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Enzyme-based approaches to control microbial biofilms in dairy processing environments: A review

S. F. Gonçalves[1], S. H. I. Lee[1], A. Mousavi Khaneghah[2], C. A. F. Oliveira[1]*

[1]Department of Food Engineering, School of Animal Science and Food Engineering, University of São Paulo, Pirassununga, SP, Brazil; [2]Department of Food Science, Faculty of Food Engineering, State University of Campinas, Campinas, SP, Brazil

***Corresponding Author:** C. A. F. Oliveira, Department of Food Engineering, School of Animal Science and Food Engineering, University of São Paulo, Pirassununga, SP, Brazil. Email: carlosaf@usp.br

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Abstract

In this review, formation of biofilms and the available data on enzyme-based preparations to control microbial biofilms in dairy processing environments are presented. Mature biofilms, especially those formed by pathogenic bacteria, have increased resistance to biocides, hence stressing the importance of stringent hygienic procedures. Proteases, amylases, cellulases and DNAses are among the most recently studied enzymes that could be associated with the main biocides used in the dairy industry to increase the effect of removal of biofilm. However, additional studies should be conducted to select the best cost-benefit combinations of selected enzymes and biocides to remove efficiently biofilms in dairy processing environments.

Keywords: biofilm; enzyme-based preparations; industrial hygiene; surface contamination

Introduction

Contaminated surfaces that have been exposed to food are potential sources of transmitting pathogenic or deteriorating microorganisms in food processing and at handling sites. Microorganisms can be found on the surfaces of equipment and utensils in the form of planktonic or sessile cells, which may form biofilms that increase the risk of food contamination (Fleming et al., 2016). Biofilms are defined as communities of microorganisms, of sessile microbial life, with adhesion to solid supports and production of extracellular polymeric substances (EPS), representing about 50–95% (w/w), thus ensuring cell protection (Hooshdar et al., 2020). Biofilms can contain many bacterial and fungal cells that may coexist and establish a cooperative/aggressive phenotype in which they can form a three-dimensional layer or structure (Oxaran et al., 2018). Biofilms are considered as highly organised forms, allowing the exchange of nutrients and metabolics in the same ecosystem in order to guarantee survival (Shiand Zhu, 2009). Among foodborne microorganisms that form biofilms on surfaces (Figure 1), *Staphylococcus aureus* (Lee et al., 2014), *Bacillus cereus* (Ehling-Schulz et al., 2019; Gopal et al., 2015), *Listeria monocytogenes* (Lee et al., 2017a), *Escherichia coli* (Cherif-Antar et al., 2016), *Salmonella* spp. (Wang et al., 2016) and *Pseudomonas* spp. (Cherif-Antar et al., 2016; Rossi et al., 2018) are of major importance for the dairy industry because of their frequent occurrence and potential health and economic effects.

S. aureus is a Gram-positive coccus, anaerobic facultative, coagulase and catalase positive bacterium and is considered as one of the main causes of bacterial foodborne diseases in humans (Gutiérrez et al., 2012). Food poisoning by *S. aureus* is characterised by symptoms such as nausea, vomiting, cramps and diarrhoea, which can be triggered if concentration of *S. aureus* in food is more than 10^5 colony forming units/gram (CFU/g) (Jamali et al., 2015). *S. aureus* is often found in milk and dairy products, which are excellent substrates for the growth of the pathogen (Lee et al., 2014). *B. cereus* is a rod-shaped, facultative aerobic and Gram-positive bacterium that can form spores, with significant impact on human health

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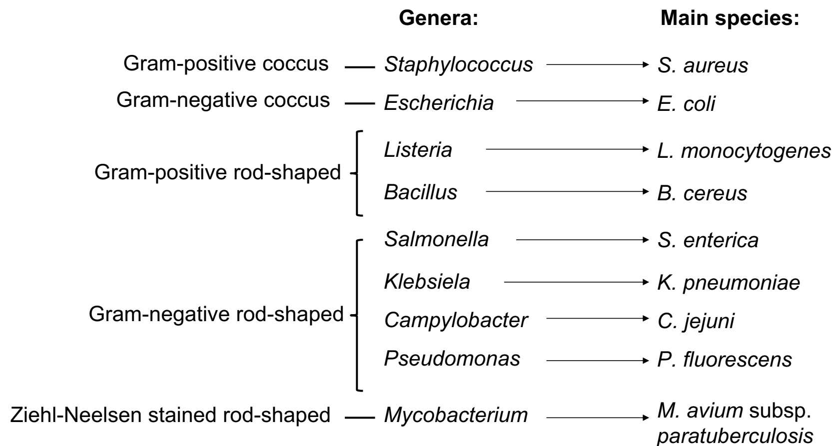

Figure 1. Main biofilm-forming pathogenic microorganisms found in dairy processing environments.

as a causative agent of foodborne diseases (Owusu-Kwarteng et al., 2017). Dairy foods contaminated with *B. cereus* are among the most frequent vehicles of its toxins responsible for diarrhoeal or emetic syndromes (Vidic et al., 2020). The diarrheal syndrome caused by *B. cereus* is characterised by abdominal pain, watery diarrhoea and rectal tenesmus, which develop between 8 and 16 h of eating contaminated foods containing levels above 10^5 CFU/g (Bottone, 2010). The concentration required for developing emetic syndrome is around 10^7 CFU/g, which is characterised by nausea and vomiting after 1 to 5 h of ingestion of contaminated food (Bottone, 2010).

L. monocytogenes is another Gram-positive, rod-shaped bacterium found in milk and dairy products that causes human diseases, including listeriosis, after consuming contaminated foods (Akrami-Mohajeri et al., 2018). *L. monocytogenes* causes meningitis, endocarditis, encephalitis, septicemia, spontaneous abortion, immune depression and death in extreme cases (Rodríguez-López et al., 2018). Several risk assessments have concluded that levels $>10^6$ CFU/g are responsible for the majority of cases of invasive listeriosis (Ryser, 2011). *L. monocytogenes* has been reported in raw milk of cow, sheep and goat (Mansouri-Najand et al., 2015) as well as in ready-to-eat dairy products such as cheese (Jakobsen et al., 2011) and ice cream (Chen et al., 2016). Other bacteria frequently found in milk and dairy products, with economic and health interest, as well as biofilm formation ability, include several species of *Pseudomonas* such as *P. fluorescens* (Meng et al., 2017), *Klebsiela pnemoniae* (Mohamed et al., 2018), *Mycobacterium avium* subsp. *paratuberculosis* (Mullan, 2019), *Campylobacter jejuni* (Song et al., 2020), *S. enterica* and *E. coli* O157:H7 (Ahmed and Shimamoto, 2014).

Since biofilms are formed, dispersed, and then reformed on the same surface with the remains of the previously formed biofilms, regular and effective cleaning methods are usually useful to prevent bacterial cells' initial adhesion on surfaces in the dairy industry. For this reason, food quality programmes, such as Good Manufacturing Practices, Sanitation Standard Operating Procedures, and Hazard Analysis and Critical Control Points, are fundamental tools to ensure effective cleaning and disinfection in food production environments (Cusato et al., 2014), thus avoiding the adhesion of bacterial cells and subsequent formation of biofilms on surfaces (Dominciano et al., 2016). In this context, the process of cleaning-in-place (CIP) is considered a central point in the biofilm control, provided that sanitisers are used to ensure the inactivation of microorganisms (Srey et al., 2013). The correct application of biocides during the CIP process is crucial for reducing the bacterial load at the end of the disinfection procedure (Dominciano et al., 2016). However, pipe connections, valves and other places with difficult access for biocides are the main obstacles for proper cleaning of dairy processing lines, thus representing potential biofilm formation (Marchand et al., 2012). Moreover, microorganisms adhered to surfaces after biofilm formation are more resistant to biocides, such as chlorine, than non-adherent microorganisms (Lee et al., 2016, 2017b).

In food processing environments, bacterial species typically reside in mixed-species biofilm, leading to several interactions among species, which markedly influence the growth of biofilms (Oxaran et al., 2018). In this context, microorganisms that often co-occur in dairy environments such as some species from the genera *Lactobacillus* and *Streptococcus* have been tested regarding their potential anti-biofilm activity (Jeong et al., 2018). In addition, biofilms formed by *L. rhamnosus* GG were successfully used for detoxification of aflatoxin M_1 in milk (Assaf et al., 2019). Yeganeh et al. (2017) reported that *L. plantarum*, *L. casei* and *L. acidophilus* were effective against a ciprofloxacin-resistant uropathogenic

strains of *E. coli* in pasteurised milk, resulting in an inhibitory effect on formation of its biofilm. According to Kim et al. (2019), the use of bacteriocins produced by *L. brevis* significantly reduced biofilms formed by *E. coli* and *S. enterica* serovar Typhimurium, mainly in the initial stages of biofilm formation. However, practical applications of microorganisms against biofilms formed by foodborne pathogens in real food processing environments have not been assessed.

In recent years, several research works have demonstrated that some enzyme-based preparations are effective against microbial biofilms on surfaces by degrading the biofilm matrix components (Coughlan et al., 2016). Nahar et al. (2018) published an extensive review of the enzyme-based biofilm impairment strategies with potential food industry applications. The scientific interest in these issues has markedly increased in the past years, leading to newly published data on enzyme-based approaches to tackle the biofilm problem. This article aims to review the biofilm formation and the recently published data on enzyme-based preparations to control biofilms formed by pathogenic bacteria in dairy processing environments.

Biofilm formation and structure

The formation and development of biofilms on surfaces depend on several factors, such as the type of surface material, pH, temperature and availability of organic and inorganic materials (Di Ciccio et al., 2015; Fleming et al., 2016; Srey et al., 2013). Biofilms have a porous structure with channels where water and nutrients are distributed, providing a homogeneous architecture of the environment that ensures a consistent growth with effective transport of water, nutrients and oxygen to its interior (Oxaran et al., 2018). The formation of biofilms begins on surfaces when nutrients are available, and the first stage of formation is considered as adhesion of microorganisms through the matrix of exopolysaccharides favouring the appearance of microcolonies (Lee et al., 2016). The irreversible adhesion stage results from high specificity and short-distance interactions between pili, flagella and EPS production, by which dipole–dipole interaction bonds, hydrogen bonds, covalent and ionic bonds are strengthened (Høiby, 2017). These factors provide initial adhesion onto surfaces, leading to the beginning of cell mass growth, which ultimately forms bacterial biofilm (Cherif-Antar et al., 2016; Marchand et al., 2012). The biofilm assumes an organised flat or mushroom shape during the maturation stage, depending on the source of nutrients found on the surfaces (Srey et al., 2013). The period between formation and maturation of biofilms is variable, usually between 3 and 6 days after the initial adhesion stage (Nahar et al., 2018). Dispersion is the last stage in the biofilm cycle, allowing cells to detach and initiate a new cycle. Detachment occurs due to internal processes, such as the release of endogenous enzymes and EPS (Srey et al., 2013).

Among foodborne pathogens, adhesion of *B. cereus* is attributed to its hydrophobic characteristics (Ehling-Schulz et al., 2019). In addition, *B. cereus* biofilms can behave as nests for the formation and release of spores in food-producing environments, which are difficult to eradicate since the spores are enveloped by the matrix adhered to guarantee its complete germination, thus making it resistant to sanitising agents (Kwon et al., 2017). The ability of *S. aureus* to form biofilms is also an important virulence factor since this mechanism guarantees its survival in a new environment (Watters et al., 2016). Materials such as stainless steel, glass and polypropylene have proven to be the sources of contamination by *S. aureus* after undergoing adhesion and subsequently forming biofilms (Lee et al., 2014; Unlu et al., 2018). *L. monocytogenes* is another pathogenic bacterium whose biofilms represent a significant problem in dairy processing areas, mainly because of the contamination of food handling surfaces, equipment and pipes (Kadam et al., 2013). Formation of biofilms of *L. monocytogenes* depends on several factors, such as pH, surface material for adhesion, availability of inorganic or organic matter, resistance to adhesion and temperature (Lee et al., 2017a, 2017b). According to Silva and De Martinis (2013), *L. monocytogenes* can adhere and form biofilms at a cell concentration of 10^4–10^7 UFC/cm^2. Moreover, Colagiorgi et al. (2017) and Oxaran et al. (2018) demonstrated that *L. monocytogenes* could form mixed-species biofilms with *S. aureus Flavobacterium* spp. on stainless steel surfaces widely used in the dairy industry. In another study, Alonso and Kabuki (2019) observed a dominant behaviour of *Enterococcus faecalis* in mono-species, as well as in multi-species, biofilms with *L. monocytogenes*, *S. aureus* and *B. cereus* on stainless steel surfaces at 25°C.

Cleaning and disinfection procedures using proper biocides are usually effective against initial stages of biofilms formed by pathogenic bacteria (Lee et al., 2017a). The main types of biocides used in the dairy industry include halogenic compounds, peroxygen, organic acids and quaternary ammonia. Importantly, these compounds' effectiveness, among other factors, depends on the initial bacterial load, application time, type of surface and spectrum against the microorganism (Dominciano et al., 2016). It has been demonstrated that chlorine-based sanitisers were effective against *L. monocytogenes* biofilms because of rapid oxidation processes in the bacterial cell metabolism (Rodríguez-López et al., 2018). However, presence of extracellular matrix in the biofilms formed by pathogenic bacteria usually provides greater resistance to biocides compared to its planktonic stage (Srey et al.,

2013). The mechanisms responsible for biofilm resistance to biocides are not entirely understood, although biofilms' specific architecture, decreased metabolic activity or the EPS composition have been hypothesised as possible reasons (Bridier et al., 2011). Therefore, routine assessments are recommended in the dairy industry environment to identify pathogens with the ability to produce biofilms on surfaces from materials commonly used for the manufacture of dairy products (Lee et al., 2014).

Enzyme-based approaches for removal of biofilms

In mature biofilms, the EPS formed comprises multiple types of molecules, including polysaccharides, DNA and proteins, although the complexity of EPS composition may vary markedly among the bacterial species (Combrouse et al., 2013). EPS has been considered an important target in sanitisation procedures for tackling biofilm problem in food processing environments (Sadekuzzaman et al., 2015). In this context, the use of enzyme-based preparations in combination with biocides offers an attractive approach to solve the problem of biofilms on surfaces in the dairy industry by the degradation of its matrix components, thus facilitating the inactivation and removal of detached cells during the industrial cleaning and disinfection procedures (Thallinger et al., 2013). Table 1 presents the primary outcomes of recent studies on the application of enzyme-based preparations for removal of microbial biofilms formed on different surfaces.

Proteases are the main class of enzymes showing high potential for removing bacterial biofilms and protein residues attached to internal surfaces of equipment such as vessels and pipes (Augustin et al., 2004). Examples include proteinase K, lysostaphin and aureolisin (Saggu et al., 2019). Proteases had greater activity on EPS degradation of biofilms formed by *P. fluorescens* than amylases (Srey et al., 2013). Glycosidases and deoxyribonuclease (DNAses) are also enzymes with potential activity for degradation of biofilms' EPS and release of planktonic cells (Saggu et al., 2019). Craigen et al. (2011) observed that DNAse I efficiently degraded the extracellular DNA of *S. aureus* biofilms, thus preventing the biofilm matrix's adhesion to the surface. However, practical applications of enzymes in the dairy industry are limited due to the high costs of enzyme-based preparations, especially when compared with traditional sanitisation methods with biocides, and variations in the enzymatic activities of different types of enzymes as reported in experimental studies (Augustin et al., 2004). By using metalloprotease secreted by *Mycobacterium* spp. SKS10, Saggu et al. (2019) observed an enzymatic degradation of nearly 62% in biofilm biomass and increased antibiotic accessibility inside the biofilm. Previously, Watters et al. (2016) demonstrated that α-amylase, papain and bromelain almost wholly removed the biofilm formed by different strains (ATCC 25923, ATCC 33591, IQ0070, SA5214, SA5123 and SA5120) of *S. aureus* on polystyrene microplate.

The effect of DNAse I on dual-species biofilms formed by *L. monocytogenes* and *E. coli* on stainless steel coupons was reported by Rodríguez-López et al. (2016). The authors observed a reduction of nearly 2 log cycles in the bacterial counts for both species by DNAse I at 400 μg/mL. The removal of biofilms by DNAses is credited to the digestion of eDNA strand, leading to the destruction of biofilm matrix and cell death (Koohy et al., 2013). However, the role of eDNA as a structural component of biofilm matrix still needs clarification to completely elucidate the mode of action of DNAses for reducing bacterial cell adhesion in mature biofilms (Grande et al., 2011).

Several types of proteases have been studied against microbial biofilms, alone or in combination with other compounds. Araújo et al. (2017) observed reductions of 1.59 and 1.93 log CFU/cm^2 after treating *P. fluorescens* biofilms with protease alone or associated with cetyltrimethylammonium bromide, respectively. Complete elimination of biofilms formed by *Macrococcus caseolyticus* after 1.5-h contact with protease (500 mg/mL) at 45°C was reported by Mnif et al. (2020). Combination of proteases with surfactants and phenoxyethanol against biofilms *L. monocytogenes* provided a cell count reduction of 6.9 log CFU/cm^2 (Mazaheri et al., 2020). The application of proteinase K against biofilms of *E. coli* O157:H7 resulted in higher efficacy than other enzymes such as DNAse I and cellulose, leading to 91–99% biofilm mass reductions, equivalent to 2.43 log CFU/cm^2 (Lim et al., 2019). However, Wang et al. (2016) observed a lower percentage reduction (55%) by proteinase K against biofilms of *Salmonella* spp., indicating species-specific variations in the efficacy of this enzyme.

Another protease frequently tested against biofilms is papain (from *Papaya carica*), which can be attributed to its high proteolytic capacity, broad spectrum against protein substrates and the ability to hydro led these proteins into small peptides and amino acids (Borrajo et al., 2020; Sáringer et al., 2019). Song et al. (2020) observed that papain at 5.0 μg/mL resulted in 26.1, 21.6 and 50.9% reductions in the biofilm mass formed by *S. aureus*, *C. jejuni* NCTC 11168 and *C. jejuni* Y23-5, respectively. Accordingly, Mohamed et al. (2018) demonstrated that 100 mg/mL of papain removed 59% of biofilms formed by *Klebsiella pneumoniae*, although no effects on planktonic cells were observed.

Amylases represent an expressive group tested in enzymatic cleaning procedures, among which the main types

Table 1. Recent studies on the application of enzyme-based preparations for the removal of microbial biofilms formed on different surfaces.

Enzyme preparation	Microorganism/biofilm formation	Experimental conditions	Main effects	Reference
β-glucanase, α-amylase, lipase and protease, associated with cetyltrimethylammonium bromide (CTAB)	*P. fluorescens* grown on bioreactor I (containing stainless steel surface) and dripped on bioreactor II at 30ºC for 7 days.	Enzymatic solutions or CTAB applied through flow cells for 1 h, and evaluated after 2, 12 and 24 h.	Protease obtained a greater cell reduction (1.59 log CFU/cm^2) than lipase, β-glucanase and α-amylase (1.34, 1.25 and 1.09 log CFU/cm^2 respectively). Protease associated with CTAB reduced 1.93 log CFU/cm.[2]	Araújo et al., 2017
Pronase, cellulase, pectinase, DNAse I, lysozyme, phospholipase, peroxidase, β-glucanase and chitinase	*L. monocytogenes* mono-species and dual-species biofilms grown on stainless steel coupons at 25ºC for 48 h.	1 mL of mixed-enzymes solution applied on the stainless steel coupons with biofilms at room temperature for 1 h.	*L. monocytogenes* cells mono-species biofilms reduced nearly 1 log cycle. In dual-species biofilms, only moderate effects were observed.	Puga et al., 2018
Papain (from *C. papaya*)	*K. pneumoniae* grown on plates at 37ºC for 24 h.	Papain at 3.125, 6.25, 12.5, 25, 50 and 100 mg/mL tested against planktonic cells and during biofilm formation.	No bactericidal effect was observed. The highest level (100 mg/mL) reduced 55–59% of biofilm mass.	Mohamed et al., 2018
Metalloprotease (from *Mycobacterium* spp. (SKS10)	*S. aureus* MTCC 11949 grown on polystyrene surface at 37ºC for 72 h.	Metalloprotein dissolved in Luria–Bertani broth at 10, 100 or 1,000 µg/mL, and incubated with biofilms for 24 h at 37°C.	The enzyme degraded ≥ 62% of biofilm biomass, starting from 10 µg/mL. Increased accessibility of antibiotics inside the biofilm was also observed.	Saggu et al., 2019
DNAse I, proteinase K and cellulase	*E. coli* O157: H7 grown on polystyrene and stainless steel at 25ºC for 24 h.	Cellulase (20 mg/mL), proteinase K (10 mg/mL) and DNAse I (1 mg/mL) incubated with biofilms on polystyrene plates (25ºC, 24 h) and stainless steel (37ºC, 1 h).	Proteinase K and cellulase reduced 91–99% and 65–98% of biofilm mass, respectively. Proteinase K treatment resulted in greater reduction of biofilm cells (2.43 log CFU/cm^2).	Lim et al., 2019
Endolysin LysCSA13 secreted by *S. aureus*	*S. aureus* RN4220 and CCARM 3090 grown on polystyrene plates, stainless steel and glass surface at 37ºC for 24 h.	Endolysin LysCSA13 solutions (100, 300 and 1000 nM) incubated with biofilms on polystyrene (2 h), stainless steel (1 h) and glass (2 h).	Endolysin LyCAS13 at levels 300 nM reduced 82–84% of biofilm mass on all surfaces tested.	Cha et al., 2019
Proteases in combination with ethoxylated sodium lauryl ether glycolate, N-oxide N, N-dimethyl-C12–C14-alkylamine, anionic and non-ionic surfactants and phenoxyethanol	*L. monocytogenes grown on* stainless steel coupons at 30°C for 7 days.	Enzyme-detergent solution (3 mL) was added to biofilms and kept at 50ºC for 15 min.	Maximum reduction of 6.9 log CFU/cm^2 in the biofilm cells, corresponding to 85–99% of biomass reduction.	Mazaheri et al., 2020
Mannanase, savinase and α-amylase in combination with thymol and cinnamaldehyde	*L. monocytogenes* grown on stainless steel coupons at 30ºC for 7 days.	Three consecutive treatments of biofilms with the enzyme-based preparation at 50ºC for 20 min.	Reduction of 75–98% in the biofilm biomass.	Ripolles-Avila et al., 2020
Papain (from *C. papaya*)	*S. aureus* and *C. jejuni* (NCTC 11168 and Y23-5) grown on polystyrene microtiter plates for 24 h and 72 h at 37ºC, respectively.	Papain (0.31–5.0 µg/mL) added on biofilms and incubated at 37ºC for 24 h (*S. aureus*) and 72 h (*C. jejuni*).	Degradation of mature biofilms by papain at 5.0 µg/mL with 22–26% and 22–51% reduction in biofilm biomass from *S. aureus* and *C. jejuni*, respectively	Song et al., 2020
Protease, lipase, amylase, CMCase and DNAse	*M. caseolyticus* grown on polystyrene microplates and stainless steel surfaces at 30ºC for 24 h	Pronase, CMCase, amylase and lipase at 62.5–500 mg/mL, and DNAse at 7.8–62.5 mg/mL, incubated with biofilms at 45ºC for 1.5 h.	DNAse, protease and lipase at their highest concentrations eliminated the biofilms. CMCase and amylase did not affect biofilms.	Mnif et al., 2020

studied are α-amylase and glucoside amylase, representing about 25% of the world market for enzyme commercialisation (Sundarram and Murthy, 2014). These enzymes catalyse hydrolysis reactions in the α-1,4-glycosidic bonds of starch, thus producing glucose and maltose (Sundarram and Murthy, 2014). Araújo et al. (2017) observed that α-amylase reduced counts in the biofilms of *P. fluorescens*in by nearly 1.1 log CFU/cm^2. Recent evidence has indicated that combinations of α-amylase with thymol, cinnamaldehyde and other enzymes, such as mannanase and savinase, were highly significant (75–98% reductions in the biomass) against the biofilms formed by *L. monocytogenes* (Ripolles-Avila et al., 2020). However, amylases at levels as high as 500 mg/mL were not effective against the biofilms formed by *Macrococcus caseolyticus* (Mnif et al., 2020).

Recently, cellulases and lipases have been studied alone or in an association against biofilms and removing residual food materials from surfaces. Guerrero-Navarro et al. (2019) found that the association of lipase, amylase and protease removed 78% of milk fouling from stainless steel surfaces after enzymatic treatment. Regarding removal of biofilms by lipases, Araújo et al. (2017) reported a reduction of 1.34 log CFU/cm^2 of biofilms from *P. fluorescens*, while complete elimination of *M. caseolyticus* biofilms was described by Mnif et al. (2020). Cellulase at a concentration of 1–20 mg/mL led to reductions of 5.0 log CFU/cm^2 of biofilm cells from several serotypes of *Salmonella enterica* (*S.* Typhimurium, *S.* Enteritidis, *S.* Infantis, *S.* Stanley, *S.* Agona, *S.* Derby and *S.* Indiana) (Wang et al., 2016). Similar results were reported by Lim et al. (2019), who found that cellulase at 20 mg/mL reduced 65–98% of the biofilm mass formed by *E. coli* O157:H7, thus confirming the potential application of this enzyme against biofilms.

Concluding Remarks

As communities of microorganisms, biofilms adhere to several types of surfaces to guarantee their survival under stress conditions in the environment. Among the main pathogenic microorganisms listed as problems for the dairy industry are *L. monocytogenes*, *S. aureus*, *B. cereus*, *Salmonella* spp. and *E.coli*. As mature biofilms have increased resistance to biocides, enzyme-based preparations have been studied aiming at their potential use as aiding agents for removal of biofilms. Among the main groups of enzymes studied, proteases, amylases, cellulases and DNAses show perspectives for degradation of biofilm matrix components, reducing 1.0–6.9 log CFU/cm^2 of the aforementioned pathogens in mature biofilms and up to 99% of their biofilm biomass. These features increase perspectives for the application of enzyme-based preparations alone or associated with biocides for cleaning equipment, utensils and other surfaces in dairy processing environments. Further studies are needed to define potential combinations of the most effective enzymes and sanitisers that are efficient and economically viable for application in dairy industries.

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