Corvinus University of Budapest

Faculty of Food Science

Department of Microbiology and Biotechnology

HIGHLIGHTED APECTS OF FOOD MICROBIOLOGY IN THE COURSE OF NATURAL MINERAL WATER AND SPRING WATER PRODUCTION

Theses

Kata Pap

Budapest

2011

PhD School/Program

Name: PhD School of Food Science

Field: Food Science

Head: Prof. Péter Fodor
University Professor, DSc
Corvinus University of Budapest
Department of Applied Chemistry

Supervisor: Csilla Mohácsi - Farkas
Associate Professor, PhD
Corvinus University of Budapest
Department of Microbiology and Biotechnology

The applicant met the requirement of the PhD regulations of the Corvinus University of Budapest and the thesis is accepted for the defence process.

.

....

Head of PhD School Supervisor

1. INTRODUCTION

The bottled spring and mineral water production is an important area of food microbiology. According to valid Hungarian law (65/2004. (IV. 27.) FVM-ESzCsM-GKM) no treatment - including any microbiological -, is allowed for such products which could cause any change of their product composition. In case of non-carbonated products, their final composition has no inhibitory effect on any possible bacterial contaminant and even though several sources reported surviving microbes in naturally carbonated waters. Therefore, food hygiene and the knowledge of microbial risks are particularly important during production of bottled spring and mineral water.

Biofilms are rather well-studied segments of water system' microbiology. The above mentioned law requires that no *Pseudomonas aeruginosa* can be present in bottled spring and mineral water products. Nevertheless, it is well known, that excellent biofilm forming *Pseudomonas* species are well spread in underground water-systems and in case of a weak protection of the water-base, the penetration of surface or sewage waters can happen too. Frequently used material of food industry, like steel, aluminium, glass, Teflon, nylon are good fundamentals for biofilm build up. The presence and the capability of biofilm formation of *Pseudomonas* species inside of production areas and equipment is a serious hygienic problem. Several other pathogenic microbes can be built into such biofilms risking food safety along hygienic problems. Moreover, biofilms can be linked to significant financial losses through inefficient heat exchange, decreased circulation speed, shorter filter usage-time or even causing material damages through their metabolic products. It is important to state, that not only the closed production systems are vulnerable for biofilms.

Inactivation of well established biofilms is generally difficult since they have high resistance against antimicrobial agents strengthened with cell surface polysaccharides (EPS). Due to inefficient disinfectants and cleaning agents, surface sticking destroyed cells and EPS are very good medium and entrance ways for planctonic cells, embracing the fast formation of newer biofilms. After inefficient disinfectant treatments, surviving particles of such biofilms can regenerate rapidly and form new, more resistant biofilms by reutilizing the just released nutrients. Therefore, a good biofilm inactivation must mean both the destruction and the complete removal of the biofilm at the same time.

An other poorly examined area of spring and mineral water production is the occurrence of moulds in bottled waters. Publications report the presence of filamentous or budding moulds

in surface and underground waters. Such mould species can be detected in both tap-water and bottled waters, referring their environmental contamination.

Filamentous mould can grow in non-carbonated or spring waters and even just their presence can rise health and food safety risks or simply can destroy product value together with numerous customer complains.

2. GOALS

Based on all that, the following targets were set:

1) Examination of biofilm formation of P*seudomonas aeruginosa* ATCC 9027 and isolated *Pseudomonas stutzeri* CC B 21 with several different methods in nutrient rich and poor medium on stainless steel surface.
2) Examination of biofilm inactivation of P*seudomonas aeruginosa* ATCC 9027 by several, commercial disinfectant in nutrient rich and poor medium on stainless steel surface.
3) Follow-up of survival and multiplication of several moulds isolated from packaged natural mineral water or spring water(*F. oxysporum* CC F 36; *C. cladosporoides* CC F 50; *P. chrysogenum* NCAIM F 00837*; A. fumigatus* NCAIM F 00673),
4) Follow-up of mycelium formation of mould through 26 weeks, in 12 different, commercial spring and mineral waters.

3. METHODES

3.1. Follow up of static biofilm formation on horizontal stainless steel surface in BHI broth and in non carbonated natural mineral water

- Microorganisms:
  - *Pseudomonas stutzeri* CC B 21 isolated from food-industrial well
  - *Pseudomonas aeruginosa* ATCC 9027 from National Collection of Agricultural and Industrial Microorganisms (NCAIM)
- All biofilm formation tests were performed on horizontal stainless steel (Wnr1.4301) surface. BHI broth and non carbonated natural mineral water was used as media. The Pseudomonas strains were inoculated at the level of 10^6 colony forming unit per ml. (CFU/ml). Biofilm formation was followed up fro 4 weeks.
- The stainless steel surfaces were stained with acridin orange and with Concavalin A lectin linked with fluorescein isotiocianate (FITC).
- Microscopic analysis was performed with Olympus BH2 epifluorescent microscope, on 455 nm, homogen immersion and KB4 and EY455 filters and D PLAN APO UV objective was used. Pictures were taken by digital camera and analysed by *Mathcad 2001 Professional* software. Digital picture analysis was performed based on RGB system. The intensity of red and green colour was measured. Data were analysed by *SPSS* and *Microsoft Excel* softwares

3.2. Inactivation and removal of biofilms from stainless steel

- Microorganism: *Pseudomonas aeruginosa* ATCC 9027 (NCAIM)
- Disinfectant agents: Biguanid Fläche 1%; Descosal 1%; DisquatL 1%; Domestos 2%; Innofluid TF Klór 2%
- Surviving CFU was detected with 3 different methods such as (a) pour plating with TGE agar; incubation at 37 ºC for 24 hours (b) impedimetric method with RABIT instrument; Don Whitley broth at 37 ˚C for 48 hours; direct detecting methodology; (c) staining with acridin orange.

3.3. Enumeration of survival moulds and visual inspection of visible growth of survival moulds inoculated in natural mineral waters and spring waters purchased from the European market

- Tested natural mineral waters and spring waters: samples were purchased from 4 countries of Europe. Altogether 12 different natural mineral water or spring water were involved into the tests. Six carbonated and six non carbonated products were chosen.
- Mould strains used for inoculation were previously identified from packaged natural mineral water or spring waters such as *F. oxysporum* CC F 36; *C. cladosporoides* CC F 50; *P. chrysogenum* NCAIM F 00837*; A. fumigatus* NCAIM F 00673
- The following inoculation levels were used: A) <10 conidia /100 ml, B), 10-50 conidia /100 ml, C) 100-500 conidia /100 ml product.
- Altogether 1164 bottles were used during the tests as detailed below:
    1. For visual inspection: *432 bottles* (4 different mould strains; 12 different products; 3 times parallel; 3 concentration),
    2. For determination of survival CFU: *648 bottles of carbonated products* (4 different mould strains; 3 concentration; 6 different products; 9 sampling time), *84 bottles of non carbonated products* (4 different mould strains; 3 concentration; 6 different products; 12 extra samples for 0,33 l bottles)
- Incubation of inoculated bottles was performed on 25 °C.
- The visual inspection was performed at standard light for 26 weeks (for the standard „best before time" of carbonated packaged waters). During that time all bottles were investigated on weekly basis. All small or cloudy floating particles were counted as positive results.
- Based on the expected results membrane filtration or pour plating methods were used for enumeration of surviving colony forming units. Surviving CFU was checked for 20 weeks in case of non carbonated water samples and for 12 weeks in case of carbonated water samples. Based on he survival of *A. fumigatus* NCAIM F 00673 in carbonated water extra sampling was carried out after 26 weeks of inoculation.
- Statistical analysis (ANOVA, and Bonferroni's Multiple Comparison) of the results were performed using Graph Pad Prism4 software.

4. RESULTS

Based on the surfaces direct microscopic investigation it was found that both *Pseudomonas* species represent intense biofilm formation. The results of the Acridin Orange staining showed typical saturation curves for both test species. The biofilm formation of *Ps. stutzeri* CC B 21 was more intense and faster. All biofilms were inhomogeneous at the beginning and become a homogenous robust biofilm at the end of the test period. Biofilm formation of both investigated *Pseudomonas* species was significantly weaker in mineral water than in the nutrition-reach BHI broth.

The EPS secretion of all investigated *Pseudomonas* biofilms did increased by time. The EPS secretion in biofilm of *Ps. stutzeri* CC B 21 in BHI broth was higher than in case of *Ps. aeruginosa* ATCC 9027. Meanwhile, *Ps. stutzeri* CC B 21 did not show any EPS secretion in mineral water.

According to the results of biofilm inactivation tests Domestos 2% was the most effective disinfectant against *Pseudomonas aeruginosa* biofilms. In all other cases, older biofilms did show resistance against the tested disinfectant agents.
Comparing the traditional pour plating and impedimetric method the RABIT instrument was more effective to detect viable but not cultivable cells after disinfection.

During mould challenge study 12 different natural spring-and mineral water were inoculated with moulds previously identified from packaged waters (*F. oxysporum* CC F 36, *C. cladosporoides* CC F 50, *P. chrysogenum* NCAIM F 00837 and *A. fumigatus* NCAIM F 00673). The visual monitoring results did show no visual growth in carbonated samples, however, all together 216 bottles were investigated (6 different carbonated water inoculated with 4 different moulds). On the other hand, visual growth was detected in all (216 bottles - 6 different still water inoculated with 4 different moulds) non-carbonated water samples.

The membrane filtration and pour plating results of the inoculated spring- and mineral waters did show that the investigated moulds can survive even multiply in still natural spring- and mineral waters. Despite of the results of still waters the investigated *F. oxysporum* CC F 36, *C. cladosporoides* CC F 50, *P. chrysogenum* NCAIM F 00837 did not survive the first 12

weeks in the carbonated samples. Only the facultative human-pathogen *A. fumigatus* NCAIM F 00673 was detected after 26 weeks of incubation in carbonated spring- and mineral water. The inoculated mould species had an effect on the detected surviving CFU numbers, meanwhile no correlation was found between the survival CFUs and the type of the spring- and mineral water or the packaging material

5. NEW SCIENTIFIC RESULTS

1. The bioflim formation of *Ps. aeruginosa* ATCC 9027 (NCAIM strain) on the tested stainless steel (Wnr1.4301) surface in non carbonated natural mineral water media is weaker than the biofilm formation of the investigated *Ps. stutzeri* CC B 21 (isolated from food-industrial well).

2. The biofilm formation of both strains (*Ps. aeruginosa* ATCC 9027 - NCAIM strain and *Ps. stutzeri* CC B 21 - isolated from food-industrial well) on the tested stainless steel (Wnr1.4301) surface show inhomogeneous biofilm at the first days and homogen biofilms at the mature biofilm status. In natural mineral water media both strains show weaker EPS formation than in the ideal BHI broth.

3. This study demonstrates that for detecting resistance of biofilms against disinfection agents the impedimetric RABIT instrument is more effective than the pour plating methodology.

4. My dissertation has demonstrated that the investigated moulds (*F. oxysporum* CC F 36, *C. cladosporoides* CC F 50, *P. chrysogenum* NCAIM F 00837, *A. fumigatus* NCAIM F 00673) can survive and in some case even show increase in CFU numbers in **non carbonated** bottled natural mineral waters and spring waters. Visual grows of all investigated moulds were detected in each non carbonated packaged water samples during the investigated 26 weeks time period. Based on that results it is demonstrated that the risk of visual growth during the best before time period of a non carbonated packaged water product (52 weeks) is significant.

5. This study demonstrates that there is no significant difference (at confidence level 95%) in timing of visual growth neither in different non carbonated packaged water samples nor between different investigated mould strains. The timing of the visual growth is independent on the inoculation level.

6. My dissertation has demonstrated that there is significant difference in survival of *A. fumigatus* NCAIM F 00673 and other investigated mould strains in **carbonated** bottled natural mineral water and spring water samples. *F. oxysporum* CC F 36, *C. cladosporoides* CC F 50, *P. chrysogenum* NCAIM F 00837 moulds did not show survival after 12 weeks meanwhile *A. fumigatus* NCAIM F 00673 survived the investigated 26 weeks time period. Visual growth of inoculated moulds was not detected during the investigated 26 weeks.

6. CONCLUSION AND PROPOSALS

The biofilm formation of *Pseudomonas stutzeri* CC B 21 and *Pseudomonas aeruginosa* ATCC 9027 is robust both in the nutrition-reach BHI broth and in nutrition-poor natural mineral water. The biofilm formation of *Ps. stutzeri* CC B 21 identified from a water source is quicker than in case of Ps. *aeruginosa* ATCC 9027. These results show that the significance of environmental *Pseudomonas* species is high. Therefore for mineral- and spring water bottling factories it is proposed to extend microbiological monitoring to environmental *Pseudomonas* species to top of *Ps. aeruginosa* monitoring.

Older biofilms has higher resistance against disinfectant agents than young biofilms. Non-viable biofilm cells and trace of EPS can remain on the surfaces after an inefficient cleaning and disinfection. These remaining materials can help the next coming planctonic cells to build a robust antibiotic-resistance biofilm formation. For closed pipeline systems it is proposed to set up regular preventive cleaning, sanitation to avoid biofilm formation. It is also proposed to validate disinfectant agents efficiency against biofilms.

Based on the visual monitoring results of spring and mineral waters inoculated with moulds it is clear that moulds can survive and multiply in non-carbonated mineral- and spring water and can create visual growth in final product. In the industrial practice it means that the risk of consumer complains related to mould growth exists for these products. In carbonated water moulds can not create visual growth; however, the facultative pathogen *A. fumigatus* can survive even 26 weeks in carbonated packaged water. For spring- and mineral water bottling factories it is proposed therefore, to set a microbiological monitoring system against mould and declare product release criteria as well as alert limits. It is also proposed to identify the moulds detected in final products or in environmental samples to be able to apply disinfection and sanitation procedure in case of facultative pathogen moulds are detected.

LIST OF PUBLICATION

Journals

In journals with impact factor

1. Pap, K., Kiskó, G., 2008. Disinfection test with static biofilms. Acta Alimentaria 37 (1), 1–7

2. Pap, K., Kiskó, G., Szilli, M., 2006. Testing antimicrobial efficiency of seven disinfectants against bacteria and fungi with surface test. Acta Alimentaria, 35 (2), 163-170

In journals without impact factor

1. Pap, K., Tornai- Lehoczki, J., Syposs, Z., 2008. Mold challenge study in bottled waters. Acta Microbiologica et Immunologica Hungarica 55 (2), 145–154

Conference proceedings

Hungarian (full text)

1. Pap, K., Kiskó, G., 2003. Nagykonyhai technológiában jelentős baktériumok biofilm képző tulajdonságának, és a biofilmek eltávolításának vizsgálata. V. Medi-Clean Tisztítástechnológiai Konferrencia és Kiállítás anyagának gyüjteménye, Gyula, Magyarország, 2003. október 30-31.

2. Pap, K., 2005. Biofilmek kialakulása és az ellenük való védekezés. GTE XV. Tisztítástechnológiai Konferencia – I. Fürdők higiéniája Konferencia kiadványa, Gyopárosfürdő, Magyarország, 2005. április 29.

Hungarian (abstract)

1. Pap, K., Kiskó, G., Gillay, Z., 2004. Biofilmek fényképezése epifluoreszcens mikroszkóp segítségével. Modern Sejtanalitikai módszerek, a IV. Magyar Sejtanalitikai Konferencia kiadványa, Budapest, Magyarország, 2004. május 6-8. p 164

International conference (abstract)

1. Pap, K., Kiskó, G., 2003. Biofilm removing effect of five desinfactants on bacteria and fungi attached to stainless steel. Abstract book 1st FEMS Congress of European Microbiologists, Ljubljana, Slovenia June 29-July 3 2003, p 408

2. Pap, K., Kiskó, G., 2003. Resistance of *Pseudomonas aeruginosa* biofilms against different disinfectants. Abstracts CESAR 2003 Central European Symposium on Antimicrobial Resistance, Brijuni, Croatia July 4-7 2003 p28

3. Pap, K., Kiskó, G., 2003. Effects on different disinfectants on *Candida albicans* biofilms. Book of Abstracts 23rd International Specialised Symposium on Yeasts, Budapest, Hungary, 26-29 August, 2003, pp. 127

4. Pap, K., Kiskó, G., 2003. Disinfection test with static biofilms. Abstracts of the 14th International Congress of the Hungarian society for Microbiology, Balatonfüred, Hungary, 9-11 October, 2003, pp 92

5. Pap, K., Tornai- Lehoczki, J., Syposs, Z., 2008. Detecting of mould growing in carbonated and non carbonated bottled water samples Book of Abstracts FOOD Micro 2008. The 21st International ICFMH Symposium „Envolving microbial food quality and safety", Aberdeen, Scotland, 1-4, September 2008. pp. 464.

6. Pap, K., Tornai- Lehoczki, J., Syposs, Z., 2008. Comparison of visual growth and surviving cell numbers of molds in different bottled waters. Book of Abstracts the XII. International Congress of Mycology IUMS, 5-9 August, 2008. Istambul, Turkey, pp. 86.

7. Tornai-Lehoczki, J., Pap, K, Syposs, Z., Survival Of Moulds In Bottled Waters. 2011. IAFP European Symposium on Food Safety, 18-20 May 2011, Ede, The Netherlands. Approved for publication