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Efficacy of chemical agents and power ultrasound on biofilms formed by *Asaia* spp. – spoilage bacteria in beverage industries

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Abstract: Spoilage bacteria were isolated from spoiled fruit-flavoured bottled water with a visual defect in the form of floating flocks. The bacteria were identified as *Asaia lannensis* using the PCR technique. The efficacy of five chemical agents commonly used in the beverage industry and of power ultrasound (PUS) on *Asaia* biofilms was studied. A static cultivation procedure on stainless steel plates was used and the efficacy of the chemical agents was tested in two stages. First, only the chemical agents were used. Second, the effect of the application of PUS for 1 min prior to the application of the chemical agents was tested. The most effective chemical agent was the one based on peracetic acid. Its use without PUS proved to be more effective than a combination of any of the other chemical agents with PUS. The least effective methods included the physical sanitation by PUS, the chemical agent based on a 10% solution of sodium hypochlorite and sodium hydroxide, and the chemical agent containing a 0.3% solution of chlorine dioxide.

Keywords: *Asaia lannensis*; biofilm; cleaning agent; disinfectant; non-alcoholic beverage

Where the supply of nutrients is sufficient in the beverage industry, microorganisms grow and develop. Microorganisms can be found as planktonic cells or as attached cells covered by a film or a slime layer known as a biofilm. Biofilms in pipelines act as reservoirs of microorganisms, which are occasionally released into the final product (Florjanič & Kristl 2011). This sporadic release of cells from biofilms results in a major increase in cell density in the final product. As a consequence, a different number of bottles, from individual pieces to a whole production batch, can be contaminated and the bottled product spoiled. In addition to well-known spoilage microbes, *Asaia* spp. spoilage bacteria have recently appeared in the beverage industry where are able to form biofilms on the surface of production lines (Horsáková *et al.* 2009; Sedláčková *et al.* 2011). The *Asaia* genus belongs to the family of acetic acid bacteria (*Acetobacteraceae*), together with the *Acetobacter* and *Gluconobaceter* genera, which are known to cause quality defects in brewery and cider production (Juvonen *et al.* 2011). The origin of *Asaia* spp. as a contaminant in the beverage industry is unknown; supposedly, it comes from a natural part of recipes (*e.g.* tea extracts) (Moore *et al.* 2002). The bacteria were isolated e.g. in Poland and the Czech Republic from spoiled fruit-flavoured bottled water with a visual defect in the form of flocks (Moore *et al.* 2002; Horsáková *et al.* 2009; Kregiel *et al.* 2012).

Biofilms can form for several months and biofilm cells are more resistant to standard sanitation processes due to various extracellular polysaccharides (Moore *et al.* 2002; Horsáková *et al.* 2009; Kregiel *et al.* 2012). As a result, the determination of the effect of chemical agents on planktonic cells has proved

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to be inadequate in the case of biofilms (Mariscal *et al.* 2007). The ability of cleaning agents to remove the extracellular polymeric substance of biofilms, and thus help ensure the maximum efficacy of biocides, sanitizers and disinfectants, can be more important than the removal of the microorganisms themselves (Fratamico *et al.* 2009). The food and beverage industries usually use sanitizers based on active components, such as hypochlorite, peroxides, aldehydes, quaternary ammonium compounds, acids and chlorine dioxide. New methods for biofilm control are also being studied and developed, *e.g.* power ultrasound (PUS, 20–100 kHz) (Fratamico *et al.* 2009). PUS produces cavitation that results in the disintegration of particles and destroys the rigid structure of biofilms (Erriu *et al.* 2014), thus can clean blind and dead spots in facilities and complex shaped objects with a higher probability of biofilm formation.

The aim of this study was to determine the effect of common chemical agents used in the beverage industry and PUS on biofilms formed by the *Asaia* bacteria.

MATERIAL AND METHODS

Bacterial strains. Bacteria of the *Asaia* genus were previously isolated in our laboratory from a spoiled beverage and afterwards identified by the PCR amplification of a partial region of the 16S rRNA gene (Horsáková *et al.* 2009). *Asaia lannensis 1* were isolated from a still beverage containing fruit components (strawberry); *Asaia lannensis 2* were isolated from a sweetened green tea with peach flavour. The stock cultures were maintained in Sabouraud 4% dextrose broth. For preparation of 1 l was used 10 g of peptone (Merck, Germany), 40 g of glucose *p.a.* (Lach-ner, Czech Republic), and 1000 ml of water, and glycerol (1:1) at –20°C.

Cultivation of biofilms. A static cultivation procedure was chosen for biofilm cultivation. AISI 304 stainless steel (SS) plates certified for the production of heat exchangers used in the food industry (40 × 40 × 1 mm) were used as test surfaces for biofilm cultivation. The SS plates were sterilized, than were placed in a sterile Petri dishes and 15 ml of Sabouraud 4% dextrose broth with the tested bacterial culture (approximately 10^3 CFU/ml) was added to each. Then the Petri dishes were cultivated at 25°C for 7 days. According to Kregiel (2013), a 7-day incubation period is sufficient for the adhesion of *Asaia* spp. to materials commonly used in the beverage industry. After removing the remaining broth and drying for 2 days at 25°C, the biofilms were ready for sanitizer testing. The drying of biofilms was included in the process to simulate an interruption in operation in the beverage industry, when biofilms can adhere to the surface of pipelines.

Power ultrasound. The cleaning effect of PUS with and without combination of chemical agents on the biofilms was tested. The ultrasonic bath (Kraintek Czech, Czech Republic) operating at 40 kHz and 200 W was used.

Tested chemical agents. Five industrial chemical agents (called A–E agent due to a conflict of interest with the production company) were obtained from a Czech beverage manufacturer and tested under laboratory conditions. Their solutions were prepared in distilled water prior to the application; the used concentrations corresponded to the usage in the beverage industry (Table 1). Treatment duration was determined based on discussions with beverage manufacturers and their standard practices.

Testing procedures. The efficacy of PUS was tested as follow: the SS plates with the attached biofilms were rinsed, immersed in sterile Petri dishes with 10 ml of sterile water and placed in an ultrasonic bath for a set period of time (1, 3, 5, 10 and 15 min).

The efficacy of chemical agents was tested according the modified methods of Belessi *et al.* (2011). First, only the chemical agents were tested. Prior to the application of the agents, the SS plates with the attached biofilms were carefully rinsed with sterile saline solution to remove free planktonic cells. Then the SS plates were immersed in 10 ml of each of the chemical agents for a set period of time (Table 1). The plates were then aseptically removed, rinsed and placed in new Petri dishes with 10 ml of a sterile saline solution and scraped off by a sterile microbial loop to detach any surviving cells from the plates. SS plates with attached biofilms treated only with sterile water were used as controls.

In the second stage, the effect of the combination of PUS and the chemical agents was tested. The SS plates with the attached biofilms were immersed in 10 ml of a sterile saline solution in sterile Petri dishes and placed for 1 min in an ultrasonic bath. The plates were then carefully rinsed and subsequently treated with the chemical agents using the same process as in the first stage. SS plates with attached biofilms treated for 1 min with PUS served as controls.

Quantification was performed by overflowing 1 ml aliquot of a ten-fold dilution by Sabouraud 4% dex-

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Table 1. List of tested chemical agents

Agent	Composition	Usage	Effective	Concentration (%) (treatement duration)	
				recommended	tested
A	strongly acidic cleaner (descaler) based on nititric (> 30%) and phosphoric (< 1%) acids	wide range of CIP applications	removing inorganic scale deposits	0.6–1 (10–20 min)	1 (1, 3, 5 and 10 min)
B	strongly alkaline cleaning agent (> 30% sodium hydroxide) containing special sequestering additives	CIP cleaning; spray cleaning and cleaning bottles in the food industry	removing organic and inorganic scale deposits	0.5–4 (10–30 min)	2 (1, 3 and 5 min)
C	an oxidizing disinfectant based on stabilized peracetic acid (15%)	cold disinfection in all areas of the food and beverage industries	against all types of microorganisms	0.1–0.15 (5–15 min)	0.1 (1, 3, 5 and 7 min)
D	a disinfectant agent based on chlorine dioxide (0.3%)	used in beverage industries, dairy, brewing, fruit and vegetable processing	removing biofilm and against all types of microorganisms	0.3*	0.3 (1, 3 and 5 min)
E	a disinfectant agent, a mixture of sodium hypochlorite (< 5%) and sodium hydroxide (< 1%)	used in food industries and household	against bacteria and viruses	10*	10 (1, 3, 5 and 7 min)

*time not indicated by a manufacter

trose agar (Merck, Germany) on a Petri dish. After cultivation at 25°C for 2 days, viable cells were counted and the results were expressed in log CFU/cm^2.

Statistics. All testing was carried out in triplicate, averages and standard deviations are presented. Comparisons between the averages were performed using the one-way ANOVA test (Statistica 10; StatSoft Inc., Czech Republic). The $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

The resistance of *A. lannensis 1* and *A. lannensis 2* biofilms to chemical agents was tested using biofilms cultivated on SS plates. The numbers of the adhered cells on the SS plates after 7-day cultivation at 25°C were converted to log CFU/cm^2. The average number of viable microorganisms in the control samples amounted to 7.3 log CFU/cm^2 (6.9–7.7 log CFU/cm^2); the standard deviations of the three parallel measurements were between < 0.01 and 0.57 log CFU/cm^2, with an average of 0.52 log CFU/cm^2 for all the samples (data not shown). To compare the results of the individual chemical agents, control samples were always used. Treating the biofilms on the SS plates with the chemical agents reduced the number of surviving cells; this reduction increased with the time of exposure. However, the level of biofilm destruction differed for each of the chemical agents and cultures tested. The effects of each of the chemical agents on the biofilms at various treatment times are shown in Figure 1A–D.

For both the strains, *A. lannensis 1* and *A. lannensis 2*, the most effective chemical agent tested was the C-agent. Peracetic acid is a reactive disinfectant substance; its microbicidal effect is based on the production of hydroxyl radicals, which damage basic cell structures by oxidation of lipids, proteins and DNA (Kitis 2004). Compared to hydrogen peroxide, which has similar effects, peracetic acid is active at much lower concentrations and is not subject to peroxidases inactivation (McDonnell & Russel 1999). Using the C-agent without PUS turned out to be more effective than using any of the other chemical agents together with PUS ($P < 0.05$). As a result, the use of the C-agent in combination with PUS was not tested. In case of *A. lannensis 1*, the amount of the biofilm viable cells was reduced by 4.8 log CFU/cm^2 after 5 min of action and by 6.2 log CFU/cm^2 after 7 min of action. In case of *A. lannensis 2*, the amount of the biofilm viable cells was reduced by 4.3 and 6.2 log CFU/cm^2, respectively.

The least effective chemical agent tested was the E-agent (10% solution of a mixture of sodium hypochlorite and sodium hydroxide): after 5 min of action, the number of the biofilm viable cells decreased by 2.1 log CFU/cm^2 for *A. lannensis 1* and 2.0 log CFU/cm^2 for *A. lannensis 2*; after 7 min, it decreased by 3.1 log CFU/cm^2 for *A. lannensis 1* and 3.0 log CFU/cm^2 *A. lannensis 2*. No significant improvement was observed ($P > 0.05$) when the biofilm was exposed

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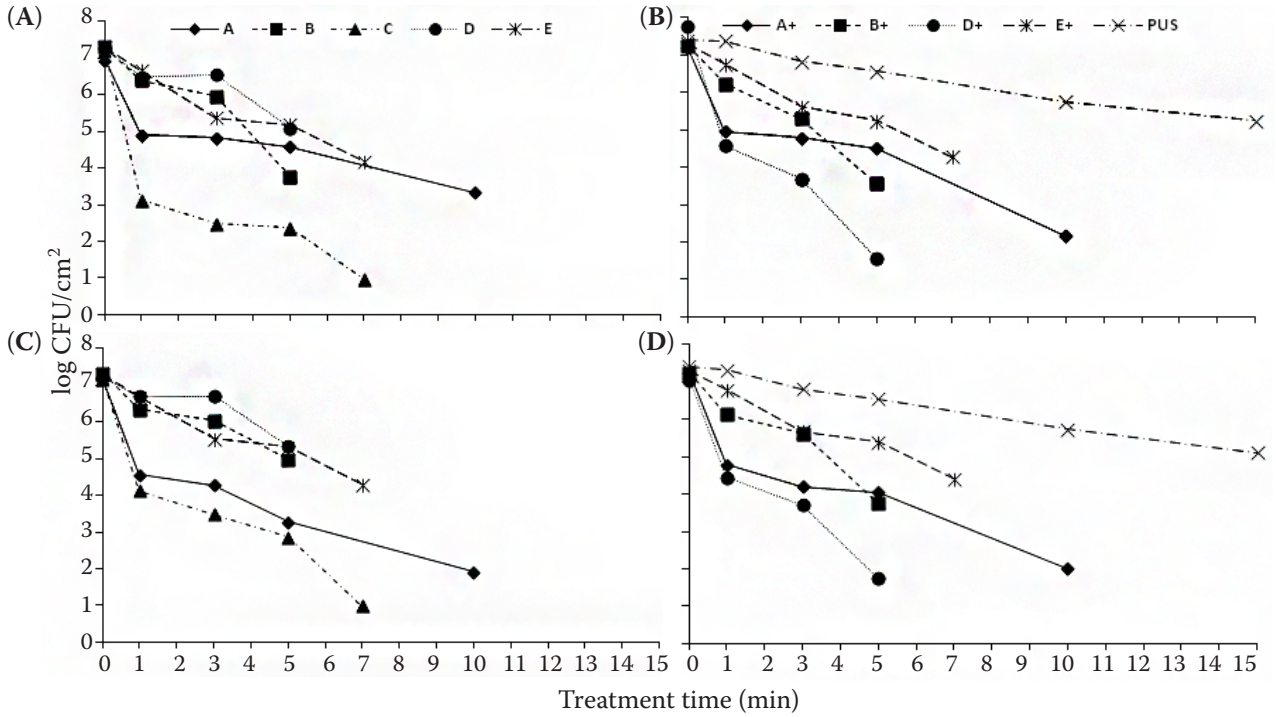

Figure 1. Efficiency of chemical agents alone (**A**) and (**C**) and with power ultrasound itself (**B**) and (**D**) on biofilm formed by *A. lannensis 1* (**A**) and (**B**) or *A. lannensis 2* (**C**) and (**D**)

A–E – chemical agents;PUS – power ultrasound; +chemical agents in combination with power ultrasound; values are presented as a mean (n = 3); s.d. < 0.01–0.57 log CFU/cm^2

to PUS prior to the E-agent application. When the biofilm was treated with both the E-agent and PUS, the reduction amounted to 2.1 log CFU/cm^2 for *A. lannensis 1* and 1.9 log CFU/cm^2 for *A. lannensis 2* after 5 min, and to 3.0 log CFU/cm^2 for *A. lannensis 1* and 2.9 log CFU/cm^2 for *A. lannensis 2* after 7 min of action. The low efficacy of the E-agent complies with the results of Purkrtová *et al.* (2010) on the efficacy of a similar product on *Listeria monocytogenes* biofilms, which concluded that the number of biofilm viable cells was not significantly reduced even after 30 min of exposure.

Similarly, in the case of the A-agent (a strongly acidic cleaner based on nitric and phosphoric acids), there was no difference in biofilm reduction efficacy in either of the tested strains between the treatment without PUS and the treatment with PUS used for 1 min before the cleaner was applied (P > 0.05). In the food industry, the A-agent is mainly used to remove inorganic deposits, such as limescale and milk and beer deposits, disrupting organic impurities only by an oxidative treatment. Despite this, when the A-agent was used alone for 5 min, the amount of the adherent cells was reduced by 2.3 log CFU/cm^2 for *A. lannensis 1* and by 3.9 CFU/cm^2 for *A. lannensis 2*, when it was used with PUS, the amount of the adherent cells decreased by 2.7 log CFU/cm^2 for *A. lannensis 1* and by 3.2 log CFU/cm^2 for *A. lannensis 2*.

The D-agent (0.3% chlorine dioxide solution) is a strong oxidizing disinfectant eliminating algae, fungi, endospores, endotoxins and, according to its manufacturer, is very effective in removing biofilms by disrupting bacteria cellular structure. The reduction of the *A. lannensis 1* viable cells after 5 min of treatment amounted to 6.1 log CFU/cm^2 (P < 0.05) when the D-agent was used with PUS; when it was used without PUS, the amount of the viable cells decreased by only 2.2 log CFU/cm^2 (P > 0.05). Similar results were obtained for *A. lannensis 2* (5.4 and 1.8 log CFU/cm^2, respectively).

The B-agent (a strongly alkaline cleaning agent containing > 30% sodium hydroxide and special sequestering additives) is effective for organic soil elimination and scale deposit prevention, however, some authors reported the inefficacy of even hot sodium hydroxide to remove biofilm (Simões *et al.* 2010). Typically, a sodium hydroxide solution at 65–80°C is used in CIP equipment. Due to safety of manual handling during testing, the efficacy of B-agent was tested only at 30°C. The amount of the *A. lannensis 2* adherent cells was reduced by 2.3 log CFU/cm^2 after 5 min application of the B-agent and

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by 3.6 log CFU/cm^2 ($P < 0.05$) when the B-agent was used together with PUS; for *A. lannensis* 1, the difference was only 3% (reduction by 3.5 and 3.6 log CFU/cm^2, respectively; $P > 0.05$).

The physical cleaning agent (PUS) turned out to be less effective than sanitation by the chemical agents. The amount of the *A. lannensis 1* viable cells was reduced by 0.8 log CFU/cm^2 after 5 min treatment and by 2.2 log CFU/cm^2 ($P > 0.05$) after 15 min treatment; for *A. lannensis 2*, it was 0.9 and 2.3 log CFU/cm^2, respectively ($P > 0.05$). When compared with the chemical agents, PUS is effective for biofilm removal only when applied for longer periods of time, which is unsuitable in terms of usability in the industry.

CONCLUSIONS

The bacteria of the *Asaia* genus are able to form biofilms on surfaces of pipelines and factory equipment inthe beverage industry; single cells can be then released from the biofilms and reach the final product where they occur as a floating flock. The biofilms are very stable due to extracellular polymeric substances and therefore very resistant to normal sanitation processes.

Despite its limitations (monospecies biofilms, static conditions), the study concluded that there are differences between chemical agents and between cultures tested. For both the tested strains, the most effective chemical agent turned out to be the C-agent (based on peracetic acid). Its use without PUS was more effective than combining any of the other chemical agents with PUS. The least effective method was the physical cleaning by PUS; the least effective chemical method was using the E-agent (10% solution of sodium hypochlorite and sodium hydroxide), both alone and in combination with PUS, and the D-agent (0.3% chlorine dioxide solution), when applied alone.

References

Belessi Ch.-E.A., Gounadaki A.S., Psomas A.N., Skandamis P.N. (2011): Efficiency of different sanitation methods on *Listeria monocytogenes* biofilms formed under various environmental conditions. International Journal of Food Microbiology, 145: S46–S52.

Erriu M., Blus C., Szmukler-Moncler S., Buogo S., Levi R., Barbato G., Madonnaripa D., Denotti G., Piras V., Orrù G. (2014): Microbial biofilm modulation by ultrasound: Current concepts and controversies. Ultrasonics Sonochemistry, 21: 15–22.

Florjanič M., Kristl J. (2011): The control of biofilm formatin by hydrodynamice of purified water in industrial distribution system. International Journal of Pharmaceutics, 405: 16–22.

Fratamico P.M., Annous B.A., Gunther N.W. (eds): Biofilm in the Food and Beverage Industries (1st Ed.). Cambridge, Woodhead Publishing: 600

Horsáková I., Voldřich M., Čeřovský M., Sedláčková P., Šicnerová P., Ulbrich P. (2009): *Asaia* sp. as a bacterium decaying the packaged still fruit beverages. Czech Journal of Food Sciences, 27: S366–S368.

Juvonen R., Virkajärvi V., Priha O., Laitila A. (2011): Microbiological Spoilage and Safety Risks in Noon-beer Beverages Produced in a Brewery Environment. Espoo, VTT Tiedotteita-Research Centre of Finland: 107.

Kitis M. (2004): Disinfection of wastewater with peracetic acid: a review. Environment International, 30: 47–55.

Kregiel D., Rygala A., Libudzisz Z., Walczak P., Oltuszak-Walczak E. (2012): *Asaia lannensis* – the spoilage acetic acid bacteria isolated from strawberry-flovored bottled water in Poland. Food Control, 26: 147–150.

Kregiel D. (2013): Attachment of *Asaia lannensis* to materials commonly used in beverage industry. Food Control, 32: 537–542.

Mariscal A., Carnero-Varo M., Gutierrez-Bedmar M., Garcia-Rodriguez A., Fernandez-Crehuet J. (2007): A fluorescent method for assessing the antimicrobial efficacy of disinfectant against *Escherichia coli* ATCC 35218 biofilm. Applied Microbiology and Biotechnology, 77: 233–240.

McDonnell G., Russel A.D. (1999): Antiseptics and disinfectants: activity, action, and resistance. Clinical Microbiology Reviews, 12: 147–179.

Moore J.E., McCalmont M., Xu J., Millar B.C., Heaney N. (2002): *Asaia* sp., an unusual spoilage organism of fruit-flavoured bottled water. Applied and Environmental Microbiology, 68: 4130–4131.

Purkrtová S., Turoňová H., Pilchová T., Demnerová K., Pazlarová J. (2010): Resistance of *Listeria monocytogenes* biofilms to disinfectants. Czech Journal of Food Sciences, 28: 326–332.

Sedláčková P., Čeřovský M., Horsáková I., Voldřich M. (2011): Cell surface characteristic of *Asaia bogorensis* – spoilage microorganism of bottled water. Czech Journal of Food Sciences, 29: 457–461.

Simões M., Simões L.C., Vieira M.J. (2010): A review of current and emergent biofilm control strategies. LWT-Food Science and Technology, 43: 573–583.

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