and has many hydroxyl groups and hydrogen bondings. Physically, the *β*-glycosidic bonds between the *β*-D-glucose residues cause a stiff elongated conformation with little flexibility (Horton *et al.* 2002). Cellulose weigh ranges from 300,000 to over 1,000,000, and is generally insoluble in water, but dissolves in concentrated zinc chloride solution and caustic alkali containing carbon disulphide (Davidson *et al.* 2007). Cellulose forms subfibrils and crystallizes into microfibrils which form insoluble layered sheets and hydrogen bonding network (Jonas and Farah 1998; Ross *et al.* 1991). Bacterial cellulose has higher degree of polymerization and crystallinity, which has greater mechanical strength comparing to plant cellulose.

The best known species of bacteria producing cellulose are *Acetobacter*, *Agrobacterium*, *Rhizobia* and *Sarcina* (Delmer 1999). The production of cellulose is recognized in various *Enterobacteriaceae* such as *Citrobacter* spp. and *Enterobacter* spp. isolated from the human gastrointestinal tract as well as *Salmonella enteritidis*, *S. typhimurium* and *E. coli* which are sometimes accompanied by the expression of curli on the surface of the bacteria cells (Bokranz *et al.* 2005; Römling 2005; Solano *et al.* 2002; Zogaj *et al.* 2001; Zogaj *et al.* 2003). Zogaj *et al.* (2001) reported that cellulose non-covalently binds with curli and forms a highly inert, hydrophobic extracellular matrix which confers structural stability to *Salmonella* and *E. coli*. Expression of cellulose by bacterial cells may be influenced by temperature, pH, atmospheric conditions and nutrient composition in media (Zogaj *et al.* 2003). The operon *bcs* is responsible for cellulose biosynthesis (Ross 1987), and the operon encodes 4 proteins including cellulose synthase (*bcsA*, catalytic subunit), regulator of cellulose synthase (*bcsB*, cyclic di-GMP binding protein), the oxidoreductase subunit (*bcsC*) and the cellulose encoding subunit (*bcsZ*, endoglucanase) (Ross *et al.* 1991; Wong *et al.* 1990).

CsgD and AgfD regulate cellulose biosynthesis in *E. coli* and *Salmonella*. The expression of CsgD stimulates the synthesis of cellulose by transcriptional activation of a GGDEF domain protein, AdrA (Gerstel and Römling 2001; Römling *et al.* 1998; Römling *et al.* 2000; Zogaj *et al.* 2001). The AdrA then stimulates production of c-di-GMP (cGMP) binding to bcsB, which results in stimulating the activity of bcsA to polymerize cellulose using UDP-glucose as precursors (Mayer *et al.* 1991; Zogaj *et al.* 2001). These cascaded reactions result in the formation of extracellular matrix that includes curli and cellulose (Zogaj 2001).

Cellulose produced by bacterial cells can be quantified using a colorimetric assay designed by Updegraff (1969). Firstly, cellulose is isolated by extracting non-cellulosic materials such as lignin, hemicelluloses and xylosans with the acetic acid/nitric acid reagent, and cellulose is then quantified with anthrone in a sulfuric acid solution. Because the antorne molecules can bind to the glucose units, they generate colored compound via the hydrolysis of the glycosidic bonds of cellulose under the heated strong acid. The resulting colored compound is measured at a wavelength of around 625 nm (Viles and Silverman 1949). For qualitative measurement of cellulose, calcofluor White Stain (4,4′-bis[4-anilino-6-bis(2-hydroxyethyl)amino-*s*-trizin-2-ylamino]-2,2′-stilbenedisulfonic acid) is used. Calcofluor white stain is a fluorescent brightener and binds to β(1-4) and β(1-3) glycosidic bonds of polysaccharides, found in cellulose and chitin (Haigler *et al.* 1980). It identifies nascent cellulose in *Acetobacter xylinum*, cellular slime molds, *Dictyosteliumand*, and algae within the genus Oocystis (Haigler *et al.* 1980; Harrington and Raper 1968; Roberts *et al.* 1982). Rezende *et al.* (2007) showed the ability of calcofluor to detect biofilms formed by *Aeromonas*, *Salmonella*, *Vibrio* and *E. coli* by binding to the EPS in biofilm. Cellulose produced by *Salmonella* and *E. coli* has been identified by the fluorescence of colonies grown on calcofluor plates (Bokranz *et al.* 2005; Solano *et al.* 2002; Zogaj *et al.* 2001)

Cellulose is not associated with virulence of *S. Enteritidis* but plays a significantly role in protecting bacterial cells (Solono *et al.* 2002). The bacterial cellulose has the viscosity and hydrophilicity to protect bacterial cells against the changes in moisture content, acidity, and presence of toxic substrates in the environment (Steinbüchel *et al.* 2005). Bacterial cellulose has the capability to hold water over 100 times of its weight due to its entangled network structure

and hydrophilic properties (Ross *et al.* 1991; Schrecker and Gostomski 2005). Cellulose produced by *Acetobacter xylinum* in rotting fruits protected the cells of the bacterium from the detrimental effect of UV light (Williams and Cannon 1989). Cellulose along with curli, protect *Salmonella* from desiccation and treatment with sodium hypochlorite at concentrations up to 30 ppm (White *et al.* 2006). Cellulose deficient mutants of clinically isolated *Salmonella enteritidis* are more sensitive to chlorine, suggesting that cellulose is directly responsible for the survival of cells (Solano *et al.* 2002).

d. Other cell surface structures

E. coli are generally motile by virtue of multiple peritrichous flagella, which serve as adhesive structure to assist cells in attaching to abiotic surfaces (Moens and Vanderleyden 1996). When flagella in *E. coli* was absent or paralyzed, initial biofilm formation was severely delayed. It was proposed that motility of the cells promotes initial contact of the cells by overcoming the repulsive forces between the cells and the surface (Pratt and Kolter 1998).

Fimbriae of *E. coli* are 0.5 to 10 µm long and 7-11 nm thick (Ofek and Doyle 1994). Among them, type 1 fimbriae commonly found in both commensal and pathogenic *E. coli* are 7 nm wide, 1 µm long and rod shaped adhesive surface organelles (Klemm and Krogfelt 1994). The structural components of fimbrial organelle are encoded by the *fim* gene cluster (Fernandez *et al.* 2000). *E. coli* cells with a deletion in *fim* is defective in initial attachment to abiotic surfaces such as polyvinylchloride (PVC) surface under stagnant culture conditions (Pratt and Kolter 1998) or Pyrex slides in minimal culture medium of a continuous flow culture bioreactor

(Beloin *et al.* 2004). The interactions between Type 1 fimbriae and cell surface induce the structural changes in the outer membranes of attached cells, leading to a reduced expression of outer membrane proteins (Otto *et al.* 2001).

Autotransporter proteins are protein required for secretion across the cytoplasmic and the outer membrane to bacterial cell surface. Among the autotransporters, antigen 43 (Ag43) and the AIDA (adhesion involved in diffuse adherence) contribute to adhesive phenotype of *E. coli* (Houdt and Michiels 2005). Ag43 is an adhesion containing receptor, which promotes the formation of bacterial biofilm particularly when capsular polysaccharides or Typ1e 1 fimbriae are expressed (Danese *et al.* 2000; Kjacrgaard *et al.* 2008). Ag43 also induce both inter and intraspecies cell aggregation between *E. coli* and *Pseudomonas fluorescens*, resulting in enhanced biofilm formation (Kjaeragaard *et al.* 2008). The AIDA produced by diarrheagenic *E. coli* is a glycosylated adhesion which promotes adherence to human and mammalian cells, biofilm formation and autoaggregation (Benz and Schmidt 1989; Sherlock *et al.* 2004).

Conjugative pili expressed by F+ strains of *E. coli*, are filaments with ~8 nm in width and a 2-nm axial lumen (Willetts and Skurrary 1987). The F plasmid integrated into plasmids of *E. coli* K12 promote biofilm formation through accelerating the initial adhesion and biofilm development on pyrex slides in a continuous flow bioreactor with a high input rate of fresh medium (Ghigo 2001; Reisner *et al*. 2003). It was suggested that the conjugative pili promoted the initial biofilm formation. The attachment of donor bacteria expressing the conjugative pili initiates the process of biofilm formation by accelerating adhesion to the abiotic surface and supporting the three dimensional growth of the biofilm (Ghigo 2001).

4. Control of biofilm

Cell attachment on a surface may take a few hours (Meyer 2003) while formation of a biofilm occurs within several days to several weeks (Zottola and Sasahara 1994). Therefore, cleaning using effective detergent or mechanical force (Mattila-Sandholm and Wirtanen 1992; Meyer 2003) is of great significance to avoid both accumulation of particulates and bacterial cells on surfaces and subsequent biofilm formation (Kumar and Anand 1998; Mattila-Sandholm and Wirtanen 1992; Meyer 2003). However, cells in biofilms have greater resistance against cleaning and sanitizing chemicals than do planktonic cells (Brown and Gilbert 1993). For instance, cells of *Listeria monocytogens* on glass and stainless steel are more resistant to sodium hypochlorite, quaternary ammonium compounds, benzalkonium chloride and anionic sanitizers, compared to planktonic cells (Frank and Koffi 1990; Mustapha and Liewen 1989). Ryu and Beuchat (2005) reported that biofilm cells of *E. coli* O157:H7 on the surface of stainless steel were more resistant to chlorine than did the planktonic cells. Extracellular polymeric substances produced by bacteria cells serve as protective barriers against antimicrobials (Costerton *et al.* 1995; Lewis 2001). Moreover, polysacchardies produced by biofilm cells confer resistance to bacterial cells against cleaning or sanitizing chemicals by inhibiting penetration of biocides into biofilm structure (Meyer 2003). Therefore, it is important to set cleaning procedures that dissolve the biofilm and organic materials before applying disinfectants which inactivate and kill the microorganisms on the surface or after detachment (Zottola and Sasahara 1994). Poulsen (1999) suggested that insufficient cleaning may leave remaining organic matter which prevents the pass of disinfectants through the polymer layer, resulting in an ineffective disinfection.

a.Organic acids – acetic and lactic acid

Various antimicrobials have been used to treat meat products for the purpose of decreasing microorganisms (USDA-FSIS 2010, FSIS Directive 7120.1). For example, acetic and lactic acid are used for decontamination in the meat processing environment (Berry and Cutter 2000; Gordon Grrer and Dilts 1995). The antimicrobial activity of such organic acids is related to pH and undissociated form of the acid (Davidson and Taylor 2007); most organic acids can be used in food with pH < 5.5 and pKa 3.0–5.0 (Doores 2005). Organic acids influence synthesis of prokaryotic cell wall, interfere with protein synthesis and genetic mechanism. The undissociated form of organic acids penetrates lipid bilayer of the cell membrane, and become dissociated form inside the cell with generating protons because the cell interior of bacterial cells maintains neutral pH. The protons acidify the cytoplasm, and are extruded to the exterior of cells, which generates an electrochemical potential across the membrane (Mitchell and Moyle 1969). This interruption of cell membrane function may result in interference with the function of membrane-bound protein, which inhibits cell growth. High concentrations of anions in the cytoplasm of the cells increase osmolarity and interference with metabolic processes (Paul and Hirshfield 2003).

Acetic acid (CH_3COOH) is one of the oldest antimicrobials used in food with pKa 4.75 and is effective to inhibit yeasts and bacteria including *Bacillus* spp., *Campylobacter jejuni*, *Clostridium* spp., *E. coli*, *L. monocytogenes*, *Pseudomonas* spp., *Salmonella*, and *Staphylococcus aureus* (Davidson and Taylor 2007). Lactic acid ($CH_3CHOHCOOH$; pKa 3.79) is used as an antimicrobial, pH control agent, and flavoring agent in food products, and has the efficacy to reduce or eliminate pathogens on meat and poultry carcasses (Barboza de Martinez *et al.* 2002;

Davidson and Taylor 2007; Smulders et al. 1986, Snijders et al. 1985). Quantitatively, use of 2% lactic acid resulted in 0.5 log reductions in *E. coli* O157:H7 on lean beef, while 1, 3, 5% acetic or lactic acid caused 1 to 2 log reduction of same bacteria on lean beef (Cutter and Siragusa 1994; Siragusa and Dickson 1993). Another study showed that *E. coli* O157:H7 inoculated on beef trim pieces was reduced by 0.1 log CFU/g by spray with 2% acetic or lactic acid in a model ground beef production scheme (Conner *et al.* 1997). Treatments with acetic acid reduced the cell population of *Listeria* biofilm cells on the fiberglass disks by 3.8 logs (Ammor *et al*. 2004). In addition, the biofilm cells of *Salmonella* and *Listeria* on stainless steel were reduced by 1.31 and 0.47 log CFU/cm^2 when they were treated with lactic acid (8 mmol/liter, pH 3) for 60 min (Chorianopoulos *et al*. 2008).

b.Detergents

In food processing and handling operations, routine cleaning programs utilize detergents which are composed of mixtures of ingredients reacting with food soils, unwanted matter on food contact surface. Detergents make food soils more soluble and easier to be removed (Schmidt 1997). Chemically active ingredients in detergents can be classified into four basic categories including alkalis, acids, water conditioners and oxidizing agents (Schmidt 1997) (Table 2.3).

Alkaline detergents can be caustic or non-caustic. Caustic alkalis are sodium hydroxide or potassium hydroxide. Sodium hydroxide is a typical alkaline detergent possessing dissolution and saponification properties, which can act both on protein and greases and has bacteriocidal

power (Holah 2003). Under incorrect usage, it is corrosive to metals or cause severe chemical burns on skin. The exposure to 100% sodium hydroxide and potassium hydroxide at 23ºC for 30 min reduced the population of *E. coli* O157:H7 by 6.44 and 3.78 log CFU/ml (Sharma and Beuchat 2004). The wash with 1% potassium hydroxide having pH 13 for 1 min reduced the cell population of *E. coli* on poultry carcasses from 3.14 ±0.42 to none detectable level (Hinton and Ingram 2006). Sodium hydroxide at pH 10 on the surface of stainless steel inhibits the attachment of *Pseuddomonas fragi*, and removes the attached cells at a significant level (Herald and Zottola 1989). Antoniou and Frank (2005) observed that the treatment with 6% sodium hydroxide at 66 ºC for 3 min reduced the coverage of polysaccharide produced by *Pseudomonase putida* on stainless steel surface, and the polysaccharide coverage was reduced from 22.8-23.3 % area to 0.02-1.3 % area. Non-caustic detergents such as sodium carbonate and trisodium phosphate showed good dissolving and saponification properties, but had weak bactericidal activity (Sansebastiano *et al.* 2007).

Inorganic acids have very weak dispersing, emulsifying, and saponification properties. Strong inorganic acids such as nitric, hydrochloric, hydrofluoric, and sulphuric acid, however, are highly corrosive, thus of the limited use in the food industry. Weaker acids such as phosphoric acid and sulfamic acid are less corrosive. So, they are effective to remove mineral incrustations such as milkstone formed during pasteurizers (Hayes 1992; Sansebastiano *et al.* 2007). In contrast, organic acids are weaker and safe to handle, and also have bacriostatic activity (Sansebastiano *et al.* 2007).

Table 2.3. Classification of detergents by chemically active ingredients.

Category of detergents		Examples of detergents
Alkaline	Caustic	Sodium hydroxide , Potassium hydroxide
	Non-caustic	Sodium, potassium or ammonium salts of phosphates, silicates, carbonates
Acid	Organic	Hydroxyacetic, citric, gluconic
	Inorganic	Phosphoric, nitric, sulfamic, sodium acid sulfate, hydrochloric
Water conditioners	Sequestering agents	Sodium tripolyphosphate, tetra-potassiumpyrophosphate, organo-phosphates, polyelectrolytes
	Chelating agents	Sodium gluconate, ethylene diamine tetracetic acid (EDTA)
Oxidizing agents	Chlorinated detergents	

Modified from Schmidt (1997).

Water conditioners are used to prevent not only the precipitation of mineral salts such as magnesium and calcium salts in water, but also the formation of incrustations. They can be classified into sequestering agents forming soluble complexes with calcium and magnesium and chelating agents (Schmidt 1997). Among sequestering agents, sodium polyphosphates have good emulsifying properties and capabilities of dissolution and dispersion. Tetrasodium pyrophosphate can remove magnesium, while sodium tripolyphosphate and sodium tetraphosphate remove calcium and magnesium (Sansebastiano *et al.* 2007). In chelating agents, EDTA (ethylendiaminotetracetic acid) and NTA (nitrilotriacetic acid) are organic water

conditioners and are widely used because they have high solubility in liquid detergents and are cost effective (Sansebastiano *et al*. 2007).

Oxidizing agents frequently used in detergents are chlorine compounds, particularly hypochlorite. Chlorine is the most commonly used sanitizer in food processing, and its compounds includes liquid chlorine, hypochlorites, inorganic chloramines and organic chloramines (Schmidt 1997). The antimicrobial activity of hypochlorite acts on cell wall (Pulvertaft and Lumb 1948), membrane (Chang 1944), DNA (Haas and Engelbrecht 1980), as well as sulfhydrylenzyme (Green and Stumpf 1946). Chlorine (HOCl) liberates oxygen combining with components of cell protoplasm, causing the oxidation of protoplasm in the bacterial cells (Chang 1944). Chlorine also combines with proteins of cell membranes, which interfere with cell metabolism (Baker 1959), and its oxidative action affects enzymatic reactions such as SH group of enzyme, inhibiting glucose oxidation (Green and Stumpf 1946; Knox *et al*. 1948). Chlorine is more effective in the undissociated form (HOCl) than the dissociated form (OCl-) (Morris, 1966), and the antimicrobial activity of chlorinated water depends on the amount of HOCl (Beuchat and Ryu 1997). In addition, the dissociation of hypochlorous acid depends on the pH of the solution. The presence of HOCl is approximately 75% at pH 7.0, and the percentage decreased at increasing pH (Baker 1959). In alkaline detergent, OCl- is a contributing factor in disinfection by forming HOCl (Chang 1944).

$$Cl_2 + H_2O \rightarrow HOCl + H^+ + Cl^-$$

$$HOCl \leftrightarrow H^+ + OCl^-$$

HOCl liberates oxygen radicals, which in turn combines with components of cell protoplasm and thus destroys the microorganisms through oxidation of protoplasms (Chang 1944). It has been reported that chlorine reduced the populations of *E. coli* O157:H7, *Salmonella* and *L. monocytogens* (Rice *et al*. 1999; Ukuku and Fett 2002). Chlorine can also inactivate biofilm cells (Frank *et al*. 2003; Rossoni and Gaylade 2000; Sharma *et al*. 2005). For instance, Rossoni and Gaylarde (2002) reported that treatments with hypochlorite (100 or 200 mg/L) for 10 min reduced adhered cells of *E. coli*, *Pseudomonas*, and *Staphyloccoccus aureus* on stainless steel surfaces from 118.5, 52.0, and 28.0 to 1.0, 0.0 and 0.0 (cells/mm^2), respectively. Sharma *et al*. (2005) observed significant reduction in population of *E. coli* O157:H7 on stainless steel by treatments with 50 and 100 µg/ml of free chlorine for 1 min. Sodium hypochlorite (200 ppm) reduced populations of *Listeria monocytogenes* biofilm coated with 365 mg fat and 4 mg protein on the surface of stainless steel by 4.75 log/ 50 cm^2 in average (Frank *et al*. 2003).

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CHAPTER 3

INFLUENCE OF SANITIZING AND SUBSEQUENT NEUTRALIZATION TREATMENTS ON THE FATE OF SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* [1]

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ABSTRACT

This study evaluated the efficacies of Dey-Engley (DE) neutralizing broth (single or double strength), morpholinepropanesulfonic acid (MOPS) buffer, phosphate buffered saline (PBS) and sodium thiosulfate buffer on deviating the activities of 2% acetic or lactic acid and a manufacturer-recommended concentration of an alkaline or acidic detergent against Shiga toxin producing *Escherichia coli* (n = 9). To evaluate the possible toxicity of the neutralizing agents, cells of STEC strains were treated with each of the neutralizing agents, respectively at room temperature for 10 min. Neutralizing efficacies of the agents were evaluated by placing the cells of individual STEC in the mixture of a sanitizer and a neutralizer under the same condition. The evaluated neutralizing agents did not have detectable toxicity against selected STEC cells, and PBS was least effective in neutralizing the activities of selected organic acids and detergents. Single strength DE and sodium thiosulfate neutralized the antimicrobial activities of 2% acetic and lactic acid against all nine STEC strains used in the study. Although MOPS buffer neutralized the activity of 2% acetic acid, it was only partially effective in neutralizing the activity of 2% lactic acid. The evaluated agents failed to neutralize the antimicrobial activities of the commercial detergents against all the STEC strains used in the study. Further study revealed that double strength DE broth effectively neutralized the antimicrobial activities of the two commercial detergents with no detectable toxicities. Results suggest that the buffers/broth intended for neutralizing the antimicrobial activities of sanitizers should be appropriately evaluated before being used.

Shiga toxin-producing *Escherichia coli* (STEC) is a group of pathogens that are capable of producing potent cytotoxins know as Shiga toxins or verotoxins. The symptoms of STEC-related diseases range from mild diarrhea to hemorrhagic colitis and hemolytic uraemic syndrome (*12, 16*). The pathogen is commonly found in farms and wild animals, as well as infected humans. Although the major reservoir of STEC has been reported as cattle (*1*), various types of food in addition to beef have been contaminated with STEC during processing and subsequence handling (*15*).

Sanitizers, such as organic acids and detergents are often used for cleaning and decontaminating surfaces in food processing environments. Before industry application, the efficacies of the sanitizers in inactivating bacterial cells are usually evaluated in laboratory settings. In order to precisely estimate the antimicrobial activity of a sanitizer, a bacterial cell suspension is mixed with a neutralizing agent immediately after the inactivation treatment. The neutralizer discontinues the chemical reaction that leads to the inactivation of bacterial cells. The antimicrobial activity of the sanitizer can be overestimated if the neutralizer is not immediately applied after the inactivation treatment.

Various chemical agents have been used to neutralize the antimicrobial activity of sanitizers (*26*). A suitable neutralizer should be free of toxicity towards bacterial cells and be able to deviate the antimicrobial activity of a particular chemical when co-present in an assay system (*23*, *25*). The objective of this study was to evaluate the potential toxicity and capacities of four different buffers/broth in neutralizing the antimicrobial activities of selected organic acids and commercial detergents.

MATERIALS AND METHODS

STEC strains and growth conditions. Nine STEC strains were used in the study, which included 5-11, 6-8, 6-35, 7-17, 7-49, 7-50, 7-51, 7-52 and 7-57. These strains were of human origin and produced at least one Shiga toxin. Some of the strains were positive for *eaeA* and *hlyA*, two important virulence genes of STEC. Cell cultures of the STEC strains were grown for 18 h at 37ºC on tryptic soy agar (TSA; Difco Laboratories, Detroit, MI). The cultures were harvest with 15 ml of 0.85% of saline. Cell suspensions were diluted with 0.85% of saline to a concentration of 10^5 - 10^6 CFU/ml before being used in the following experiments.

Neutralizing agents. Four neutralizing agents were initially used in the study, which included single strength Dey-Engley (DE) neutralizing broth, morpholinepropanesulfonic acid (MOPS) buffer, phosphate buffered saline (PBS) and sodium thiosulfate buffer. Double strength DE neutralizing broth was later added into the study. Thirty nine or seventy eight grams of DE broth base (Sigma Inc., St. Louis, MO, U.S.) was suspended in 1 liter of distilled water to prepare single or double strength DE buffer, respectively. The pH of the DE buffer was adjusted to 7.6 using 1 N NaOH. The MOPS buffer (0.05 M) was prepared by suspending 2.23 g of MOPS (Fisher Scientific, Fair Lawn, NJ U.S.) in 500 ml of distilled water. The PBS contained sodium chloride (7.65 g/liter), disodium phosphate (0.72 g/liter) and monopotassium phosphate (0.21 g/liter). The 0.1% sodium thiosulfate buffer was prepared by adding 4 ml of a 0.1 N stock solution into 1 liter of buffered peptone water (10 g/liter of peptone, 5 g/liter of sodium chloride, 3.5 g/liter of disodium phosphate, and 1.5 g/liter mono potassium phosphate). The pH of the MOPS, PBS and sodium thiosulfate buffers was adjusted to 7.4 using 1 N NaOH. All the

chemicals used to prepare the PBS and sodium thiosulfate buffers were purchased from Fisher Scientific.

Organic acids and commercial detergents. Two organic acids were used in the study, which included acetic acid glacial (99.9%) (JT Barker, Phillipsburg, NJ) and DL-lactic acid (87.9%) (PURAC America Inc. IL). Both the acids were diluted in distilled water to reach a concentration of 2% (v/v), and the pH of the acetic and lactic acid solutions was 2.54 and 2.10, respectively. Two commercial detergents were also used, and one of the detergents was alkaline (pH 13; Ecolab, St. Paul, MN) while the other was acidic (pH 1.0-1.5; ZEP Manufacturing Co., Atlanta, GA). Alkaline detergent included potassium hydroxide and potassium hypochlorite, while the acidic detergent had phosphoric acid. The working solutions of the etergents were prepared by following the manufacturers' instructions. Ten ml of the alkaline product was diluted in 368 ml of distilled water. The working solution of the acidic detergent was prepared by diluting the commercial product 4 fold with distilled water.

Potentail toxicity of the neutralizing agents. To verify that the neutralizing agents evaluated in the study had no bactericidal activities, 1 ml of 0.85% saline was mixed with 9 ml of neutralizing buffer. The samples were mixed well using vortexing and left at room temperature for 10 min. Subsequently, 0.1 ml of each STEC cell suspension described above was added. The final concentration of cells was approximately 10^3- 10^4 CFU/ml. The neutralizing buffer and STEC cell mixtures and their controls were kept for at room temperature for additional 10 min. Cells suspended in 10 ml of 0.85% saline were used as controls. The samples were then diluted in 0.1% buffered peptone water. The last three dilutions (0.1 ml) were plated in duplicate

on TSA plates, and the inoculated plates were incubated for 24 h at 37^{o}C before colonies were enumerated.

Neutralizing capacity of selected agents. To evaluate the neutralizing capacity of selected neutralizing agents, 1 ml of each organic acid or commercial detergent described above was mixed with 9 ml of each neutralizing buffer, respectively. The neutralizing buffer and sanitizer mixture was kept at room temperature for 10 min before 0.1 ml of each STEC cell suspension was added. Cells of STEC in the mixture of neutralizing buffer and tested sanitizer were kept for 10 min at room temperature before serial dilutions was made in 0.1% buffered peptone water. The samples (0.1 ml) were then plated in duplicate on TSA plates. Colonies of STEC cell were enumerated after a 24 h inoculation period at 37^{o}C. Samples tested negative in the plate count assay were further analyzed, and two 0.5 ml samples were plated on TSA plates, and the inoculated plates were incubated and enumerated according to what was described above (Detection limit < 2 CFU/ml or < 0.30 log CFU/ml). Two control samples were included in the study. The first one contained 1 ml of a tested sanitizer, 9 ml of 0.85% saline, and 0.1 ml of a STEC cell suspension, while the second one contained 10 ml of 0.85% saline and 0.1 ml of a STEC cell suspension.

Statistical analysis. Each sample in the conducted experiments had a duplicate, and each experiment was repeated at least three times. Toxicity and neutralizing capacity of a evaluated agent were estimated by comparing the cell populations of a STEC strain exposed to a neutralizer alone and the mixture of neutralizer and sanitizer, respectively with the cell populations of the STEC strain in the control samples. The differences in cell populations among various samples were analyzed using one way analysis of variance (ANOVA) followed

by Turkey's HSD test using the JMP software (version 8) (SAS Institute, Inc. NC, USA) based on a 95% confidence interval.

RESULTS

Cell populations of all STEC strains in the evaluated neutralizing buffers/broth were not significantly different ($P < 0.05$) from those in the control samples (Data not shown), indicating that the neutralizing agents did not have toxicity towards the STEC. The populations of STEC cells exposed to MOPS buffer, PBS, Sodium thiosulfate buffer or single strength DE broth were approximately 0.01-0.09 log CFU/ml different from the cell populations in the control samples (Data not shown).

Treatment with 2% acetic acid significantly ($P < 0.05$) reduced the cell populations of six STEC strains used in the study from 3.08-3.22 log CFU/ml to 1.38-2.76 log CFU/ml (Table 3.1). It was found that sodium thiosulfate buffer, single strength DE broth and MOPS buffer successfully neutralized the antimicrobial activities of 2% acetic acid used in the present study, and populations of cells treated with 2% acetic acid and neutralized with the three agents were not significantly different from the cell populations in the control samples (Table 3.1). The PBS buffer however, only significantly neutralized the antimicrobial activities of 2% acetic acid against six of the STEC strains used.

Treatments with 2% lactic acid significantly reduced the cell populations of seven of the nine STEC strains used in the study (Table 3.2). Single strength DE broth and sodium thiosulfate buffer neutralized the antimicrobial activities of 2% lactic acid against all the STEC

strains used in the study. MOPS buffer neutralized the activities of 2% lactic acid against seven out of the nine STEC strains used in the study. The PBS however, only neutralized the antimicrobial activities of 2% lactic acid against two of the STEC.

Treatments with both the alkaline and acidic detergents significantly inactivated the cells of STEC strains (Table 3.3 and 3.4). Single strength DE broth, MOPS buffer and sodium thiosulfate buffer neutralized the antimicrobial activities of the alkaline detergent against six, four, and six out of the nine STEC strains used in the study (Table 3.3). The PBS however, only neutralized the antimicrobial activities of the alkaline detergent against one of the STEC strains. Single strength DE broth and sodium thiosulfate buffer neutralized the antimicrobial activities of the acidic detergent against three and two of the STEC strains, respectively (Table 3.4). The other two evaluated agents each neutralized the activity of the acidic detergent against one of the STEC strains.

2% lactic acid was more effective to inactivate the selected STEC strains comparing to 2% acetic acid. STEC 7-49 and 7-50, especially, were the most vulnerable against the antimicrobial activities of 2% acetic and lactic acid (Table 3.1 and 3.2). However, two commercial detergents were enough strong to inactivate all STEC strains (Table 3.3 and 3.4). In PBS buffer, STEC 6-8, 7-17 and 7-51 were vulnerable against the antimicrobial activities of the selected organic acids and commercial detergents used in the current study. Otherwise, in MOPS buffer, STEC 7-51 and 7-57 did not recovered from the antimicrobial activities of both 2% lactic acid and acidic detergents having low pH (Table 3.1 and 3.2). Two buffer including MOPS and PBS buffers did not neutralize the antimicrobial activity of alkaline detergent against 5 and 8 STEC strains, and STEC 6-8 and 7-17 inactivated to undetectable level (Table 3.4).

Further study revealed that double strength DE broth effectively neutralized the antimicrobial activities of the two commercial detergents (Table 3.5). The cell populations in mixed solutions of one of the commercial detergents and double strength DE were not significantly different from those in the control samples (Table 3.5). Furthermore, the double strength neutralizing broth did not have detectable toxicity to STEC cells used in the study (Table 3.5).

DISCUSSION

Organic acids in undisassociated form can penetrate cell membrane lipid bilayer, converting to disassociated form after the penetration, and making cell cytoplasm relatively more acidic (*4*). As a result, the functions of bacterial membrane-bound proteins are inhibited (*17*). Phosphoric acid is the key antimicrobial component in the acidic detergent used in the study. The acid chelates metal ions in cell membranes, leading to the loss of membrane integrity and inhibition of cell division (*27*). It also lowers the pH of the detergent, acidifies cell cytoplasm, and interferes cell metabolisms as described above (*14*). Potassium hydroxide in the alkaline detergent raises the pH of the detergent. It changes cell membrane potential, pH gradient, internal pH, and proton motive force of bacterial cells (*18*). Potassium hypochlorite is another active component in the alkaline detergent, and the chemical oxidizes bacterial enzymes and nucleic acids (*20*).

The use of an appropriate neutralizer is critically important for evaluating the antimicrobial efficacies of a sanitizer. The neutralizer inactivates the antimicrobial effect of the

sanitizers, allowing an accurate estimation of the recovery of surviving bacterial cells in a treatment system (*5*). If a neutralizing system is not used or ineffective, the carried-over sanitizer may inhibit the recovery of the tested microorganisms (*5).*

It is necessary that a neutralizing agent does not cause physicall or chemicall damage to the tested microorganisms because healthy and injured microorganism requires different conditions to grow (*23*). The present study selected and evaluated different agents as possible systems to neutralize the activities of selected organic acids and commercial detergents used in the study. Some of these systems have been previously used in biological, non food related research (*5, 8, 13, 25*). MOPS buffer has previously been used as a buffering system to neutralize organic acids such as acetic, malic, citric, lactic and peroxyacetic acid (*2, 9*). The amine group in the morpholine of MOPS reacts with organic and inorganic acids and forms morpholinium salts. Results of current study revealed that MOPS buffer successfully neutralized the antimicrobial activity of 2% acetic acid against all STEC strains used in the study (Table 3.1). However, it only neutralized the activity 2% lactic acid against some tested strains of STEC (Table 3.2). MOPS has been used as an excellent buffer for biological systems with near-neutral pH, and morpholine composing of MOPS can neutralize by reacting with chlorine, resulting in forming 4-chloromorpholine (*10*). However it was not adequate in neutralizing the extreme pH in alkaline and acidic detergents used in the current study. PBS is a commonly used buffer to maintain pH and osmolarity in biological research projects (*7*, *19*). It has been used by Shakeri et al. (*21*) to neutralize hydrogen peroxide (1-1000 ppm, for 1 h at 30ºC treatments) against biofilm formed by *Acinetobacter* sp. In the present study, PBS was proven to be less effective comparing with other systems in neutralizing the antimicrobial activity of organic acids and

essentially ineffective in neutralizing the antimicrobial activities of the commercial detergents used in the study. In current study, sodium thiosulfate buffer was prepared in buffered peptone water (BPW), for serving as buffering systems against an effect of low/ high pH of organic acids or detergents (*13*). Kemp and Schneider (*13*) evaluated the ability of 0.1% thiosulfate prepared in BPW chemically to inactivate acidified sodium chlorite (ASC). The pH of mixture solution with ASC and 0.1% thiosulfate in BPW was remained ranging 7.33-6.55 when chlorite concentration of ASC was increased from 0 to 133 ppm. In addition, thiosulfate in BPW neutralized chlorite of ASC and the residual chlorite was 0%, despite the concentration of ASC was increased. Sodium thiosulfate buffer has been used as a neutralizer to inactivate the antimicrobial activities of hypochlorite (*21*), acidified sodium chlorite (*13*) and alkaline hypochlorite (*24*) through the oxidation of thiosulfate to sulfate (*11*). The DE broth contains sodium thiosulfate in addition to sodium thioglycollate, sodium bisulphate, soya lecithin and polysorbate (*5*). The broth has been utilized to neutralize disinfectants such as chlorite (*5, 22*), phenolics (*5*) and quaternary ammonium (6*, 22*). Caldwell et al. (*3*) showed that pH for each sanitizer solution including chlorine (0-500 µg/ml), hydrogen peroxide, acetic acid, citric acid, and lactic acid (0-2%) were neutralized from 2.16-6.95 to 5.03-8.71 by addition with DE neutralizing broth. Results of the current study revealed that although the single strength DE broth could neutralize the antimicrobial activity of 2% acetic and lactic acid and commercial detergents against some of the STEC strains used in the study, complete neutralization of evaluated sanitizers required the presence of DE broth in double strength. The study concluded that selection of a suitable neutralizer is important and the neutralizer should be evaluated before being used.

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Table 3.1. *Cell populations of STEC (log CFU/ml) in the mixture of acetic acid (2%) and neutralizing agents, respectively after a treatment at 28°C for 10 min*

Treatment	Neutralizers	Cell populations (log CFU/ml)								
		STEC strains								
		5-11	6-8	6-35	7-17	7-49	7-50	7-51	7-52	7-57
Controls†		3.08a	3.11a	3.21a	3.16a	3.22a	3.15a	3.19a	3.10a	3.17a
	Nuetralizer controls††	2.48b	1.79b	2.54a	1.38b	2.76a	2.52a	1.89b	2.48b	2.50b
	Dey-Engley broth	3.10a	3.06a	2.85a	3.16a	3.15a	3.11a	3.10a	3.10a	3.14a
2% Acetic acid	Morpholinepropanesulfonic acid	3.00a	3.03a	3.06a	3.11a	3.15a	3.08a	3.11a	3.04a	3.01a
	Phosphate buffered saline	2.95a	1.82b	3.05a	2.36ab	2.21a	2.39a	1.94b	2.97a	2.93a
	Sodium thiosulfate	3.00a	3.00a	3.08a	3.12a	3.13a	3.16a	3.10a	2.97a	3.03a

† Controls are the samples which were not treated with neutralizer and acetic acid.

†† Neutralizer controls are samples which were treated with acetic acid but not treated with neutralizers.

Means in the same column not followed by the same letters are significantly different ($P < 0.05$).

Table 3.2. . *Cell populations of STEC (log CFU/ml) in the mixture of lactic acid (2%) and neutralizing agents, respectively after a treatment at 28°C for 10 min*

Treatments	Neutralizers	Cell populations (log CFU/ml)								
		STEC strains								
		5-11	6-8	6-35	7-17	7-49	7-50	7-51	7-52	7-57
Controls†		3.07a	3.11a	3.21a	3.16a	3.22a	3.15a	3.19a	3.10a	3.17a
	Nuetralizer controls††	1.75b	1.29b	1.35b	0.97b	2.42a	2.81a	1.92b	2.08b	1.67b
	Dey-Engley broth	3.05a	3.13a	3.18a	2.19a	2.32a	3.12a	3.14a	3.05a	3.06a
2% Lactic acid	Morpholinepropanesulfonic acid	3.03a	3.07a	3.12a	3.18a	3.17a	3.17a	3.01ab	3.00a	2.34ab
	Phosphate buffered saline	3.01a	1.11b	2.09ab	2.12ab	1.95b	1.19b	1.23b	2.93a	2.95ab
	Sodium thiosulfate	2.99a	3.07a	3.14a	3.06a	3.09a	3.08a	3.08a	3.01a	3.05a

† Controls are samples which were not treated with neutralizer and lactic acid.

†† Neutralizer controls are samples which were treated with lactic acid but not treated with neutralizers.

Means in the same column not followed by the same letters are significantly different ($P < 0.05$).

Table 3.3. *Cell populations of STEC (log CFU/ml) in the mixture of alkaline detergent and neutralizing agents, respectively after a treatment at 28°C for 10 min*

Treatments	Neutralizers	Cell populations (log CFU/ml)								
		STEC strains								
		5-11	6-8	6-35	7-17	7-49	7-50	7-51	7-52	7-57
Controls[†]		3.08a	3.11a	3.21a	3.16a	3.22a	3.15a	3.19a	3.10a	3.17a
	Nuetralizer controls[††]	< 0.30[†††]b	< 0.30c	< 0.30c	< 0.30b	< 0.30b	< 0.30c	< 0.30b	< 0.30c	< 0.30c
Alkaline detergent	Dey-Engley broth	3.06a	2.96a	2.20abc	2.11ab	2.03ab	3.00a	3.07a	3.10a	3.11a
	Morpholinepropanesulfonic acid	2.99a	2.31b	2.12abc	1.12b	2.10ab	2.10ab	3.02a	2.95a	2.99a
	Phosphate buffered saline	2.74a	0.46c	1.23bc	0.93b	1.47ab	1.08ab	0.81b	2.25b	2.31b
	Sodium thiosulfate	2.99a	2.73ab	2.75ab	2.00ab	2.47a	2.72a	2.68a	2.99a	2.99a

[†] Controls are samples which were not treated with neutralizer and alkaline detergent.
[††] Neutralizer controls are samples which were treated with alkaline detergent but not treated with neutralizers.
[†††] Detection limit of plate count assay (< 0.30 log CFU/ml).
Means in the same column not followed by the same letters are significantly different ($P < 0.05$).

Table 3.4. *Cell populations of STEC (log CFU/ml) in the mixture of acidic detergent and neutralizing agents, respectively after a treatment at 28°C for 10 min*

Treatments	Neutralizers	Cell populations (log CFU/ml)								
		STEC strains								
		5-11	6-8	6-35	7-17	7-49	7-50	7-51	7-52	7-57
Controls†		3.08a	3.11a	3.21a	3.16a	3.22a	3.15a	3.19a	3.10a	3.17a
	Nuetralizer controls††	< 0.30†††b	< 0.30b	< 0.30b	< 0.30b	< 0.30b	< 0.30c	< 0.30b	< 0.30c	< 0.30c
	Dey-Engley broth	3.04a	1.41ab	2.00ab	1.83ab	1.95ab	2.81a	1.944ab	3.07a	3.04ab
Acidic detergent	Morpholinepropanesulfonic acid	2.60a	< 0.30b	0.78ab	< 0.30b	0.65b	0.46c	0.46b	2.78ab	2.61b
	Phosphate buffered saline	2.54a	< 0.30b	1.11ab	< 0.30b	0.60b	1.08bc	1.09ab	2.60b	2.55b
	Sodium thiosulfate	3.01a	1.54ab	1.84ab	1.59ab	1.19ab	2.45ab	1.91ab	3.03a	3.06ab

†Controls are samples which were not treated with neutralizer and acidic detergent.

†† Neutralizer controls are samples which were treated with acidic detergent but not treated with neutralizers.

†††: Detection limit of plate count assay (< 0.30 log CFU/ml).

Means in the same column not followed by the same letters are significantly different ($P < 0.05$).

Table 3.5. *Cell populations of STEC (log CFU/ml) in the mixture of alkaline and acidic detergents and double strength Dey-Engley broth (DE), respectively after a treatment at 28°C for 10 min*

Treatments	Cell populations (log CFU/ml)								
	STEC strains								
	5-11	6-8	6-35	7-17	7-49	7-50	7-51	7-52	7-57
Control†	3.61a	3.57a	3.56a	3.62a	3.63a	3.57a	3.61a	3.63a	3.66a
Neutralizer control-Alkaline detergent††	<0.30b	<0.30b	<0.30b	<0.30b	<0.30b	<0.30b	<0.30b	<0.30b	<0.30b
Neutralizer control-acidic detergent	<0.30b	<0.30b	<0.30b	<0.30b	<0.30b	<0.30b	<0.30b	<0.30b	<0.30b
DE†††	3.56a	3.53a	3.53a	3.62a	3.63a	3.53a	3.56a	3.58a	3.60a
Alkaline detergent and DE	3.54a	3.52a	3.54a	3.63a	3.61a	3.51a	3.55a	3.57a	3.59a
Acidic detergent and DE	3.54a	3.52a	3.59a	3.60a	3.59a	3.51a	3.51a	3.55a	3.57a

†Controls are samples which were not treated with detergents and double strength Dey-Engley broth.
†† Neutralizer controls are samples which were treated with detergents but were not treated with neutralizers.
††† Sample treated with double strength DE showed the toxicity of neutralizers to STEC cells.

CHAPTER 4

INFLUENCE OF SELECTED ENZYMATIC AND CHEMICAL TREATMENTS IN CELLULOSE AND INACTIVATING SHIGA TOXIN PRODUCING *ESCHERICHIA COLI* [1]

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ABSTRACT

This study evaluated the effectiveness of selected enzymatic and chemical treatments in degrading cellulose and inactivating Shiga toxin producing *E. coli* (STEC). Six cellulose producing STEC strains were treated with cellulase (0.51 and 3.83 U/15 ml), acetic and lactic acid (2 and 4%), and an acidic and alkaline commercial detergent (manufacturer recommended concentrations), respectively under appropriate conditions. Following each treatment, residual amounts of cellulose and surviving populations of STEC were determined. Treatments with acetic and lactic acid significantly reduced ($P < 0.05$) the average populations of STEC, and treatments excluding 2% acetic acid also significantly decreased the amounts of cellulose produced by STEC cells. The residual amounts of cellulose positively correlated to the surviving populations of STEC strains after the treatments with organic acids (r = 0.64-0.94), and significance of the correlations ranged from 83 to 99%. Treatments with cellulase and detergents both degraded cellulose. However, treatments with cellulase had no influence on the survival of STEC cells and those with the detergents reduced the cell populations of STEC to undetectable levels. Thus, by these two treatments, correlations between the residual amounts of cellulose and the surviving populations of STEC could not be observed. The results demonstrated that the selected enzymatic and chemical agents reduced cellulose, and the treatments with organic acids and detergents also inactivated the cells of STEC. The efficacies of organic acids in inactivating cellulose producing cells of STEC might be related to their ability to reduce cellulose which remained around cell surface of STEC.

Shiga toxin producing *Escherichia coli* (STEC) are enteropathogens producing one or more toxins related to Shiga toxins of *Shigella dysenteriae* serotype 1 (16, 21, 22). The pathogens cause human illness ranging from mild diarrhea to severe hemorrhagic colitis and hemolytic uremic syndrome (3, 11, 15, 23). The reservoirs of STEC are ruminants such as cattle, sheep, goats, and etc., but cattle have been identified as the predominant source of STEC (1, 17). STEC infection can be transmitted through contaminated foods, especially raw and undercooked foods of animal origin (7, 18, 20, 36).

Cells of STEC produce cellulose as an extracellular component (5, 48). The cellulose is a long chain polymer of glucose, which is insoluble, inelastic, and has a high tensile strength (25, 46). The polymer forms subfibrils and crystallizes into microfibrils (12). The fibrils subsequently form insoluble layered sheets and build hydrogen bonding networks (25). Cellulose producing bacterial cells can be entrapped into the networks formed by cellulose (25, 43).

Cellulose is viscous and hydrophilic, and it protects bacterial cells from changes in moisture content, acidity, and the presence of toxic substrates in the environment (25). Bacterial cellulose has the capability to hold water over 100 times of its weight by the entangled network structure and hydrophilic properties (25, 29). Williams and Cannon (43) reported that the cellulose produced by *Acetobacter xylinum* grown on rotting fruits protected the cells of the bacterium from the detrimental effect of UV light. Cellulose along with curli, a protein projection on cell surface, protected the cells of *Salmonella* from desiccation and treatment with sodium hypochlorite at a concentration up to 30 ppm (41). Similar results were also observed by Solano et al (33). The mechanical and chemical protection exerted by cellulose to bacteria cells

may make the sanitary or cleaning practices in the food processing environment a greater challenge. The objectives of this study were to evaluate the effectiveness of selected enzymatic and chemical agents in degrading cellulose and inactivating the cells of STEC, and to determine the correlations between the residual amounts of cellulose and the surviving cell populations of STEC after the enzymatic and chemical treatments.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Six strains of STEC (6-8, 6-35, 7-17, 7-49, 7-50, and 7-51), were used in the study. Cultures of the STEC strains were grown on Luria Bertani no salt agar (LBNS; 10 g tryptone, 5 g yeast extract, and 15 g agar per liter) at 28ºC for 72 h. The resulting cultures were used in the following experiments.

Enzymatic hydrolysis of cellulose produced by STEC cells. Cellulase of *Aspergillus niger* (1.02 U/mg; Sigma-Aldrich Inc., St. Louis, MO) was dissolved in sterile distilled water (5 g in 10 ml). The enzyme solution was dialyzed in sterile distilled water at refrigeration temperature for 18 h. The concentration of the dialyzed cellulase solution was determined to be 6.25%. The STEC cultures on the surface of LBNS agar plates were treated with 15 ml of 0.05 M sodium acetate buffer (pH 5.0) containing different concentrations of cellulase (0.51, 2.12, or 3.83 U/15 ml). STEC cultures treated with 15 ml of sodium acetate buffer without cellulase were included in the study. The treatments were conducted at 37ºC for 2 h with gentle shaking.

Acidic treatments of cellulose produced by STEC cells. The acids used in the study included acetic (JT barker, Phillipsburg, NJ) and lactic (Purac America inc. Lincolnshire, IL) acid. Each of the STEC cultures on the surface of LBNS agar plates was treated with 15 ml of 2

or 4 % acetic and lactic acid, respectively. STEC cultures treated with 15 ml of sterile distilled water were used as controls. The treatments were conducted at room temperature for 20 min with gentle shaking.

Treatments using commercial detergents of cellulose. Two commercial detergents were used to reduce cellulose which were produced by STEC cells. One of the detergents was alkaline (pH 13; Ecolab, St. Paul, MN) while the other one was acidic (pH 1.0-1.5; ZEP Manufacturing Company, Atlanta, GA). The active ingredients in the alkaline detergent included potassium hydroxide, phosphoric acid, and potassium hypochlorite, while the acidic detergent had phosphoric acid as the active component. The working solutions of the detergents were prepared according to the manufacturers' recommendations. The alkaline detergent was diluted by mixing 10 ml of the commercial product with 368 ml of sterile water. The working solution of the acidic detergent was prepared by diluting the commercial product 4 fold with sterile water. Cells of the STEC cultures were treated with 15 ml of the diluted detergent solutions at room temperature for 7 and 15 min, respectively with gentle shaking. STEC cultures treated with the same volume of sterile distilled water were used as controls.

Quantification of cellulose. After each treatment, 10 ml of each treated and un-treated control cell suspension were collected for cellulose quantification using a colorimetric method developed by Updegraff (37) with modifications. The STEC cultures were placed in glass centrifuge tubes (17 x 118 mm) with conical bottoms. The cultures were then centrifuged at 3,400 *g* for 25 min. The cell pellet of each culture was collected after the supernatant fluids were discarded. Three ml of an acetic-nitric reagent (150 ml 80% acetic acid and 15 ml concentrated nitric acid) were added to the cell pellet of each culture in the glass centrifuge tube and mixed

properly. The test tubes were covered with aluminum foil and placed in a boiling water bath for 30 min, after which the contents in the test tube were re-centrifuged at 3,400 *g* for 15 min. Following centrifugation, the supernatants were discarded and the pellets were washed twice with sterile distilled water. One ml of 67% sulfuric acid was then added to each centrifuge tube and allowed to stand for 1 h at room temperature. The acid solution in each centrifuge tube was diluted with 4 ml of distilled water and placed in an ice bath when 10 ml of cold anthrone reagent (0.2 g anthrone in 100 ml concentrated H_2SO_4) (Acros Organics, New Jersey, NJ, USA) was added. The centrifuge tubes were inverted gently and then placed in a boiling water bath for 16 min after which they were placed into an ice bath. The absorbance of each sample at 620 nm (A_{620}) was recorded using the Novaspec II Spectrophotometer. A standard curve of absorbance as a function of cellulose concentration was prepared. The quantities of cellulose remaining on the surface of STEC cells were calculated by comparing the absorbance values of the standard with the values of the tested samples. All reagents used in the quantification of cellulose were purchased from Fisher Scientific unless otherwise specified.

Influence of cellulose degradation treatments on the survival of STEC cells.

Immediately after the treatments described above, 1 ml of each treated STEC cell suspension and untreated control was mixed with 9 ml of double strength Dey-Engley (DE) buffer by following a previously reported protocol (28). The cell suspensions were left in the DE buffer at room temperature for 15 min before serial dilutions were made in 0.1% buffered peptone water. The last three dilutions of each cell suspension were plated in duplicate on tryptic soy agar (TSA) plates. The colonies on the surface of TSA plates were enumerated after 24 h incubation at 37°C.

Statistical analysis. All samples had duplicates and appropriate controls, and each experiment was repeated in three independent trials. Data obtained were analyzed using the general linear model of the Statistical Analysis Software (SAS) (28), following by t-test at a 95% confidence interval. Significant differences in the residual amounts of cellulose in the cells suspensions of the six STEC strains reflecting the efficacies of cellulose degradation achieved by different treatments were calculated by comparing the overall mean absorbance of the anthrone solutions at 620 nm. The influence of the treatments on the survival of the STEC cells was also determined using the same statistical protocol. Correlation coefficients between residual amounts of cellulose and surviving cell populations of individual STEC strains after each treatment were calculated using the JMP statistical software (10).

RESULTS

Expression of cellulose by the cells of selected STEC strains. The six STEC strains used in the study produced different amounts of cellulose. The amounts of cellulose produced by 6-8, 7-49, 7-50, and 7-51 were significantly ($P < 0.05$) higher than the amount of cellulose produced by 7-17 (Fig.4.1). Cells of 6-35 produced an average of 1.32 μg cellulose per 10^{10} cells, which was significantly ($P < 0.05$) lower than the amounts of cellulose produced by the cells of 6-8, 7-49, 7-50, and 7-51, but numerically ($P > 0.05$) higher than the amount of cellulose produced by the cells of 7-17 (Fig. 4.1). Cells of 7-51 produced the highest amount of cellulose among the six STEC strains evaluated in the study, and the amount of cellulose produced by the cells of 7-51 was approximately 5.58 times greater than the amount of cellulose produced by the cells of 7-17 (Fig. 4.1).

Treatments with cellulase. Treatments with cellulase significantly reduced ($P < 0.05$) the average amounts of cellulose which were produced by the six STEC strains (Table 4.1). The efficacy of the treatments increased as the concentration of cellulase increased (Fig 4.2). The average amounts of cellulose produced by the cells of 7-51 were reduced by 4.38, 4.94, and 5.09 μg per 10^{10} cells, respectively when the cells were treated with 0.50, 2.16, or 3.83 U/15 ml of celluase at 37°C for 2 h. The reductions in the average amounts of cellulose produced by 7-49 were relatively smaller, 0.39 and 2.10 μg per 10^{10} cells, respectively by the treatments with 0.50 or 2.16 U/15 ml of cellulase and 2.44 μg per 10^{10} cells by the treatments with 3.83 U/15 ml of celluase (Fig. 4.2). Furthermore, the treatments with 2.16 and 3.83 U/15 ml of cellulase reduced the average amount of cellulose produced by the cells of 7-50 by 3.00 and 3.09 μg per 10^{10} cells, respectively. Similar declining trends were also observed with the amounts of cellulose produced by strains of 6-8, 6-35, and 7-17 (Fig. 4.2). Treatments with cellulase did not have any influence on the survival of STEC cells (Table 4.2).

Treatments with organic acids. Treatments with 2 and 4% acetic as well as lactic acid significantly ($P < 0.05$) reduced the average populations of the six STEC strains (Table 4.1) as well as the populations of individual STEC strains used in the study except for 6-35 and 7-51 treated by 2% acetic acid (Table 4.2). Treatments with 4% acetic acid and both concentrations of lactic acid significantly reduced ($P < 0.05$) the average amounts of cellulose which were produced by the six STEC strains (Table 4.1). The average amounts of cellulose produced by the the six STEC strains were reduced by 0.61 and 1.07 μg per 10^{10} cells, respectively by the treatments with 2% acetic or lactic acid, and by 1.10 and 1.73 μg per 10^{10} cells, respectively by the treatments with 4% acetic or lactic acid (Table 4.1).

The average amounts of cellulose produced by the cells of 7-51 were reduced by 2.36 and 3.00 μg per 10^{10} cells, respectively by the treatments with 2% acetic or lactic acid (Fig. 4.3A and 4.3B). Treatments with 2% acetic acid reduced the average amounts of cellulose produced by the strains of 7-49 and 7-50 by 0.46 and 0.82 μg per 10^{10} cells, respectively (Fig. 4.3A) while the treatments with the same concentration of lactic acid reduced the average amounts of cellulose produced by the strains of 7-49 and 7-50 by 0.69 or 1.67 μg per 10^{10} cells (Fig. 4.3B). Treatments with 2% acetic or lactic acid reduced the average amounts of cellulose produced by 6-8 by 0.21 or 0.83 μg per 10^{10} cells (Fig. 4.3A and 4.3B). The average amounts of cellulose produced by 6-35 were reduced by 0.17 and 0.58 μg per 10^{10} cells, respectively by the treatments with 2% acetic or lactic acid (Fig. 4.3A and 4.3B). The average amounts of cellulose produced by 7-17 were reduced to 0.24 and 0.22 μg per 10^{10} cells by the treatments with 2% acid and lactic acid, respectively (Fig. 4.3A and 4.3B). Increasing the concentration of acetic and lactic acid from 2 to 4% further reduced the amounts of cellulose produced by the cells of the six individual STEC strains used in the study (Fig. 4.3A and 4.3B).

Treatments with detergents. Treatments with the two detergents reduced the average cell populations of the six STEC strains to undetectable levels (< 1.00 CFU/ml or 0.00 log CFU/ml) (Table 4.2). Treatment with the detergents was numerically ($P < 0.05$) more effective than those with the alkaline detergent in reducing cellulose (Table 4.1). The average amounts of cellulose produced by the six STEC strains were reduced by 0.85 and 1.35 μg per 10^{10} cells, respectively after the cells were treated by the acidic or alkaline detergents at room temperature for 7 min (Table 4.1). When the treatments were extended to 15 min, the average amounts of

cellulose produced by the six STEC strains were reduced by 1.62 and 2.01 μg per 10^{10} cells, respectively (Table 4.1).

Treatments with the alkaline detergent at room temperature for 7 min reduced the amounts of cellulose produced by 7-49 and 7-50 by 0.29 and 1.00 μg per 10^{10} cells, respectively. The treatments also reduced the average amounts of cellulose produced by the strains of 6-8, 6-35, and 7-51 by 0.62, 0.36, or 2.70 μg per 10^{10} cells (Fig. 4.4A). The amounts of cellulose produced by the cells of five out of the six STEC strains were below 1.0 μg per 10^{10} cells when the cells were treated with the acidic detergent for 15 min (Fig. 4.4B). Treatments with acidic detergent for 7 min reduced the average amounts of cellulose produced by 7-49 and 7-50 by 2.16 and 2.25 μg per 10^{10} cells, respectively. After the same treatment, the average amounts of cellulose produced by 6-8, 6-35, and 7-51 were reduced by 1.49, 0.39, and 1.61 μg per 10^{10} cells, respectively (Fig. 4.4B). Extending the treatment time from 7 to 15 min with the acidic and alkaline detergent did not significantly improved ($P > 0.05$) the efficacy of cellulose degradation (Table 4.1).

Correlations between residual amounts of cellulose and the surviving cell populations of STEC. As stated previously, treatments with cellulase and commercial detergents both degraded the cellulose produced by the cells of STEC. However, treatments with cellulase had no influence on the survival of STEC cells, and those with the commercial detergents decreased the populations of STEC cells to undetectable levels (Table 4.2). Correlations between the residual amounts of cellulose and surviving cell populations of STEC resulted from these treatments were therefore, not observed. Under the treatments with acetic and lactic acid however, the residual amounts of cellulose positively correlated to the surviving

cell populations of individual STEC strains, and the correlation coefficients (r) between the two parameters from the treatments with the two organic acids were in the range of 0.64 – 0.94 with confidence levels ranged from 97 to 99% (Table 4.3). The correlation coefficients (r) between the residual amounts of cellulose and surviving cell populations of 6-8, 7-50, and 7-51 under the treatments with the two organic acids were 0.85 – 0.94 with 97 – 99% confidence levels (Table 4.3). The confidence levels of correlations between the residual amounts of cellulose and surviving cell populations of 6-35 and 7-49 under the treatments with lactic acid were also in the range of 97 – 99%, while the confidence level of correlation was 94% for 7-17 under the treatment with lactic acid. The significances of correlations between the residual amounts of cellulose and surviving cell populations of 6-35, 7-17, and 7-49 under the treatments with acetic acid were 83-90% (Table 4.3).

DISCUSSION

Cellulose is a long polymeric chain of D-glucose monomers which are linearly linked together by β-(1-4) glucosidic bonds (25). The glycosidic bond is generated by the reaction between the hydroxyl group on a glucose molecule and the hemiacetal goup of another glucose molecule accompanying with the water loss (39). According to Stainslaw et al. (35), bacterial cellulose is relatively purer comparing to plant cellulose which is often associated with hemicelluloses and lignin. Previous studies have shown that bacterial cellulose produced by *Acetobacter xylinum* was hydrolyzed by cellulase from *Trichoderma* and chemical agents such as sodium hydroxide, hydrochloric acid (13, 26, 31, 32). The results of the present research showed that the cellulose produced by STEC were reduced by enzymatic cellulose hydrolysis, and

chemical treatments with acetic and lactic acids, as well as selected commercial acidic and alkaline detergents.

Cellulase is a cellulolytic enzyme which breaks down cellulose to glucose units by acting on glycosidic bonds. The enzyme is produced by several fungi including *Trichoderma* and *Aspergillus* as well as cellulolytic bacteria such as *Sporocytophga myxococcoides*, *Clostridium*, and *Acetivibrio* (44). There are three different classes of cellulose hydrolytic enzymes including endo-1,4- β-D glucanase (endo-cellulase), exo-1,4- β-D glucanase (exo-cellulase) , and β-glucosidase based on the modes of chemical reactions (2). The cellulase used in the present study was an endocellulase from *A. niger*, which randomly hydrolyses the chains of cellulose, reducing the length of cellulose chain or the degree of polyermization and releasing cellobiose and glucose (2, 4, 9). Hurst et al. (8) reported that purified cellulase from *A. niger* (0.25 μg) could hydrolyze 5 mg of cellulose to reducing sugars in 0.1 M sodium acetate buffer (pH 4.0) at 40°C. In the present study, treatment of STEC cell suspensions with the cellulase of *A. niger* significantly reduced the amounts of cellulose produced by STEC cells (Table 4.1). The efficacies of the enzymatic hydrolysis increased as the concentration of cellulase increased (Fig. 4.2).

Organic acids are commonly used as cleaning or sanitizing agents by the food industry (34). The results of present study showed that treatments with 4% acetic acid and both concentrations of lactic acid at pH 2.10- 2.54 significantly reduced the average amounts of cellulose produced by the cells of the six STEC strains. Acids break down the glycosidic bonds in cellulose polymers (45). Acid hydrolysis is initiated with the interaction between a proton from the acid and the glycosidic oxygen, forming a conjugate acid. The C-O bond is then

cleaved followed by the breakdown of the conjugate acid. Subsequent addition of water releases a free sugar and a proton (24, 45). Because of the stable structure of cellulose by hydrogen boding network and van der Waals interactions, however, acid hydrolysis require elevating temperature or concentration of acids. Otherwise, the relatively mild condition in the weak acid degrades the vulnerable glcosidic bond, resulting in weakeing the bond in polymers, and increasing solubility (47). It assumed, in the current study, that the reduction of cellulose produced by STEC strains may be occurred by degradation using the mild acid. Dupont and Tétreault (6) demonstrated that acetic acid (20 and 200 mg/m^3) in vapor depolymerized cellulose polymer of paper by acid hydrolysis resulting in shorter and weaker chain of cellulose fibres. Acetic acid (96% at 25ºC) hydrolyzed the regenerated cotton cellulose from cupriethylenediamine (27). Simiarly, viscosity of exopolysaccharide isolated from *Cordyceps sinensis* was decreased in dilute sulphuric acid solution (pH 1, 25, 50 and 90ºC) in 30 min by the degradation of exopolysaccharide (47).

The alkaline detergents used in the present study contained a mixture of potassium hydroxide and potassium hypochlorite, and the treatments with the detergent either significantly or numerically reduced the amounts of cellulose produced by the cells of the STEC strains (Table 4.1). Under alkaline conditions, chemical isomerization occurs at the reducing end of the cellulose molecule, which causes the carbonyl groups in the cellulose molecules to move along the carbon chain. Cellulose de-polymerization subsequently occurs through a peeling off reaction, and glucose units in the cellulose molecule are released one by one (14, 38). Shibazaki et al (32) reported that a treatment with 18% NaOH for 60 min at 22°C effected ontoreducing the crystallite size of bacterial cellulose from 75 to 25 nm. Phosphoric acid in the acidic detergent is

the active component against cellulose or STEC cells. The phosphoric acid has been used for cellulose depolymerization or decrystallization (40, 42, 49). Under the treatment with phosphoric acid (> 80%), cellulose swelled (42), depolymerized (40), and became more soluble. Wei et al. (40) showed the linear relationship the cellulose depolymeration (log DP) by reacting time with phosphoric acid (>85%), and the depolymeration rate constant was 4.79×10^{-3}/h at 25ºC.

Cellulose are extruded as short precellulosic polymers (ca. 10 to 15 chains) from discrete structures of the lipopolysaccharide layer within the outer membrane and these polymers covalently attach to cell membrane components (protein, lipid or sugar) with hydrogen bond (26). The hydrogen bonds are weak that can be readily broken although strongly associated with structural stability of biochemical molecules. Acids used in the study are polar being capable served as hydrogen-bond acceptor (carbonyl) and hydrogen-bond doners (hydroxyl) and detergent are oxidizing agents. The treatments used in the study also had very low or high pH. Under these conditions, hydrogen bonds between cellulose molecule and cell surface molecules may be broken, form hydrogen bonds with water and cause the reduction of cellulose from STEC cells.

Linear model regression analysis performed in the study revealed a positive correlation between the residual amounts of cellulose and the surviving cell populations of individual STEC strains. When lesser amounts of cellulose were present in the STEC suspensions, cells were more easily inactivated by the treatments with the two organic acids. As a result, relatively lower numbers of STEC cells survived the treatment process. In the contrary, higher numbers of STEC survivors were recovered from the cell suspensions that had relatively higher amounts of

cellulose after the treatments with the two organic acids. According to our knowledge, correlations between the existence of cellulose and survival of STEC cells have not been made previously. However, studies have shown that cellulose protects bacterial cells against treatments with antimicrobial agents (33, 41). Solano et al (33) reported cellulose deficient mutant cells of *Salmonella* Enteritidis were more sensitive to 30 ppm NaOCl for 20 min. Cellulose deficient strain of *Salmonella* had less than 0.01% survival with greater than a 4-log cfu of reduction when the cell was grown for 3 month and exposed to sodium hypochlorite for 20 min (41).

The enzymatic and chemical agents used in the present study had different abilities in hydrolyzing or degrading cellulose and inactivating the cells of STEC. In correlation analysis, as more amounts of cellulose were remained, the more populations of STEC were survived following the treatments with acetic and lactic acids (Table 4.3). The abilities of organic acids in inactivating cellulose producing cells of STEC may be related to their efficacies in hydrolyzing or degrading the glucose polymer.

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Table 4.1. Average residual amounts of cellulose on the cell of six STEC strains grown on LBNS agar after treatments with selected enzymatic and chemical agents

Treatments		Average residual amounts of cellulose ($\mu g/10^{10}$cells)	Average surviving cell populations (log CFU/ml)
Cellulase (37°C, 2 h)	Control[†]	2.83a	8.80a
	0.50 U/15 ml	1.63b	8.74a
	3.83 U/15 ml	0.37c	8.67a
Organic acids (room temperature, 20 min)	Control	2.72a	8.80a
	Acetic acid (2%)	2.11ab	7.67b
	Acetic acid (4%)	1.62bc	6.82b
	Lactic acid (2%)	1.65bc	5.01c
	Lactic acid (4%)	0.99c	3.70d
Commercial detergents[a] (room temperature, 7 / 15 min)	Control	2.72a	8.80a
	Alkaline (7 min)	1.87ab	NDb[††]
	Alkaline (15 min)	1.10bc	NDb
	Acidic (7 min)	1.37bc	NDb
	Acidic (15 min)	0.71c	NDb

[†]Untreated samples. The cell populations or the amounts of cellulose presented are the average cell populations or the average amounts of cellulose of six STEC including 6-8, 6-35, 7-17, 7-49, 7-50, and 7-51, without any treatment or after treatments with enzymes and selected chemicals.
[††]ND means values below detection limit (< 4.00 CFU/ml)
Values that are not followed by the same letters within the same treatment category (cellulase, organic acids or commercial detergents) are significantly different ($P < 0.05$). [a]; Manufacturers recommended concentrations were used

Table 4.2. Cell populations of STEC on LBNS agar after treatments with cellulase, selected organic acids and commercial detergents

Treatments		Cell populations (log CFU/ml)					
		STEC strains					
		6-8	6-35	7-17	7-49	7-50	7-51
Untreated control		9.00aAB	9.09aA	8.77aAB	9.07aA	9.09aA	8.83aAB
Cellulase (37°C, 2 h)	0.51 U/15 ml	8.62aA	8.47aA	8.69aA	9.07aA	9.09aA	8.50aA
	3.83 U/15 ml	8.65aABC	8.97aA	8.55aBC	8.90abAB	8.61abABC	8.34aC
Organic acids (room temperature, 20 min)	Acetic acid (2%)	7.49bA	8.59aA	5.70bB	7.61bcA	8.27bA	8.34aA
	Acetic acid (4%)	7.09bA	6.63bB	5.94bC	6.97cAB	7.17bA	7.12bA
	Lactic acid (2%)	5.20cA	5.70bA	0.85cB	5.28dA	6.74cA	6.75bA
	Lactic acid (4%)	4.10dB	4.00cB	0.73cC	4.23dAB	4.59dA	4.57cA
Commercial detergents (room temperature, 7 / 15 min)	Alkaline (7 min)	NDeA[†]	NDdA	NDcA	NDeA	NDeA	NDdA
	Alkaline (15 min)	NDeA	NDdA	NDcA	NDeA	NDeA	NDdA
	Acidic (7 min)	NDeA	NDdA	NDcA	NDeA	NDeA	NDdA
	Alkaline (15min)	NDeA	NDdA	NDcA	NDeA	NDeA	NDdA

Means in the same column not followed by the same lowercase letters are significantly different in terms of STEC strains ($P < 0.05$).
Means in the same row not followed by the same uppercase letters are significantly different in terms of treatments ($P < 0.05$).
[†]ND means values below detection limit (< 4.00 CFU/ml)

Table 4.3. Correlations between the residual amounts of cellulose and the surviving cell populations of STEC treated with acetic or lactic acid for 20 min at room temperature using the linear least-squares regression and linear models (n = 6)

STEC strain	Treatments with acetic acid (2, 4%)			Treatments with lactic acid (2, 4%)		
	Linear models	Correlation coefficients (*r*)	*P* values[†]	Linear models	Correlation coefficients (*r*)	*P* values
6-8	y=2.39x+2.88	0.88	0.02	y=2.17x+2.87	0.90	0.02
6-35	y=2.72x+4.74	0.72	0.10	y=3.57x+3.50	0.91	0.01
7-17	y=3.97x+5.18	0.64	0.17	y=9.30x+0.46	0.79	0.06
7-49	y=1.85x+2.34	0.68	0.14	y=2.31x+0.15	0.85	0.03
7-50	y=0.78x+5.55	0.94	0.01	y=1.22x+3.30	0.93	0.01
7-51	y=0.44x+6.57	0.85	0.03	y=0.85x+4.42	0.88	0.02

The linear models explain the relationships between the residual amounts of cellulose (x) and the surviving cell populations of individual STEC strains (y).

[†]*P* values indicate the significance of the correlations. $P < 0.05$ means that *r* exceeded the critical value with 95% confident.

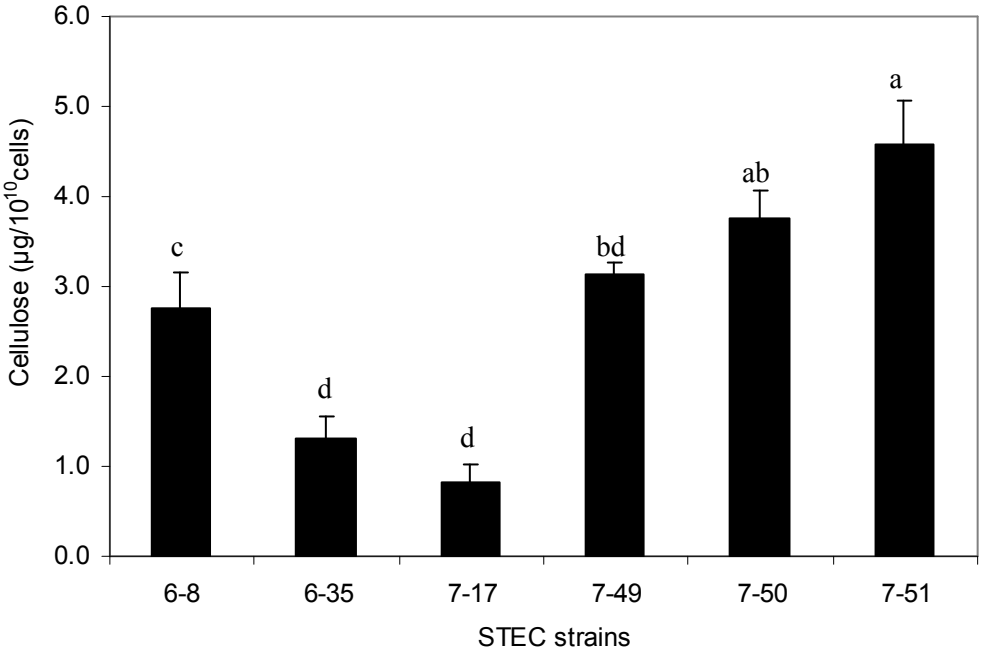
FIGURE LEGENDS

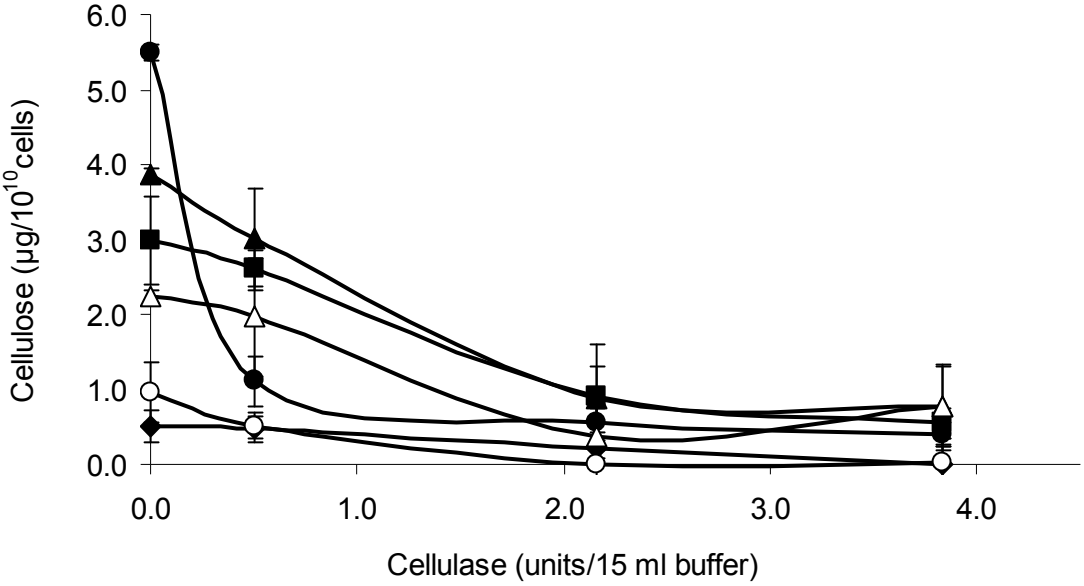
Fig. 4.1. Cellulose production by STEC strains on LBNS agar. The amounts of cellulose produced by STEC were measured using a colorimetric assay and presented as the absorbance values of anthrone solutions at 620 nm.

Fig. 4.2. Influence of enzymatic hydrolysis on cellulose produced by the cells of STEC strains on LBNS agar. STEC cells on LBNS agar were treated with 0.5, 2.16 and 3.83 units of cellulase per 15 ml of sodium acetate buffer for 2h at 37°C*, and the cellulose remaining in the cell suspensions of STEC after the treatments was measured using a colorimetric assay . ◆7-17 ■ 7-49 ▲ 7-50 ● 7-51 △ 6-8 ○ 6-35*

Fig. 4.3. Influence of acid treatment on cellulose produced by STEC cells. Cultures of STEC on LBNS agar were treated with 2 and 4% acetic acid (A) or lactic acid (B) solutions for 20 min at room temperature*, and the residual amounts of cellulose in the STEC cell suspensions were measured using a colorimetric assay. ◆7-17 ■ 7- 49 ▲ 7-50 ● 7-51 △ 6-8 ○ 6-35*

Fig.4.4. Influence of commercial detergents on cellulose produced by STEC cells. Cultures of STEC on LBNS agar were treated with a commercial alkaline detergent (A) or acidic detergent (B) for different lengths of time at room temperature. Each detergent was prepared at manufacturer recommended concentrations. ◆7-17 ■ 7- 49 ▲ 7-50 ● 7-51 △ 6-8 ○ 6-35


6.0
5.0
4.0
3.0
2.0
1.0
0.0
Cellulose (µg/10^10cells)
c
d
d
bd
ab
a
6-8
6-35
7-17
7-49
7-50
7-51
STEC strains


6.0
5.0
4.0
3.0
2.0
1.0
0.0
Cellulose (µg/10^10cells)
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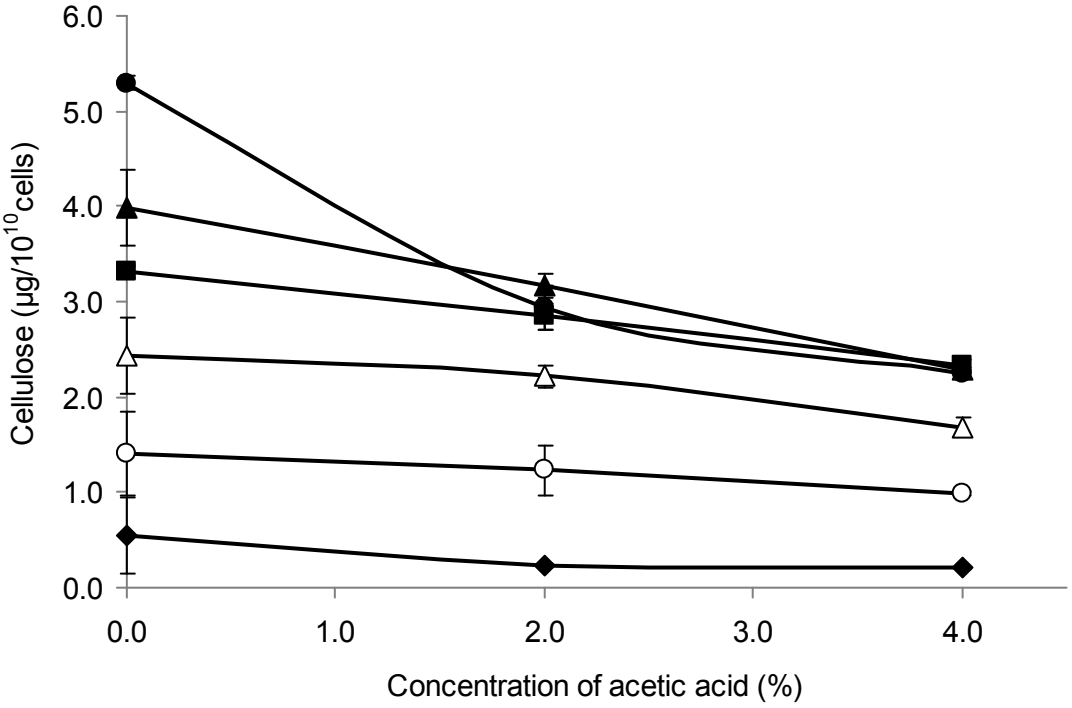
1.0
2.0
3.0
4.0
Cellulase (units/15 ml buffer)

A


6.0
5.0
4.0
3.0
2.0
1.0
0.0
Cellulose (µg/10^10cells)
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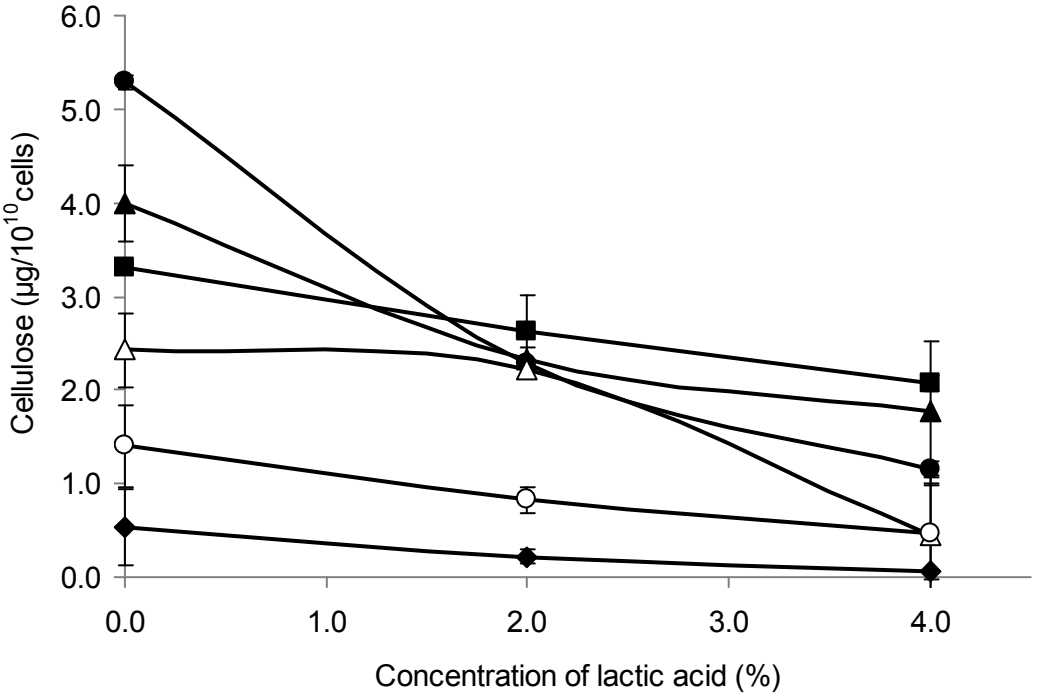
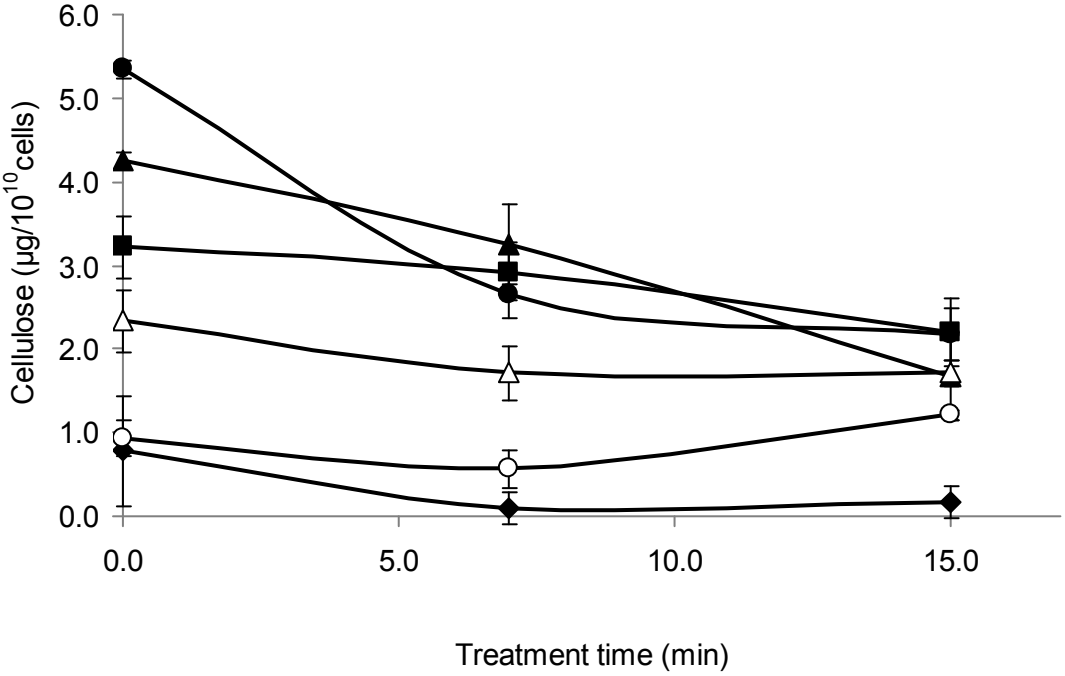
1.0
2.0
3.0
4.0
Concentration of acetic acid (%)

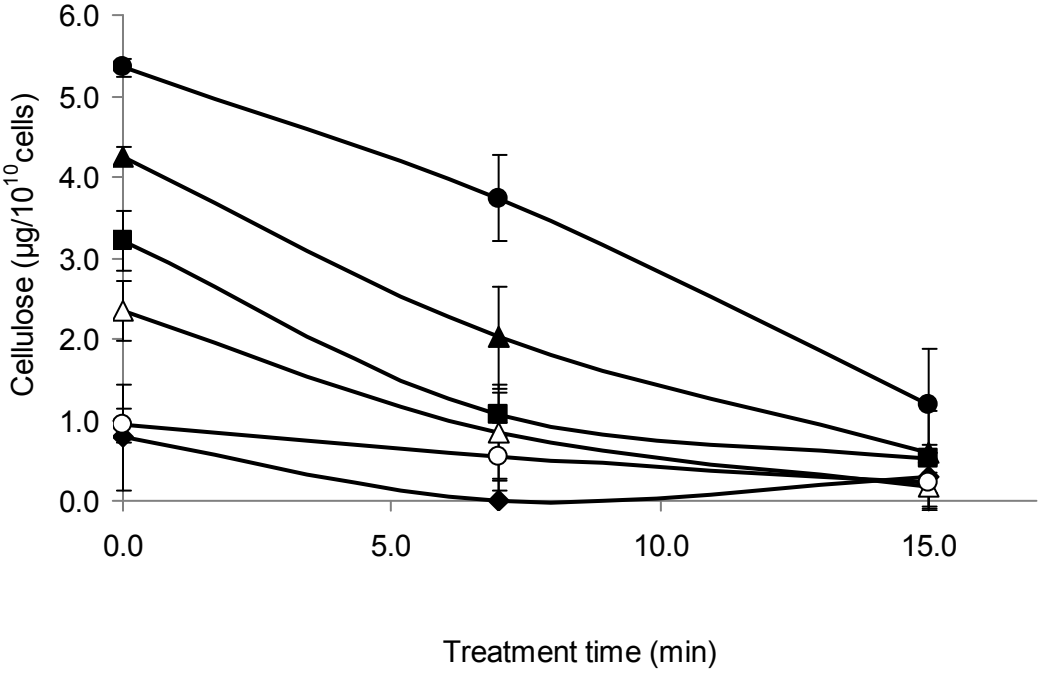
B


6.0
5.0
4.0
3.0
2.0
1.0
0.0
Cellulose (µg/10^10cells)
0.0
1.0
2.0
3.0
4.0
Concentration of lactic acid (%)

A


6.0
5.0
4.0
3.0
2.0
1.0
0.0
Cellulose (µg/10^10cells)
0.0
5.0
10.0
15.0
Treatment time (min)

B


6.0
5.0
4.0
3.0
2.0
1.0
0.0
Cellulose (µg/10^10cells)
0.0
5.0
10.0
15.0
Treatment time (min)

CHAPTER 5

REDUCTION OF CURLI AND THE INACTIVATION OF SHIGA TOXIN PRODUCING *ESCHERICHIA COLI* CELLS BY TREATMENTS WITH SELECTED ENZYMES AND CHEMICALS[1]

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ABSTRACT

To degrade curli and to inactivate Shiga toxin producing *E. coli* (STEC) cells, this study evaluated the effectiveness of protease, organic acids, and commercial detergents. Specifically, protease (1 and 2 U/ml), acetic and lactic acids (2 and 4%), and acidic and alkaline detergents (manufacturer recommended concentrations) were separately treated on cells of six STEC that involve three curli-expressing strains and their non curli-expressing counterparts. The residual amount of curli and surviving cell population were determined using Congo red binding assay and plate counting, respectively. The result showed treatments with acetic and lactic acid significantly reduced the amount of curli and surviving cell populations of STEC ($P < 0.05$). Under the treatments with these organic acids, the residual amounts of curli correlated positively to the surviving cell populations of STEC strains except 7-57-, and the correlation coefficients varied in the range of 0.55 – 0.92. However, treatments with protease also degraded curli but no influence on the survival of STEC cells. In addition, detergents had an efficacy on degrading curli and inactivating the STEC cells to undetectable levels. Hence, the correlations between residual amounts of curli and surviving populations of STEC cells were unobservable. It was demonstrated that the selected enzymatic, chemical agents degraded curli and that organic acids and detergents inactivated the STEC cells. However, the efficacy of organic acids in inactivating curli expressing STEC cells might be positively associated with that in degrading curli.

Shiga toxin-producing *Escherichia coli* (STEC) are a group of enteropathogen that have an ability to produce one or more Shiga toxins (Stx). The human infection of STEC may cause illness range from mild diarrhea to hemolytic uremic syndrome (HUS), hemorrhagic colitis (HC), and thrombotic thrombocytopaenic purpura (TTP), so STEC has been a serious concern for public health problem (Karmali 2003; Konowalchuk et al. 1977; O'Brien et al. 1984). As the major reservoir of STEC are ruminants including cattle, sheep, goats, water buffalo and wild ruminant species, most of serotypes isolated from human originate in beef and cattle which are the predominant reservoirs of STEC in North America (Beutin et al. 1993; Mainil and Daube. 2005). Moreover, the human infections of STEC are mostly through the consumption of contaminated foods by the bacteria-containing faeces or through cross-contamination during food production, transport, and slaughter (Fairbrother and Nadeau 2006). For example, 52% of outbreaks of *E. coli* O157:H7 infection in United States during 1982-2002 (Rangel et al. 2005) were associated with contaminated foods.

Many pathogenic isolates of *E. coli*, particularly STEC, express curli on the cell surface (Cookson et al. 2002; Gophna et al. 2001; Olsén et al. 1989; Uhlich et al. 2001). The similar structures known as thin, aggregative fimbriae are also observed on *Salmonella enteric* serovar Enteritidis and *Salmonella enteric* serovar Typhimurium (Collinson et al. 1991; Collinson et al. 1993; Romling et al. 1998a). Curli are heteropolymeric proteinaceous filamentous appendages and are associated with the adherence of cells (Olsén et al. 1989). Curli are composed with the primary structural proteins, CsgA and CsgB (Bian, Z. and Normark. 1997; Hammar et al. 1996), and appear as a tangled and amorphous matrix surrounding bacteria cell (Chapman et al. 2002). Notably, these fibers are cross β-structure which distinguishes protein amyloid (Chapman et al.

2002). These aggregated amyloid structures of curli fiber have stability and chemical strength conferred by noncovalent bonds, notably hydrogen bonds, hydrophobic interactions (Cherny and Gazit 2008).

Curli protein binds to and interacts with its contact host, the processes that facilitate bacterial infection (Barnhart and Chapman 2006). In biofilm formation by *E. coli*, curli plays an important role in the attachment process during the initial biofilm formation (Houdt and Michiels 2005). Similarly, curli expressed by *E. coli* promoted biofilm formation on the surfaces such as polystyrene, glass, stainless steel, and rubber, because the attachment of cell enhances biofilm formation and long-term survival (Pawar et al. 2005; Cookson, et al. 2002). Curli were also shown to contribute to mediate cell-to-cell contacts, and curli produced by adjacent cells formed thick bundles that bound cells together onto the surfaces (Prigent-Combaret et al. 2001). Also, Curli is identified as the major proteinaceous component for extracellular matrix in *S. typhimurium* and *E. coli*, along with cellulose as secondary component. Furthermore, this extracellular matrix surround of cells, eventually form chemically resistive three-dimensional matrix. As a result, this matrix provides a challenge to cleaning practices in the food processing industry (Zogaj et al. 2001).

Therefore, to control the pathogens in food processing environment, it is important to understand the protective role of curli produced by STEC cells and the protection mechanism of curli producing cells against cleaners and sanitizers for food contact surface. The objectives of this study were (1) to evaluate the effectiveness of enzymatic and chemical agents in the reduction of curli and (2) to determine the correlation between the residual amounts of curli and the surviving populations of STEC cells after the enzymatic and chemical treatments.

MATERIALS AND METHODS

Bacterial strains and growth conditions. STEC strains 5-11, 7-52 and 7-57 were from our laboratory collection and used in this study. Variants including curli-expressing and non curli-expressing colonies were isolated from the cells of 5-11, 7-52 and 7-57 by growing on the Luria Bertani no salt agar (LBNS) supplemented with indicator at 28ºC, and the variants of three strains were used as separate cultures. The curli-expressing variants of three strains were designated 5-11+, 7-52+ and 7-57+, and the non curli- expressing variants were designated as 5-11-, 7-52- and 7-57-. The cultures were grown on LBNS agar (10 g tryptone, 5 g yeast extract, and 15g agar per liter) supplemented with 40 µg/ml of Congo red (Sigma-Aldrich Co., St. Louis, MO.) and 20 µg/ml of Coomassie brilliant blue (Sigma-Aldrich) at 28ºC for 72 h for qualitative assessment of curli that are produced by the STEC cells. In other experiments described in this study, the STEC cultures were grown on LBNS agar without indicators and were incubated under the same conditions described above.

Enzymatic degradation of curli expressed by STEC cells. Protease of Bacillus (1 U/mg; Sigma-Aldrich) was dissolved in 10 mM sodium acetate buffer with 5 mM calcium acetate at pH 7.5 (0.1g in 10 ml). The concentration of enzyme solution was determined to be 0.1 or 0.2% (g/ml) by diluting in the same buffer. The cells of STEC strains grown on the LBNS agar plates were treated with 15 ml protease solution prepared in sodium acetate buffer with calcium acetate buffer at different concentrations (1 or 2 U/ml). As control samples, STEC cultures were treated with 15 ml of the same buffer without enzyme, and then were incubated. The enzymatic treatments were conducted at 37ºC for 30 min with gentle shaking.

Reduction of curli expressed by STEC cells using organic acids and commercial detergents. To see the influence of organic acid onto curli expressed by STEC cells, acetic (JT barker, Phillipsburg, NJ) and lactic (Purac America Inc. IL) acids were used. Each acid was prepared at 2 and 4% of concentration, and then 15 ml of the prepared acid was applied onto each cell of the STEC cultures grown on the surface of LBNS agar plates at room temperature for 20 min with gentle horizontal shacking (Orbit Shaker, Lab-Line® Melrose Park, IL).
For the reduction of curli expressed by STEC cells, two commercial detergents with acidic or alkaline properties were used. The study with commercial detergents used the working solution that was prepared by the instruction recommended by manufacturers. Alkaline detergent (pH 13; Ecolab, St. Paul, MN) included the active ingredients such as potassium hydroxide, phosphoric acid, and potassium hypochlorite, and was prepared as working solution by diluting 10 ml of the commercial product in 368 ml of sterile water. Acidic detergent (pH 1.0-1.5; ZEP manufacturing Company, Atlanta, GA) had phosphoric acid as the active component and the working solution was prepared by diluting the commercial product 4- fold with sterile water. Fifteen ml of prepared solutions was applied to the cells of the STEC cultures on the agar plate of LBNS at room temperature for 15 min.

Influence of curli degradation treatments on the survival of STEC cells. The STEC cells were treated with the solutions (enzyme, organic acids or commercial detergents) that are described in curli degradation, and then 1 ml of each treated or untreated control of cell suspension was neutralized with 9 ml of double concentration of Dey-Engley (DE; Becton, Dickinson and Company, Sparks, MD) buffer at room temperature for 15 min. Double concentration of DE was prepared by dissolving 78g of DE in 1 l distilled water, and then was

autoclaved at 121ºC for 15 min before using. The neutralized cells were plated in duplicate on tryptic soy agar (TSA; Becton, Dickinson and Company) plates after serial dilution with 0.1% buffered peptone water. The colonies were enumerated after the 24 h incubation at 37ºC, one that indicated the survival population of STEC cell.

Purification and quantification of curli expressed by STEC cells. The remained curli on the treated STEC cells and curli present on the non treated STEC cells were purified by sequential centrifugation before quantification. Three pairs of STEC strains, 5-11, 7-52 and 7-57, were grown on LBNS agar plates at 28ºC for 72 h, and then were treated with enzyme, organic acids or commercial detergents as described above, and were finally scraped off. For non-treated control, the cultures grown on LBNS agar plates were also suspended with 15 ml of distilled water and were scraped off.

Cell suspensions of each culture were palleted by centrifugation for 10 min at 13,000 rpm in Eppendorf centrifuge (Eppendorf, Centrifuge 5810R, Westbury, NJ), and then the supernatants fluids were discarded. The pellets were suspended in 1.5 ml of 2% sodium dodecyl sulfate (SDS), and then the samples were boiled for 45 min. The samples were centrifuged for 5 min at 7,000 rpm, and were washed three times with 1.5 ml of distilled water. The sample was collected by centrifugation at 13,000 rpm for 10min for the subsequent quantification.

The partially purified curli from STEC cells were quantified using the Congo red binding assay by Gophna et al (2001) with some modifications. The pallet of samples was suspended in 1.5 ml 0.02% Congo red solution (Sigma) and was left for 10 min at room temperature. The cell suspension in dye solution was centrifuged at 13,000 rpm for 10 min. The optical density of supernatant was measured against a saline background at 500 nm, and the optical density of

Congo red solution was also measured for the calculation of Congo red binding. The Congo red binding of each sample was determined as the difference in optical density of Congo red solution before and after the incubation with purified curli samples.

Statistical analysis. The Data were analyzed by the one-way analysis of variance (ANOVA), followed by T- test, using the JMP statistical software version 8.0 (SAS Institute Inc., Cary, NC, USA, 2008) at a 95% confidence interval. Significant differences in the remained curli in the cells of three pairs of STEC cultures and the efficacies of curli degradation by different treatments were calculated by comparing the overall mean absorbance of the free Congo red at 500 nm. The influence of different treatments on the survival of STEC cells was also determined by the same statistical procedure. The correlations between the residual amounts of curli and the surviving populations of STEC follwing the treatments with the selected organic acids were analyzed using the Pearson's correlation model, using the JMP statistical software (SAS Institute Inc., 2008).

RESULTS

Curli production on the cells of selected STEC strains. Congo red binding assay was utilized to quantify of curli produced by the STEC cells. The optical densities of the supernatants at 500 nm-wavelengths indicated the concentrations of the free-formed Congo red generated after binding to curli fibers. In other words, the amount of curli on the surface of cells was presented as Congo red binding units which were determined by comparing the difference of optical densities before and after the reaction with Congo red.

Based on the results in Fig. 5.1, STEC 7-57+ produced the greatest amounts of curli compare to the other strains. In addition, the 2 variants of STEC pairs, 7-52 and 7-57, showed the significant difference ($P < 0.05$) in curli production after 72 h incubation (Fig. 5.1). The curli on the cells of STEC strains, 7-52+ and 7-57+, were significantly higher in the Congo red binding units compared to that by 7-52- and 7-57- ($P < 0.05$). Also, the cells of STEC, 5-11+ produced numerically ($P > 0.05$) higher level of curli than their counterpart, 5-11- (Fig. 5.1).

Enzymatic treatments on curli and STEC cells. As presented by Congo red binding units, the average amounts of curli expressed by the three pairs of STEC strains were significantly ($P < 0.05$) reduced by the treatments with protease (Table 5.1). However, the efficacy of protease at the different concentrations (1 U/ml and 2 U/ml) was not significantly different in the average curli produced by the cells of the three pairs of STEC strains (Table 5.1).

Enzymatic treatments with 1 and 2 U/ml of protease significantly ($P < 0.05$) reduced the amounts of curli on the cells of STEC 7-52+ and 7-57+. Under the same treatments with 1 and 2 U/ml, the reductions in STEC 7-57+ were relatively higher than those in STEC 7-52+ (Table 5.3). The reduction of curli, otherwise, was relatively lower in other four STEC cells. The same protease treatments on 5-11+ cells showed the reductions of curli by 0.04 and 0.01, respectively. In contrast, in counterpart cells of strong curli-expressing STEC strains, 5-11- and 7-57-, and the treatments with protease at both concentrations reduced less than 0.01 of Congo red binding units (Table 5.3). In addition, treatments with protease did not influence on the survival of STEC cells (Table 5.2).

Treatments with organic acids on curli and STEC cells. Acetic or lactic acid treatments with 2 and 4 % concentration significantly ($P < 0.05$) reduced the average cell

populations of six STEC strains used in the study (Table 5.1). Also, the cell populations of individual STEC strains were decreased significantly ($P < 0.05$) by the treatments with acetic acids with the exception in 5-11+, and 7-52- treated with 2% acetic acid (Table 5.2). Lactic acid also significantly ($P < 0.05$) decreased the cell populations of the three pairs of STEC strains at 2 and 4% concentration (Table 5.2). Particularly, the cell populations of 5-11- was reduced to less than 1.00 CFU/ml in all acetic or lactic acid treatments except 2% acetic acid. However, for the cells of 5-11+ and 7-52-, 2% acetic acid treatment was less effective (Table 5.2).

Also, the same treatments significantly ($P < 0.05$) reduced the average of Congo red binding units (Table 5.1), one that indicated significant reduction of the amounts of curli expressed by the cells of the three pairs of STEC strains. The average amounts of curli were reduced by 0.09 and 0.15 by the 2 and 4% acetic acid treatments, respectively, and by 0.13 and 0.17 by the 2 and 4% lactic acid treatments, respectively (Table 5.1).

For the cells of 5-11+ and 7-52+, the curli amounts were decreased by 0.08 and 0.07, respectively by the treatments with 2% acetic acid (Table 5.4). Also, for the the cells of 5-11- and 7-52-, the amount of curli on STEC cells were reduced by 0.04 and 0.01, respectively, with 2% acetic acid treatment. The treatments with 4% acetic acid significantly ($P < 0.05$) reduced the amounts of curli on STEC cells, 5-11+, 5-11- and 7-52+. In contrast, for the cells of 7-57+, the curli amounts were significantly ($P < 0.05$) reduced by 0.23 and 0.31 by 2 and 4% acetic and lactic acid treatments, respectively (Table 5.4).

With the 2 and 4% lactic acid treatment, the amounts of curli produced by the three pairs of STEC strains were reduced by 0.13 and 0.17 (Table 5.1). The treatments with 2 and 4% lactic acid significantly ($P < 0.05$) reduced the amounts of curli on STEC cells, 5-11+, 7-52+ and 7-

57+ (Table 5.5). For the cells of 5-11- and 7-57-, lactic acid treatments with 2 and 4% concentration reduced the average of curli amounts by ranging 0.03-0.09. In addition, as the concentration of acetic and lactic acid increased, the average of Congo red binding units reduced numerically more (Table 5.5).

Treatments with commercial detergents on curli and STEC cells. Acidic and alkaline detergent treatments reduced the average cell populations of STEC strains to less than 4.00 CFU/ml (Table 5.1). Also, the average amount of curli was decreased significantly ($P < 0.05$) by the treatments (Table 5.1). As treatment time was increased from 7 min to 15 min, the average amounts of curli on six STEC cells were reduced by from 0.09 or 0.08 to 0.24 (Table 5.1).

For the 7-52+ and 7-57+ cells, the alkaline detergent treatments for 15 min reduced the average amounts of curli by 0.43 and 0.36, respectively (Table 5.6), and the acidic detergent treatments decreased them by 0.39 and 0.47, respectively (Table 5.7). Additionally, for 5-11+ cells, the alkaline and acidic detergent treatments for 15 min reduced the average amounts of curli by 0.29 and 0.32, respectively (Table 5.6 and 5.7). Also, for the cells of 5-11-, 7-52- and 7-57, alkaline detergent treatment for 15 min decreased the amounts of curli by 0.21, 0.17 and 0.16, respectively (Table 5.6), and acidic detergent treatment reduced them by 0.19, 0.13 and 0.14, respectively (Table 5.7).

Correlations between the remained amounts of curli and the surviving cell populations of STEC. Treatments with protease showed the influence on the degradation of curli produced by the cells of STEC, but not on the survival of STEC cells. In addition, two commercial detergents decreased the populations of STEC cells to undetectable levels (Table 5.2), thus making the correlation between the residual amounts of curli and survival cell

populations of STEC not meanigful. With the treatments with acetic and lactic acids, the residual amount of curli had positive correlation to the surviving cell populations of individual STEC strains except 7-57- (Table 5.3). In the correlation analysis, positive value of coefficients (*r*) means that STEC strains with high level of residual curli had high surviving population after treatment; and one with low level of residual curli had low surviving population. Under the treatments with acetic acid, the correlation coefficients between the residual amounts of curli and surviving cell population of STEC strains except 7-57- were positive in the range of 0.65 – 0.78 (Table 5.3). Under the lactic acid treatments onto STEC cells of curli producing strains, the correlation coefficients between the two parameters were in the range of 0.60 – 0.92. While, in non-curli producing STEC cells with the same treatments, the correlation was positive but weak, and they were in the range of 0.15 – 0.59 (Table 5.3). However, with the acetic acid treatment on cells 7-57-, the correlation was negative (Table 5.3).

DISCUSSION

Curli are amyloid fibers expressed by and on surfaces of *E. coli* (Chapman et al. 2002; Hammar et al. 1996; Olsén et al. 1989). Biochemical, biophysical, and imaging analyses identified the characteristics of amyloid fibers of curli produced by *E. coli* (Chapman et al. 2002). In general, amyloid fibers are filamentous proteins composed of aggregated β-sheets structure into an ordered fibrillar structure with straight, rigid and unbranched form (Kayed et al. 2003; Rochet and Lansbury 2000). The cross β-structure, the major structural property of amyloids, is drawn in β-strands that are extended sequences of amino acids with amide bonds. The two or more β-stranded peptides composed β-sheets that are parallel or anti-parallel

alignments by inter-strand hydrogen bonds (Gillespie et al. 1997). The β-sheets of amyloids stabilize protein architecture and are related with protein-protein interactions. In addition, the major curli subunit protein, CsgA, is soluble and unstructured protein, but nucleated to an insoluble amyloid fiber in the presence of the CsgB (Hammar et al. 1996). CsgA and CsgB are composed of the five-repeating units consisting of 19-24 amino acids, and repeating unit also contains asparagine, glycines and glutamine (Collison et al. 1999; Wang et al. 2007).

Like other amyloid proteins, curli were capable to bind Congo red that preferentially bind to the β- sheet conformation (Chapman et al. 2002; Klunk et al. 1989). Congo red binds to amyloid through bonding between the sulfuric acid groups of Congo red and amino acid group of protein molecules with β-sheet conformation, and then the bound amyloid Congo can to be quantified (Klunk et al. 1989). In this study, the amounts of curli on the STEC cells were quantified using Congo red: the binding of Congo red to curli caused the decrease in the concentrations of the free-formed dye in the supernatants, which is presented as the decrease in the optical densities at 500 nm-wavelength. The results showed the curli-expressing cells of STEC bound greater amounts of Congo red than the non curli-expressing cells (Fig. 5.1). This result agrees with the previous reports where the curli-expressing cells including 7-52+, 5-11+ and 7-57+ had significantly greater binding to Congo red compared to non curli-expressing cells (Pawar and Chen, 2004).

In the current study, the treatments with the selective enzyme and chemicals significantly ($P < 0.05$) reduced curli expressed by STEC and it was observed by the quantification assay using Congo red (Table 5.1). For the enzyme treatment, protease from *Bacillus polymyxa* was used. It is a neutral nonspecific endopeptidase (EC 3.4.24. X) that is enzyme to break peptide

bonds of nonterminal amino acids. This protease also functionally belongs to metalloprotease, which activate the lysis of peptide bond by nocovalent complexation of the carbonyl group (Bongers et al. 1994; Luchini et al. 1996). Similar activity to degrade amyloid proteins has been observed in other metalloprotease (Howell et al. 1995; Yan et al. 2006). Neprilysin, the neutral metallo-endopeptidase (EC 3.4.24.11) which is enzyme to hydrolyze peptides at the N-terminal side of hydrophobic residues had a capability to degrade β-amyloid by hydrolyzing at the cleavage sites such as Glu-Phe, Gly-Trp, Phe-Phe, Ala-Ile and Gly-Leu (Howell et al. 1995). Besides, the degradation of amyloid has been also observed in other studies using endopeptidase (Howell et al. 1995; Yan et al. 1995).

On the other hand, for the chemical treatment, acetic and lactic acids as well as acidic detergents (pH 1-2) rendered the significant ($P < 0.05$), but partially, decline of curli on STEC cells (Table 5.1). In acidic condition, amyloid protein are denaturated or dissociated (Appel et al. 2006; Newcombe and Cohen 1965). The solubility of amyloid was 10-20% between pH 2 and 4 of 0.1M Sörensen's glycinate mixtures at 0°C for 24 h by the denaturation of amyloid (Newcombe and Cohen 1965). The protein denaturation can be also achieved by organic acid (Perlmann and Kaufman 1949; Tanford 1968). Furthermore, amyloid proteins are completely hydrolyzed by strong acids or at high temperature into monomers and the amyloid hydrolysis is cuased by destroying hydrogen bond and hydrophobic forces which form polymerized structure of amyloid (Appel et al. 2006; Chapman et al 2002; Collinson et al. 1991). Otherwise, mild acidic treatments at room temperature used in the study may cause the reduction of curli by interrupting the assembly of curli protein on the bacterial membrane. The hydrophobicity of CsgB surface generates the hydrophobic association with the bacterial membrane because

nucleator subunit, CsgB are an anchor site for fimbiral assembly of curli (White et al. 2001). Acidic treatments decrease attractive force of hydrophobic association (Voet and Voet 1995) and it can be supposed to arise between CsgB and bacterial membrane cuasing a reduction of curli on STEC cells.

Alkaline also hydrolyze amyloids because bases (OH^-) may be capable to hydrolyze the peptide bonds and to penetrate the β-sheet fibril by disrupting hydrogen bonds (Appel et al. 2005). Wang et al. (2007) also showed that NaOH (1N for 1 h) and NaOCl (1% for 30 and 60 min, 2.5% for 5-60 min) destroyed 1mg/ml of amyloid protein, Sup35NM-His6 at 25ºC. Yamaguchi et al. (2001) have examined the depolymerization of β_2-microglobulin amyloid fibrils by reaction in 50mM Tris-HCl buffer (pH 7.5) at 37ºC monitored by ThT fluorescence. It showed that ThT fluorescence was lost immediately after the reaction indicating the depolymerization of amyloid fibrils. They also showed that the effect of over pH 6 on the depolymerization of most or all amyloid fibrils by the incubation for 24 h at 37ºC. Similarly, in the current study, the alkaline detergents contained potassium hydroxide and potassium hypochlorite and had pH 11. This detergent also showed significant ($P < 0.05$) reduction the amounts of curli on STEC cells (Table 5.1).

The linear regression analysis showed a positive correlation between residual amounts of curli and surviving populations of STEC cells (Table 5.3). When less amounts of curli were remained in the STEC cells, treatments with the two organic acids were more effective in inactivating STEC cells, resulting in lower numbers of surviving STEC cells by treatments. In contrast, when the higher amounts of curli was present in STEC cells, higher level of populations were recovered after the treatments with the two organic acids. It has not reported that curli were

related with the surviving of bacterial cell after the treatments with sanitizing agents. However, bacterial cell producing curli had resistance against antimicrobial agents (Ryu and Beuchat 2005). Curli-producing cells of E. coli O157:H7 were not significantly decreased by the treatment with 50 μg chlorine/ml for 5 min, while intact cell were reduced by 4.5 log CUF/coupon (Ryu and Beuchat 2005).

It is concluded that enzyme and chemical agents showed the capability in degrading curli and inactivating the cells of STEC. The residual amounts of curli have positively relation with surviving of STEC cells after the treatments with acetic and lactic acid (Table 5.3). The results of this study suggest that curli on STEC cells may be important in the resistance of cells of some STEC strains against cleaners or sanitizers which are used in food processing area. Significant degrading of curli by the treatments with organic acids or commercial detergents allowed these treatments to inactivate STEC cells. These results underline the importance of removing curli thoroughly to remove the bacterial cells or biofilm that have may interrupt sanitizing food contact surface.

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Table 5.1. The influence of the treatments with selected enzymatic and chemical agents on *curli and survi*ving cell populations of three pairs of STEC strains on LBNS agar

Treatments		Average residual amounts of curli††	Average surviving cell populations (log CFU/ml)
Protease (37°C, 30min)	Control†	0.35a	9.00a
	1 U/ml	0.30b	9.12a
	2 U/ml	0.29b	8.95a
Organic acids (room temperature 20 min)	Control	0.35a	9.00a
	Acetic acid (2%)	0.26b	6.46b
	Acetic acid (4%)	0.20bc	5.56b
	Lactic acid (2%)	0.22bc	3.36c
	Lactic acid (4%)	0.18c	2.52c
Commercial detergents[a] (room temperature, 7 / 15 min)	Control	0.35a	9.00a
	Alkaline (7 min)	0.26b	< NDb†††
	Alkaline (15 min)	0.11c	< NDb
	Acidic (7 min)	0.27b	< NDb
	Acidic (15 min)	0.11c	< NDb

†Untreated samples. The cell populations or the amounts of curli presented are the average cell populations or the average amounts of curli of STEC strains (5-11+, 5-11-, 7-52+, 7-52-, 7-57+ and 7-57-) without any treatment or after treatments with enzymes and selected chemicals. †† the amounts of curli were expressed as Congo red binding units.
††† ND means values below detection limit (< 4.00 CFU/ml). [a]; Manufacturers recommended concentrations were used
Values that are not followed by the same letters within the same treatment category (protease, organic acids or commercial detergents) are significantly different ($P < 0.05$)

Table 5.2. Cell populations of STEC on LBNS agar after treatments with protease, selected organic acids and commercial detergents

Treatments		Cell populations (log CFU/ml)					
		STEC strains					
		5-11+	5-11-	7-52+	7-52-	7-57+	7-57-
Untreated control		8.91aAB	8.90aB	9.12aA	8.91abAB	9.13aA	9.00aB
Protease (37°C, 30min)	1 U/ml	8.81aAB	9.00aAB	9.11aA	9.00aAB	9.14aA	8.90aB
	2 U/ml	8.70aA	9.15aA	8.96aA	9.00aA	9.15aA	8.58aA
Organic acids (room temperature, 20 min)	Acetic acid (2%)	7.91abA	2.55bB	7.27bAB	7.90abA	6.38bcAB	6.76bAB
	Acetic acid (4%)	6.81bA	ND†cC	7.10bA	7.08bA	6.79bA	5.00bB
	Lactic acid (2%)	3.20cA	NDcB	3.61cA	3.79cA	4.49cA	4.47bA
	Lactic acid (4%)	1.73cdAB	NDcB	4.00cA	2.87cAB	4.20cA	1.73cAB
Commercial detergents[a] (room temperature, 7 / 15 min)	Alkaline (7 min)	NDdA	NDcA	NDdA	NDdA	NDdA	NDcA
	Alkaline (15 min)	NDdA	NDcA	NDdA	NDdA	NDdA	NDcA
	Acidic (7 min)	NDdA	NDcA	NDdA	NDdA	NDdA	NDcA
	Acidic (15 min)	NDdA	NDcA	NDdA	NDdA	NDdA	NDcA

Means in the same column not followed by the same lowercase letters significantly different in terms of STEC strains ($P < 0.05$).
Means in the same row not followed by the same uppercase letters are significantly different in term of treatments ($P < 0.05$).
† ND means values below detection limit (< 0.60 log CFU/ml). [a]; Manufacturers recommended concentrations were used.

Table 5.3. Influence of emzymatic treatment (37°C, 30 min) onto the amounts of curli on the cells of six STEC strains on LBNS agar

STEC strains	Congo red binding unit (A_{500nm})†		
	Protease treatment (Units/ml)		
	0	1	2
5-11+	0.29cA	0.25aA	0.28aA
5-11-	0.25cA	0.25aA	0.27aA
7-52+	0.38bA	0.26aB	0.25aB
7-52-	0.27cA	0.27aA	0.28aA
7-57+	0.51aA	0.30aB	0.33aB
7-57-	0.28cA	0.28aA	0.27aA

†Amounts of curli on STEC cells or the residual amounts of curli on STEC cells after the enzymatic treatment were measured using a Congo red binding assay, and presented as Congo red binding units which calculated the optical absorbance values of Congo red bound to curli.
Means in the same column not followed by the same lowercase letters significantly different in terms of STEC strains ($P < 0.05$).
Means in the same row not followed by the same uppercase letters are significantly different in term of treatments ($P < 0.05$).

Table 5.4. Influence of acetic acid treatment (room temperature, 20 min) onto the amounts of curli on the cells of six STEC strains on LBNS agar

STEC strains	Congo red binding unit (A_{500nm})[†]		
	Acetic acid treatment (%)		
	0	2	4
5-11+	0.36bcA	0.28aAB	0.20abB
5-11-	0.22dA	0.18aA	0.08bB
7-52+	0.41bA	0.34aAB	0.26aB
7-52-	0.20dA	0.19aA	0.15abA
7-57+	0.55aA	0.32aB	0.24aB
7-57-	0.24cdA	0.24aA	0.26aA

[†]Amounts of curli on STEC cells or the residual amounts of curli on STEC cells after the enzymatic treatment were measured using a Congo red binding assay, and presented as Congo red binding units which calculated the optical absorbance values of Congo red bound to curli.
Means in the same column not followed by the same lowercase letters significantly different in terms of STEC strains ($P < 0.05$).
Means in the same row not followed by the same uppercase letters are significantly different in term of treatments ($P < 0.05$).

Table 5.5. Influence of lactic acid treatment (room temperature, 20 min) onto the amounts of curli on the cells of six STEC strains on LBNS agar

STEC strains	Congo red binding unit (A_{500nm})†		
	Lactic acid treatment (%)		
	0	2	4
5-11+	0.36bcA	0.22aB	0.18aB
5-11-	0.22dA	0.17aA	0.16aA
7-52+	0.41bA	0.26aB	0.18aB
7-52-	0.20dA	0.21aA	0.19aA
7-57+	0.55aA	0.26aB	0.21aB
7-57-	0.24cdA	0.21aA	0.15aA

†Amounts of curli on STEC cells or the residual amounts of curli on STEC cells after the enzymatic treatment were measured using a Congo red binding assay, and presented as Congo red binding units which calculated the optical absorbance values of Congo red bound to curli.
Means in the same column not followed by the same lowercase letters significantly different in terms of STEC strains ($P < 0.05$).
Means in the same row not followed by the same uppercase letters are significantly different in term of treatments ($P < 0.05$).

Table 5.6. Influence of commercial alkaline detergent treatment (the manufacturer's recommended concentration, room temperature) onto the amounts of curli on the cells of six STEC strains on LBNS agar

STEC strains	Congo red binding unit (A_{500nm})[†]		
	Alkaline detergent treatment (min)		
	0	7	15
5-11+	0.39bcA	0.36aA	0.10bB
5-11-	0.28cdA	0.23bcA	0.07bB
7-52+	0.53abA	0.25bB	0.10bC
7-52-	0.24dA	0.24bA	0.07bB
7-57+	0.59aA	0.26bB	0.23aB
7-57-	0.25cdA	0.20cAB	0.09bB

[†]Amounts of curli on STEC cells or the residual amounts of curli on STEC cells after the enzymatic treatment were measured using a Congo red binding assay, and presented as Congo red binding units which calculated the optical absorbance values of Congo red bound to curli.
Means in the same column not followed by the same lowercase letters significantly different in terms of STEC strains ($P < 0.05$).
Means in the same row not followed by the same uppercase letters are significantly different in term of treatments ($P < 0.05$).

Table 5.7. Influence of commercial acidic detergent (the manufacturer's recommended concentration, room temperature) onto the amounts of curli on the cells of six STEC strains on LBNS agar

STEC strains	Congo red binding unit (A_{500nm})[†]		
	Acidic detergent treatment (min)		
	0	7	15
5-11+	0.39bcA	0.34aA	0.07aB
5-11-	0.28cdA	0.23eAB	0.09aB
7-52+	0.53abA	0.28bB	0.14aC
7-52-	0.24dA	0.26cA	0.12aB
7-57+	0.59aA	0.25cdB	0.12aC
7-57-	0.25cdA	0.24deA	0.11aB

[†]Amounts of curli on STEC cells or the residual amounts of curli on STEC cells after the enzymatic treatment were measured using a Congo red binding assay, and presented as Congo red binding units which calculated the optical absorbance values of Congo red bound to curli.
Means in the same column not followed by the same lowercase letters significantly different in terms of STEC strains ($P < 0.05$).
Means in the same row not followed by the same uppercase letters are significantly different in term of treatments ($P < 0.05$).

Table 5.8. Correlations between the residual amounts of curli and the surviving populations of STEC grown on LBNS agar after treatments with selected organic acids for 20 min at room temperature using the linear least-squares regression and linear models (n = 6)

STEC strains	Treatments with acetic acid			Treatments with lactic acid		
	Linear models	Correlation coefficients (*r*) †	*P*†† values	Linear models	Correlation coefficients (*r*)	*P* values
5-11+	y=9.02x+5.34	0.78	0.01	y=39.91x-5.84	0.92	0.00
5-11-	y=38.05x-2.57	0.78	0.01	y=56.12x-7.56	0.59	0.09
7-52+	y=11.04x+4.20	0.65	0.06	y=24.30x-1.28	0.81	0.01
7-52-	y=19.02x+4.52	0.76	0.02	y=9.60x+3.13	0.15	0.70
7-57+	y=7.68x+4.67	0.66	0.05	y=11.85x+1.92	0.60	0.09
7-57-	y=-7.85x+8.72	-0.17	0.65	y=35.49x-2.34	0.55	0.13

The linear least-squares regression calculation provides linear models between the residual amounts of curli and the surviving cell populations of STEC after the treatments with protease or selected organic acids.

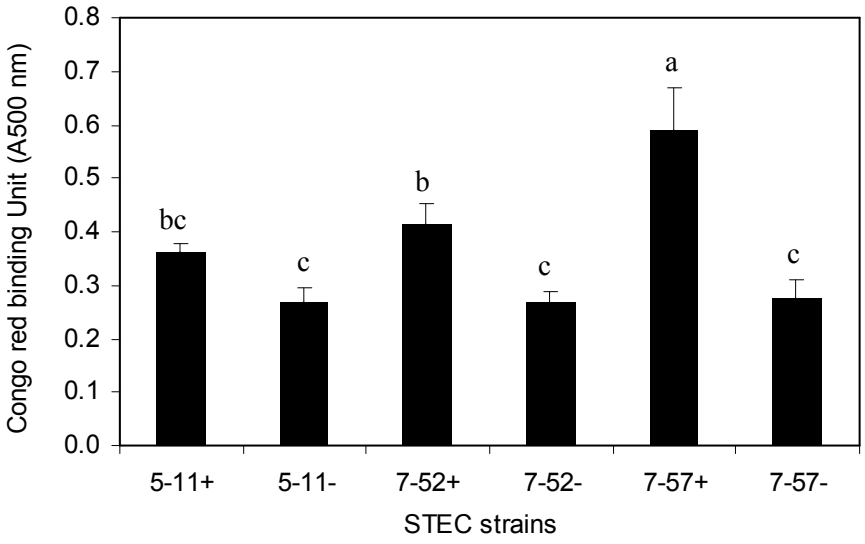
The linear models explain the relationships between the residual amounts of curli (x) and the surviving cell populations of individual STEC strains (y).

Correlations coefficients (r) measure the correlations between the residual amounts of curli and the surviving cell populations of STEC strains after the treatments with protease or selected organic acids.

† †*P* values indicate the significance of the correlations. $P < 0.05$ means that *r* exceeded the critical value with 95% confident.

FIGURE LEGENDS

Fig. 5.1. The amounts of curli expressed by different STEC strains used in the study. The amounts of curli expressed by STEC were measured using a Congo red binding assay and presented as the Congo red binding units which calculated the optical absorbance values of dye bound to curli.


Congo red binding Unit (A500 nm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
bc
c
b
c
a
c
5-11+
5-11-
7-52+
7-52-
7-57+
7-57-
STEC strains

CAPTER 6

CONTROL OF BIOFILMS FORMED BY THE CELLS OF SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* USING TREATMENTS WITH ORGANIC ACIDS AND COMMERCIAL DETERGENTS[1]

[1] Park, Y.J. and J. Chen. To be submitted to *Journal of Applied Microbiology*.

ABSTRACT

Aims: To determine the amounts of biofilm formed by different Shiga toxin producing *Escherichia coli* (STEC) on polystyrene and stainless steel surface and the effectiveness of treatments with organic acids and commercial detergents in removing the biofilms formed by STEC.

Methods and Results: Cells of STEC producing different amount of cellulose (n = 6) or curli (n = 6) were allowed to form biofilms on polystyrene and stainless steel surfaces at 28^{o}C for 7 d. The biofilms were treated with 2% acetic or lactic acid and manufacturer recommended concentrations of commerical detergents. Biofilms were quantified before and after the treatments with organic acids and commercial detergents. The result indicated that cells expressing both cellulose and curli formed the greatest amounts of biofilms. Cells expressing more cellulose and curli formed more biofilms. Polystyrene surface had more biofilm than did stainless steel surface. Acidic and alkaline detergents were more effective in removing biofilm than were organic acids.

Conclusions: Both cell components of bacterial cells and their contact surfaces are crucial for biofilm formation. Commonly used cleaners and sanitizers have different abilities to remove biofilm for surfaces.

Significance and Impact of the Study: The study provides additional knowledge for strategizing biofilm control.

Many strains of Shiga toxin- producing *Escherichia coli* (STEC) are pathogenic causing severe human illnesses such as haemorrhagic colitis and haemolytic uraemic syndrome (HUS) (Boerlin *et al*. 1999; Cleary 2004; Johnson *et al*. 1983). *E. coli* O157:H7 is the most predominant serotype of STEC which is responsible for majority of the foodborne outbreaks of human infections (Desmarchelier and Fegan 2003; Riley *et al*. 1983). Other serotypes of STEC such as O111 and O103 have also been associated with outbreaks and sporadic cases of diarrhea and HUS (Desmarchelier and Fegan 2003; Johnson *et al*. 1996; Nataro and Kaper 1998).

Human STEC infections are commonly caused by the consumption of contaminated food. Food contamination in food processing environment is often associated with biofilms (Kumar and Anand 1998). A biofilm is a population of microbial cells growing and enclosed in an amorphous matrix (Donlan 2002). Biofilm can contain various polymeric substances and includes proteins, DNA, RNA, ions, and extracellular polysaccharides (EPS) produced by bacterial cells (Sutherland 2001). Bacterial EPS such as cellulose plays a structural role for the formation of biofilm. The three-dimension biofilm matrix conferred by EPS has highly viscosity, rigid properties, and poor solubility (Hoyle *et al*. 1990; Sutherland 2001). In addition to EPS, a protein project expressed by enteric bacterial cells, curli, is also crucial for the formation of biofilm (Kumar and Anand 1998). Curli grants cells a better ability to interact with contact surfaces in nutrient restricted and low osmolarity environment and under lower than optimal growth temperatures (Austin *et al*. 1998; Olsen *et al*. 1989; RÖmling *et al*. 1998). EPS nevertheless, contributes to the architecture of biofilm and confers physical and mechanical strength to biofilm (Solano *et al*. 2002). The hydrophobicity of cell surface by hydrophobic surface components such like curli, fimbriea plays an important role in adherence or bacterial

attachment on the surface iniciating biofilm formation (Donlan 2002). The objectives of the present study were to quantify the amounts of biofilm formed by the cells of STEC on polystyrene and stainless steel surfaces, and evaluate the abilities of two organic acids and two commercial detergents in removing the biofilms from the two types of cell contact surfaces.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Two groups of STEC and a total of twelve strains from our laboratory collection were used in the study (Table 6.1). The first group consisted of six STEC strains producing different amounts of cellulose, a long chain polymer of glucose. The second group was comprised of three pairs of STEC, and each pair has a curli producer, and a non-curli producer (5-11+, 5-11-, 7-52+, 7-52-, 7-57+ and 7-57-). Confluent cell cultures of the STEC strains were grown on Luria Bertani no salt (LBNS) agar plates at 28^{o}C for 72 h. The LBNS agar was prepared by dissolving tryptone (10 g), yeast extract (5 g), and agar (15 g), all from Becton Dickinson and Co. (Sparks, MD), in 1 l of distilled water.

Preparation of STEC cultures and abiotic surfaces for biofilm studies

Each STEC culture grown on LBNS agar was transferred into 9 ml of LBNS broth (10 g tryptone and 5 g yeast extract) and incubated for 18 h at 28°C. The resulting cultures were diluted (1: 40) with fresh LBNS broth, and the diluted cultures were used in the following experiment.

Two types of surfaces were used for biofilm formation including the 24-well polystyrene tissue culture plates (Falcon 3847, Becton Dickinson Labware, Frankline Lakes, NJ) and stainless steel coupons (2 x 5 cm^2, 14- gauge and 2B finish; Impulse Manufacturing, Inc; Dawsonville, GA). The purchased polystyrene tissue culture plates were sterile and ready-to-use. The stainless steel coupons were soaked in diluted SparKLEEN solution (1:10; Fisher Scientific, Pittsburgh, PA) at room temperature for 18 h. The coupons were then cleaned with a dish-washing detergent (Cincinnati, OH) and rinsed thoroughly with deionized water before being autoclaved at 121°C for 30 min.

Biofilm formation on polystyrene surfaces

Diluted cell cultures of STEC described above (2 ml) were inoculated into the wells of the microtiter plates, respectively. The microtiter plates with un-inoculated broth were used as controls. The microtiter plates were incubated at 28°C for 7 d. The broth was withdrawn at the end of the incubation period, and loosely attached cells were removed by washing the plates twice, each with 2 ml of 0.1% peptone water. The plates with biofilm were air-dried at 60°C for 2 h before the biofilm mass was measured using the crystal violet binding assay (Pawar et al. 2005). Briefly, the tissue culture plates with the biofilm were passed over the flame of a burner (Fisher Scientific) several times. The fixed biofilm on the polystyrene surface was stained with 2 ml of 1% crystal violet (Fisher Scientific) at room temperature for 15 min and then washed with running deionized water until the wash water contained no visible strain. After drying for 2 h at 60°C and cooling to room temperature, the crystal violet from the stained STEC cells were extracted by washing each well of the tissue culture plates with 2 ml of ethanol-acetone solution

(80:20). The concentrations of crystal violet extracted from the cells were determined by measuring the absorbance of the ethanol and acetone solutions at the wavelength of 550 nm.

Biofilm formation on stainless steel surfaces

Stainless steel coupons were placed horizontally in Petri plates (Fisher Scientific). Each Petri plate had one stainless coupon and 30 ml of a diluted STEC culture described above. The Petri plates with the same number of coupon and same volume of un-inoculated LBNS broth served as controls. All Petri plates were incubated for 7 d at 28°C. At the end of the incubation, the broth was withdrawn, and the stainless steel coupons were washed once with 30 ml of 0.1% peptone water for 10 sec at the room temperature to remove loosely attached cells. Biofilms on the stainless steel surfaces were determined using a modified crystal violet binding assay. Specifically, the stainless steel coupons with the biofilms were washed, air-dried, and fixed as described above. The fixed biofilms on the stainless steel surface were stained with 5 ml of 1% crystal violet at room temperature for 15 min, and then washed with running deionized water until no visible stain in the rinsing water. The coupons were dried for 2 h at 60ºC, and then cooled to room temperature. The coupon in each Petri plate was washed with 5 ml of ethanol-acetone solution (80:20) with gentle shaking at 50 rpm to extract the crystal violet. The concentrations of the extracted crystal violet were determined as described above.

Removal of biofilm by treatments with organic acids and commercial detergents

Biofilms formed on polystyrene and stainless steel surfaces were treated with 2% acetic (99.9%, Purac America inc. Lincolnshire, IL, USA) and 2% lactic (87.9%, JT barker, Phillipsburg, NJ,

USA) acid, respectively. Two ml of 2% acetic or lactic acid was added into each well of the microtiter plates, and the microtiter plates were agitated at 50 rpm at room temperature for 20 min. Each stainless steel coupon was treated with 30 ml of 2% acetic or lactic acid, respectively, in Petri plates for 20 min at room temperature with gentle shaking at 50 rpm.

The biofilms on polystyrene and stainless steel surfaces were also treated with an acidic (pH 1.0 - 1.5; ZEP Manufacturing Company, Atlanta, GA.) and an alkaline (pH 13; Ecolab, St. Paul, MN.) detergent, respectively. The active ingredients in the alkaline detergent included potassium hydroxide, phosphoric acid and potassium hypochlorite, while the acidic detergent had phosphoric acid as the active component. The working solution of the alkaline detergent was prepared according to the manufacturer's instruction by diluting 10 ml of the commercial product in 368 ml of sterile water. The working solution of the acidic detergent was prepared by diluting the commercial product 4 fold with sterile water. Each diluted commercial detergent (2 ml) was added into the individual wells of the polystyrene microtiter plates, respectively. Each stainless coupon in the Petri plates was treated with 30 ml of commercial detergents described above, respectively. Both treatments were conducted at room temperature for 15 min with gentle shaking at 50 rpm.

After treatments with organic acids and commercial detergents, double strength Dey-Engley (DE) solution (2 ml; Becton, Dickinson and Company, Sparks, MD) was added into each well of the microtiter plates. Stainless steel coupons were nevertheless treated in 30 ml of double strength DE solution, and both treatments were conducted at room temperature for 10 min. The plates and coupons were left at room temperature for 10 min followed by air dry at 60°C for 2 h. The biofilm remaining on the tissue culture plates was determined using the

respective crystal violet binding assays described above.

Statistical analysis

Three replicates of each experiment were performed, and each experiment was conducted in duplicate. The data collected in the study were analyzed using the analysis of variance (ANOVA) followed by t-test, using the JMP statistical software version 8.0 (SAS Institute Inc., Cary, NC, USA, 2008). Significant differences between mean values of the absorbance at 550 nm were determined based on a 95% confidence level.

RESULTS

Removal of biofilm formed by Group I STEC on polystyrene surface

Treatments with 2% acetic and lactic acid only significantly ($P < 0.05$) reduced the amounts of biofilm formed by the cells of 6-8 and 7-49 (Table 6.2). Treatments with 2% lactic acid were not significantly more effective ($P > 0.05$) than those with the same concentration of acetic acid in reducing the amounts of biofilm formed by the Group I STEC strains. Treatments with the two commercial detergents significantly ($P < 0.05$) reduced the amounts of biofilm formed by the cells of all six Group I STEC cultures (Table 6.2). There is no significant difference ($P > 0.05$) between the effectiveness of the two detergents (Table 6.2).

According to the results of the control samples in Table 2, cells of 7-49 and 7-50 formed significantly ($P < 0.05$) more biofilms on polystyrene surface than all the other Group I STEC strains used in the study. The amounts of biofilm formed by the cells of 6-8, 6-35, and 7-51 were

found to be significantly ($P < 0.05$) lower than those formed by the cells of 7-49 and 7-50, but significantly ($P < 0.05$) higher than the amount of biofim formed by the cells of 7-17 (Table 6.2). The residual amounts of biofilm formed by STEC cells had the same order after the treatments with the two commercial detergents and lactic acid. Even though the order in the residual amounts of biofilm changed slightly after the treatments with acetic acid, the amounts of biofilm formed by the cells of 7-49 and 7-50 remained to be higher, and those by the cells of 6-35 and 6-8 were among the intermediates. The residual amount of biofilm formed by the cells of 7-17 remained to be the lowest in the group (Table 6.2).

Removal of biofilm formed by Group I STEC on stainless steel surface

The trends in biofilm formation by the cells of Group I STEC on stainless steel coupons were similar to those on polystyrene surfaces. In the control samples, cells of 7-50 formed greatest amount of biofilm followed by the cells of 7-49. The amounts of biofilm formed by the cells of 6-8, 6-35 and 7-51 were significantly ($P < 0.05$) higher than the amount of biofilm formed by the cells of 7-17 (Table 6.3).

Treatments with 2% acetic and lactic acids did not significantly ($P > 0.05$) reduced the amounts of biofilm developed by the cells of all Group I STEC strains on stainless steel surface except for the biofilm formed by the cells of 6-35 and treated by 2% lactic acid (Table 6.3). Treatments with lactic acid were not significantly ($P > 0.05$) more effective than those with acetic acid in removing the biofilm formed by Group I STEC on stainless steel surface. Treatments with the two commercial detergents were not significantly more effective ($P < 0.05$) than were the two organic acids in removing the biofilms formed on the surface of stainless steel. The acidic

detergent significantly ($P < 0.05$) reduced the amounts of biofilm formed by the cells of Group I STEC except for the biofilms formed by the cells of 6-8 and 7-51, and the alkaline detergent also did not significantly ($P > 0.05$) reduced the amount of biofilm formed by the cells of 6-35 (Table 6.3).

The abilities of Group I STEC to form biofilm on stainless steel surface were similar to those on polystyrene surface. After the treatment with the organic acids and commercial detergents, the residual amounts of biofilm formed by the cells of 7-17 as well as 7-49 and 7-50 remained to be the lowest and highest respectively, whereas the group of 6-8 and 6-35 had the intermediate amounts of biofilms. However, majority of these differences were not statistically significant.

Removal of biofilms formed by Group II STEC formed on polystyrene surface

Treatments with 2% acetic acid only significantly reduced ($P < 0.05$) the amounts of biofilm of 7-52- on polystyrene surface, while treatments with lactic acid significantly reduced ($P < 0.05$) the amounts of biofilms of 5-11+, 7-52+, and 7-52- (Table 6.4). The biofilms formed by other tested group II STEC strains were not significantly reduced ($P > 0.05$) by the acid treatments. Treatments with alkaline detergent significantly ($P < 0.05$) reduced the amounts of biofilm formed by all group II STEC except for the cells of 5-11- (Table 6.4). However, treatment with the acidic detergent only significantly ($P < 0.05$) reduced the biofilms of 5-11+ and 7-52-. Before the treatments with organic acids and commercial detergents, cells of 7-52+ and 7-57+ formed significantly ($P < 0.05$) more biofilms than the cells of their non curli expressing counterparts, 7-52- and 7-57- on the surface of polystyrene (Table 6.4). The amounts of biofilm formed by the cells of 5-11+ and 5-11- were however, only numerically ($P > 0.05$) different

(Table 6.4). The order of residual amount of cellulose in the cell suspensions of Group II STEC remained the same after the treatments with the two organic acids and commercial detergents (Table 6.4).

Removal of biofilms formed by Group II STEC on stainless steel surface

Treatment with acetic acid and lactic acid only significantly ($P < 0.05$) reduced the amounts of biofilm formed by the cells of 7-57- and 7-57+, respectively on stainless steel surface (Table 6.5). The two commercial detergents used in the study significantly ($P < 0.05$) reduced the amounts of biofilms formed by the cells of 7-52+, 7-57+ and 7-52-. The acidic detergent also significantly ($P < 0.05$) reduced the amount of biofilm formed by the cells of 5-11- on the surface of stainless steel (Table 6.5).

Similar to the biofilms formed on polystyrene surface, cells of 7-52+ and 7-57+ formed significantly ($P < 0.05$) more biofilms than the cells of their non curli expressing counterparts, 7-52- and 7-57- ($P < 0.05$) (Table 6.5). The amounts of biofilm formed by the cells of 5-11+ and 5-11- were however, only numerically ($P > 0.05$) different (Table 6.5). This trend remained only after the treatment with lactic acid and alkaline detergent.

Influence of contact surfaces on biofilm formation

The overall results showed that the cells of Group I STEC strains formed more biofilms on the surface of polystyrene than they did on the surface of stainless steel with the exception of 7-17 (Fig. 6.1A). Cells of Group II STEC strains also formed more biofilms on polystyrene than on stainless steel surface except for the cells of 7-52- (Fig. 6.1B).

DISCUSSION

In the present study, greater amounts of biofilm were formed by STEC cells on polystyrene, than on stainless steel, surface except for the biofilms formed by the cells of 7-17. Polystyrene surface is hydrophobic, electrostatic and has low surface energy. The surface of stainless steel is hydrophilic and possesses high surface energy (Fletcher and Loeb 1979). It can be either positively or negatively charged. Hydrophobic surfaces have been reported as the preferential materials for bacterial attachment and biofilm formation (Jain and Chen 2007; Pawar *et al*. 2005; Fletcher and Loeb 1979). Pawar *et al*. (2005) found that higher numbers of EHEC cells attached to polystyrene and rubber surface compared with those attached to stainless steel and glass surface. Uhlich et al. (2006) found that greater amounts of biofilm were formed by the cells of *E. coli* O157:H7 on polystyrene, compared to stainless steel, glass and teflon surface.

Results of the present study showed that the expression of cellulose enhanced the ability of STEC cells to form biofilms on their contact surfaces (Table 6.2 and 6.3). Similar findings have been reported previously, and cellulose was found as a component of the biofilms formed by *E. coli*, *Enterobacter sakazakii* and *Salmonella* cells (Jonas *et al*. 2007; Solano *et al*. 2002). Da Re and Ghigo (2006) reported that cellulose production contributed to the formation of biofilm by *E. coli*. Mutant cells of *E. coli* deficient in cellulose expression also lost the ability to form biofilms. Cellulose produced by the cells of *Agrobacterium* played an important role in the formation of aggregates on carrot tissue culture cells (Matthysse 1983). Overproduction of cellulose by the cells of *Agrobacterium* leads to the formation of an extensive, thick biofilm on tomato root surface (Matthysse *et al*. 2008).

Results of the present study also showed that curli-expressing STEC cells formed, a greater amount of biofilm than did noncurli-expressing cells on polystyrene and stainless steel surfaces (Table 6.4 and 6.5). This observation was consistent with previous finding. Curli and other fimbriae of *E. coli*, *Salmonella* and *Pseudomonas* play a functional role in cell attachment to abiotic surfaces during biofilm development (Austin *et al*. 1998; O'Toole and Kolter 1998; Pawar *et al*. 2005). Cells of *E. coli* deficient in curli expression lost the abilities to form pellicles and adhere to polystyrene surface (Bokranz *et al*. 2005). Expression of curli by some strains of *E. coli* enhanced the attachment of cell on the surfaces such as polystyrene, glass, stainless steel, and rubber, resulted in better biofilm forming (Pawar *et al*. 2005; Cookson *et al*. 2002). Cells of *E. coli* or *Salmonella* capable of expressing both cellulose and curli have been shown to be better biofilm formers (Bokranz *et al*. 2005; Jain and Chen 2005; Uhlich *et al*. 2006). STEC strain 7-49 and 7-50 used in the present study expressed both cellulose and curli and had significantly greater ability ($P < 0.05$) to form biofilms on polystyrene and stainless steel surfaces than all the other cultures used in the study. The hydrophobic feature of bacterial cell surface resulting from the co-expression of curli and cellulose was proposed as the key factor mediating the interaction between bacterial cells and their contact surfaces (Zogaj *et al*. 2003). In addition to protein (curli) and polysaccharide (cellulose), biofilm matrix also contains nucleic acids as well as microbial cells (Sutherland. 2001). It was estimated that polysaccharides make up *ca*. 1-2% of the biofilm mass, which is equivalent to the combined fraction of proteins and nucleic acids in biofilms, while microbial cells accounts for 2-5 % of the biofilm composition (Sutherland 2001).

Individual components of a biofilm matrix can be reduced by acidic treatments, which

subsequently cause the breakdown of biofilm structures. For instance, Chen and Stewart (2000) showed the reduction of biofilm protein by various chemical treatments. Hypochlorite at 15, 25 mg/l (pH 6.4) and 15 mg/l (pH 10.9) removed biofilm protein formed by Pseudomonas by 37-65%. Also 16 and 47% of the same biofilm protein was removed by increasing or decreasing pH to 2.9 or 11.2 (Chen and Stewart 2000).

Under acidic condition, the glycoside oxygen atom of a polysacchride molecule accepts H^+ and forms a protonated glycosidic bond. Although extreme condition with high concentration or elevating temperature is required to complete hydrolysis cellulose to monomer of glucose, weak acid in mild condition also can degrade the vulnerable glycosidic bond causing depolymerization or increasing solubility of cellulose (Yan *et al*. 2009). Acids also denaturated protein and intrupt the hydrophobic association of protein assembles on bacterial cell surface (Appel et al. 2006; Newcombe and Cohen 1965, Voet and Voet 1995). Furthermore, organic acids in undisassociated form interrupt the functions of bacterial membrane bound proteins by penetrating lipid bilayer and making cell cytoplasm more acidic (Paul and Hirshfield 2003).

The alkaline detergent used in the study contained potassium hydroxide and potassium hypocholorite. Hypochlorite depolymerizes polysaccharides through chlorinolysis of glycosidic bonds. Chlorine reacts with the glycosidic oxygens of polysaccharide forming a chloro-oxonium cation, which undergo cleavage at oxygen bond. The anion such like chloride can attack the chloro-oxonium cation, resulting in the formation a glycosyl halide which forms a unit sugar by reacting with water (Whistler and Pyler 1968). Hypochlorite is also an oxidant, majorly to protein, and such reaction results in side-chain modification, backbone fragmentation and cross linking in protein (Pattison and Davies 2001). The backbone amide groups of proteins react with

hypochlorite, leading to the formation of chloramides which can further react or oxidize by Cl^- (Thomas 1979). The hydrolysis of backbone chloramides occur in the presence of water, which cause direct cleavage of peptide bonds (Thomas 1979). Mono and poly saccharides such as cellulose can be degraded by alkaline hydrolysis. Under alkaline condition, monosaccharides such as glucose are degraded by the sequence involving protonation of anion via a migration of hydrogen atom or proton, β-hydroxycarbonyl elimination, and production of saccharinic acids (Knill and Kennedy 2003). Cellulose which $\beta(1\rightarrow4)$ linked polysaccharide can be also degraded in the alkaline condition via the subsequent reactions via a peeling off reaction, a cleavage of glycosidic linkage, and then decrease of polymerization (Knill and Kennedy 2003). The amide units of peptide bond within a protein molecule can be hydrolyzed by hydroxyl ion (Brown et al. 1992). Proteins with the β-sheet structure are reportedly hydrolyzed by saturated calcium hydroxide *via* base-catalyzed hydrolysis (Greenlee et al. 2008). The hydroxyl ion hydrolyzes the peptide bonds and penetrates the β-sheet structure by disrupting hydrogen bonds (Appel et al. 2005). Phosphodiester bonds in nucleic acid molecules can also be hydrolyzed by alkaline hydrolysis. Hydroxides such as 0.2 N $Ba(OH)_2$ and 0.5 N NaOH cause the phosphodiester bonds to rupture (Richards and Boyer 1965). Active ingredient, potassium hydroxide used also has antibacterial activity by altering cell membrane potential, pH gradient, internal pH and proton motive force of bacterial cells, and potassium hypochlorite oxidize bacterial enzymes and nucleic acids (Repaske and Adler 1981).

The results of current and several other studies demonstrated that considerable amounts of biofilm remained on the surfaces where biofilms have formed after sanitizing treatments. Frank *et al*. (2003) believed that this may be caused by insufficient contact time between biofilm

and the sanitizing agent and improper covering of surface area by the sanitizing agent. Extracellular structures produced by bacterial cells in biofilm have been reported as an important factor limiting the efficacies of biofilm control (Uhlich *et al*. 2006; Solano *et al*. 2002). The rigid network structure of biofilm makes it difficult for sanitizing agents to access key target areas. Biofilm matrix have been shown to neutralize unstable sanitizing agent such as chlorine, thereby reducing the sanitizing capacity of the agent (de Beer *et al*. 1994).

This study suggests that organic acids and detergents used in the present study are capable of controlling the biofilms formed on polystyrene and stainless steel surface, but the treatments are not sufficient for complete removal of the biofilms. It is necessary to select effective cleaning or sanitizing agents and study the treatment conditions appropriate for complete removal of biofilms formed by bacterial cells on their contact surfaces.

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Table 6.1. STEC strains used in the study.

STEC strains	Cellulose production	Curli biosynthesis
Group 1: Cellulose producing strains		
7-17	+	-
7-49	+	+
7-50	+	+
7-51	+	-
6-8	+	-
6-35	+	-
Group 2: curli producing and non curli producing strains		
5-11+	-	+
5-11-	-	-
7-52+	-	+
7-52-	-	-
7-57+	-	+
7-57-	-	-

Cellulose production by STEC cells were identified as florescent on agar plate supplemented with calcoflur under UV light.

Curli biosynthesis by STEC cells were identified as colony morphology on agar plate supplemented with congo red and coomassie brilliant blue.

Table 6.2. Absorbance (550 nm) of crystal violet staining of biofilms, formed by Group I STEC cells on the surface of polystyrene, after the treatments with organic acids and commercial detergents

Treatments with sanitizers		Absorbance (550 nm) of crystal violet					
		STEC strains					
		7-17	7-49	7-50	7-51	6-8	6-35
Control		0.07aC	1.53aA	1.62aA	1.14aB	0.95aB	0.98aB
Organic acids (2%, 20 min , room temperature)	Acetic acid	0.04abD	1.36bAB	1.41abA	1.10aB	0.80bC	0.80aC
	Lactic acid	0.06abC	1.31bcA	1.36abA	0.95abB	0.74bB	0.77aB
Commercial detergents[a] (15 min, room temperature)	Acidic	0.02bC	1.18cA	1.03cA	0.62cB	0.51cB	0.44bB
	Alkaline	0.03bC	1.21cA	1.20bcA	0.75bcB	0.54cB	0.49bB

The values in the same columns not followed by the same lowercase letter are significantly different with respect to STEC strains ($P < 0.05$).
The values in the same row not followed by the same uppercase letter are significantly different with respect to treatments ($P < 0.05$).
[a]: Manufacturers recommended concentrations were used.

Table 6.3. Absorbance (550 nm) of crystal violet staining of biofilms, formed by Group I STEC cells on the surface of stainless steel, after the treatments with organic acids and commercial detergents

Treatments with sanitizers		Absorbance (550 nm) of crystal violet					
		STEC strains					
		7-17	7-49	7-50	7-51	6-8	6-35
Control		0.43aC	1.34aA	1.49aA	1.04aB	0.89aB	0.86aB
Organic acids (2%, 20 min, room tempearture)	Acetic acid	0.40aB	0.97abA	1.06abA	1.02aA	0.59aB	0.60abB
	Lactic acid	0.29abC	0.98abA	0.91abAB	0.60aBC	0.41aC	0.42bC
Commercial detergents[a] (15 min, room temperature	Acidic	0.14cA	0.47bA	0.49bA	0.49aA	0.34aA	0.23bA
	Alkaline	0.16bcB	0.61bAB	0.85bA	0.61aAB	0.54aAB	0.53abAB

The values in the same columns not followed by the same lowercase letter are significantly different with respect to STEC strains ($P < 0.05$).
The values in the same row not followed by the same uppercase letter are significantly different with respect to treatments ($P < 0.05$).
[a]: Manufacturers recommended concentrations were used.

Table 6.4. Absorbance (550 nm) of crystal violet staining of biofilms, formed by Group II STEC cells on the surface of polystyrene, after the treatments with organic acids and commercial detergents

Treatments with sanitizers		Absorbance (550 nm) of crystal violet					
		STEC strains					
		5-11+	5-11-	7-52+	7-52-	7-57+	7-57-
Control		1.24aBC	1.04aC	1.47aAB	0.66aD	1.54aA	0.57aD
Organic acids (2%, 20 min, room temperature)	Acetic acid	1.19aA	0.98aA	1.20abA	0.36bB	1.36aA	0.36abB
	Lactic acid	0.62bCD	0.85aBC	1.06bAB	0.31bD	1.25aA	0.42abD
Commercial detergents[a] (15 min, room temperature)	Acidic	0.57bBC	0.96aAB	1.18abA	0.24bC	1.19abA	0.36abC
	Alkaline	0.60bAB	1.04aA	0.98bA	0.15bB	0.78bA	0.20bB

The values in the same columns not followed by the same lowercase letter are significantly different with respect to STEC strains ($P < 0.05$).
The values in the same row not followed by the same uppercase letter are significantly different with respect to treatments ($P < 0.05$).
[a]: Manufacturers recommended concentrations were used.

Table 6.5. Absorbance (550 nm) of crystal violet staining of biofilms, formed by Group II STEC cells on the surface of stainless steel, after the treatments with organic acids and commercial detergents

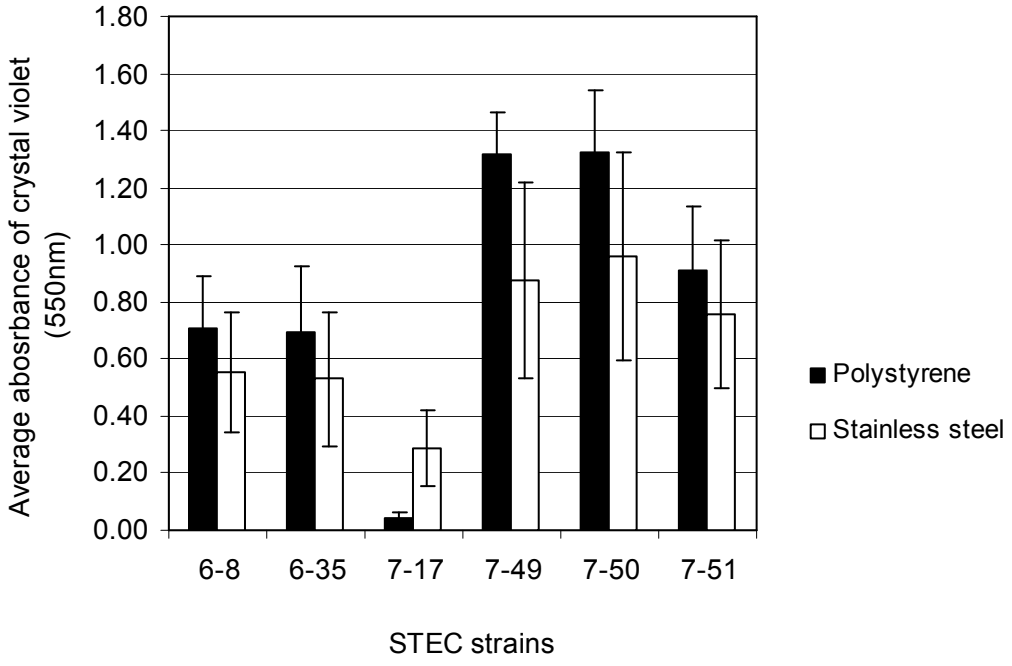
Treatments with sanitizers		Absorbance (550 nm) of crystal violet					
		STEC strains					
		5-11+	5-11-	7-52+	7-52-	7-57+	7-57-
Control		1.03aAB	1.08aAB	1.24aA	0.62aBC	1.42aA	0.44aC
Organic acids (2%, 20 min, room temperature)	Acetic acid	0.69abAB	0.69aAB	1.01abA	0.72aAB	0.87bAB	0.37abB
	Lactic acid	0.76abA	0.87aA	0.93abA	0.49aB	0.82bA	0.30bcC
Commercial detergents[a] (15 min, room temperature)	Acidic	0.29bB	0.45aAB	0.61bA	0.29aB	0.32cB	0.20cB
	Alkaline	0.68abA	0.74aA	0.70bA	0.43aAB	0.73bA	0.22cB

The values in the same columns not followed by the same lowercase letter are significantly different with respect to STEC strains ($P < 0.05$).
The values in the same row not followed by the same uppercase letter are significantly different with respect to treatments ($P < 0.05$).
[a]: Manufacturers recommended concentrations were used.

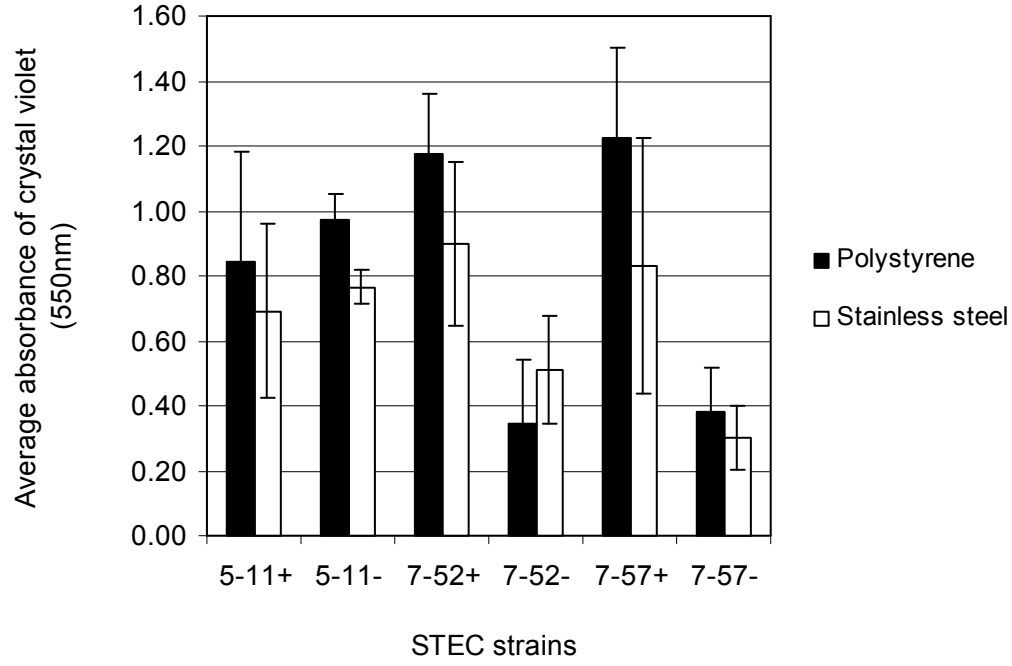
Figure Legend

Fig. 6.1. Overall absorbances (550 nm) of crystal violet staining of biofilm formed by Group I (A) and Group II (B) STEC cells on polystyrene vs. stainless steel surface. Residual biofilm was quantified after the treatments with organic acids and commercial detergents used in the study using the crystal violet assay and the absorbance was determined at the wavelength of 550 nm.

(A)


1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Average abosrbance of crystal violet (550nm)
6-8
6-35
7-17
7-49
7-50
7-51
STEC strains
Polystyrene
Stainless steel

(B)


1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Average absorbance of crystal violet (550nm)
5-11+
5-11-
7-52+
7-52-
7-57+
7-57-
STEC strains
Polystyrene
Stainless steel

CHAPTER 7

SUMMARY AND CONCLUSIONS

The following is a summary of results and conclusions drawn from the research reported in Chapter 3-6 of this dissertation:

1. Use of phosphate buffered saline, morpholoinepropanesulfonic acid, sodium thiosulfate buffers and single strength Dey-Engley broth as neutralizing systems did not significantly affect the number of STEC cells recovered from the treatments with 2% acidic and lactic acid as well as a manufacturer-recommended concentration of an alkaline detergent. The evaluated neutralizing agents did not have toxicity to the evaluated nine STEC strains. Sodium thiosulfate buffer, single strength DE and MOPS buffer neutralized the activities of 2% acetic acid, and single strength DE broth and sodium thiosulfate buffers neutralized the activities of 2% lactic acid against nine of the STEC strains. MOPS, Sodium thiosulfate and single strength DE broth neutralized the activities of the alkaline detergent against four to six of the STEC strains, but the evaluated neutralizing agents neutralized the activities of the acidic detergents against one to three of the STEC strains. Additional studies showed double strength DE broth effectively neutralized the activities of the acidic and alkaline detergents. Using this double strengthe DE broth to neutralize the activities of selected organic acids and commercial detergents, the recovering populations of STEC and residual amounts of curli and biofilm produced by various strains of STEC on can be determined and compared.

2. Treatments with 2% acetic and lactic acid significantly reduced the average populations of six STEC strains, and treatments with 2% lactic acid also significantly decreased the amounts of cellulose which were produced by STEC cells ($P < 0.05$). As the positive correlation (r = 0.64-0.94) between the surviving cell populations and the residual cellulose, the STEC strains with higher level of the residual amounts of cellulose had better ability to survive after treatments with the two organic acids. Treatments with cellulase and detergents both degraded cellulose. However, only the treatments with the two commercial detergents reduced the populations of individual STEC to undetectable levels from 8.77- 9.09 log CFU/ml. As a result, correlations between the residual amounts of cellulose and the surviving populations of STEC from the treatments with the two detergents were not observed.

3. Treatments with 2% acetic and lactic acid significantly reduced the amounts of curli and the average populations of six STEC strains ($P < 0.05$). Under these treatments, the residual amounts of curli positively correlated to the surviving cell populations of STEC strains with only one exception, and the correlation coefficients were 0.55 – 0.92. Treatments with protease degraded curli but had no influence on the survival of STEC cells. Treatments with the two commercial detergents degraded curli and reduced the populations of STEC to undetectable levels from 8.90 - 9.13 log CFU/ml. Correlations between surviving cell populations and residual amounts of curli under the treatments with the two commercial detergents were not observed.

4. Cells expressing cellulose and/or curli formed the greater amounts of biofilms. More biofilm was formed on polystyrene than on stainless steel surface. The acidic and alkaline detergents evaluated in the study were more effective than the organic acids in removing biofilms from their contact surfaces.

The approach to reduce cell surface components such like cellulose and curli has not been implicated for effective control biofilms. However, the protective affect of cell surface components for biofilm forming STEC cells exists, because cellulose and curli forms tertiary extracellular structure that would inhibits the asscess of sanitizers or cleaners. These components also associated with biofilms as biomass that may contribute to build up biofilm by fresh microbioal cells. Results in this dissertation confirm that the selected organic acids and commercial detergents which effectively reduced cellulose and curli on STEC also showed better ability to inactivate STEC cells. Cellulose and/or curli producing STEC had better ability to form biofilms, and these components on cell surface assisted STEC cells to form higher level of biofilms on polystyrene surface comparing to stainless still surface. More biofilms biomass formed by STEC cells was removed by organic acids and commercial detergent being capable to reduce cellulose and curli were particularly.

Findings emphasize the importance of cellulose and curli produced by STEC in control biofilm biomass formed by STEC. The effectiveness of sanitizers for the reduction of these cell surface components assisted to assess the successful sanitizing systems used for the food contact surfaces for the purpose of biofilm control.