Asaia sp. as a Bacterium Decaying the Packaged Still Fruit Beverages

I. HORSÁKOVÁ[1], M. VOLDŘICH[1], M. ČEŘOVSKÝ[1], P. SEDLÁČKOVÁ[1], P. ŠICNEROVÁ[1] and P. ULBRICH[2]*

[1]Department of Food Preservation and Meat Technology and [2]Department of Biochemistry and Microbiology, Institute of Chemical Technology in Prague, 166 28 Prague, Czech Republic
**E-mail: michal.voldrich@vscht.cz*

Abstract: Several cases of consumer complaints to the still soft beverages – fruit drinks and ice teas were analysed. The visible impurities and slight sensory changes were observed, sediment was formed by microbial cells. The bacteria were isolated and identified as *Asaia* sp. The identification was confirmed by PCR technique. The sensitivity of bacteria to preservative agents especially to the sodium benzoate, potassium sorbate and dimethyldicarbonate was evaluated in model samples and in the real conditions of the beverage. The minimal inhibitory concentrations were estimated as well as the factors affecting the sensitivity of bacteria. Besides the stabilisation of drinks the general possibilities of decay prevention were considered, the efficiency of cleaning and sanitation procedures were evaluated, including the comparison of various sanitation agents.

Keywords: *Asaia* sp.; fruit drink; preservative; benzoate; sorbate; dimethyldicarbonate

INTRODUCTION

The bacterial genus *Asaia* was isolated firstly from *Bauhinia purpurea* flowers, which occurs mostly in the tropical climate, and up to date contains four species *Asaia bogorensis, Asaia siamensis, Asaia krungthepensis* and *Asaia lannaensis* (Yamada & Katsura 2000; Yamada & Yukphan 2008). These bacteria are catalase positive, oxidase negative fermenting bacteria, generating small (1–2 mm in diameter) incised light pink colonies. The optimal temperature ranges from 22 to 30°C, at the temperature of 37 C occurs the significantly decreasing of grow rate, up to growth cessation. Earlier observations described the inability of *Asaia bogorensis* to reproduce at the temperature of 37°C, thus a minimal influence of these bacteria on the human health because of inability to reproduce at the human body temperature was supposed (Katsura & Kawasaki 2001). Later was described bacteremia caused by *Asaia bogorensis* (Tuuminen *et al*. 2006). The bacterial genus *Asaia* is able to survive even in the environment with pH of 3.

It is difficult to determine the origin of the contamination with the *Asaia* sp. The occurrence was approved in the natural juices only, but in the synthetic analogues it was not verified. Thereupon, the fruits are regarded as the source of the contamination (Moore *et al*. 2002).

The strain studied in this paper was isolated from the reclaimed fruit beverages. It was found, that these bacteria occurs in the processing equipment in the form of biofilm, which is persistent and hardly removable by the common sanitation. The spoilage occurred also in the acid products preserved by the benzoate, sorbate and dimethyldicabonate. The aim of the study was to evaluate the resistance of isolate to the mentioned preservatives.

MATERIALS AND METHODS

Isolation of bacteria. Contaminated drink (1 ml) was inoculated on the Petri dish and overflowed of the Plate Count Agar. After cultivation at 25°C 2 days it was identified. The identification was confirmed by PCR technique.

Sequencing of bacterial 16S rDNA gene. Bacterial chromosomal DNA was isolated by using Genelute bacterial genomic DNA kit, Sigma, Cat. Nr. NA2100. The amount of isolated DNA was estimated by DNA electrophoresis on agarose gel.

For amplification of 16S rDNA gene PCR method was performed on Touchgene Gradient cycler (Techne). PCR reaction mixture was prepared by mixing ~ 50 ng of bacterial genomic DNA, 5 µl of PCR AccuTaq LA 10× buffer (Sigma, Cat. Nr. B0174), 2.5 µl of dNTP mix (10mM each), 1 µl of 27F forward primer AGAGTTTGATCMTGGCTCAG (10 pmol/µl), 1 µl of 1492R reverse primer GYTACCTTGTTACGACTT (10 pmol/µl), 1 µl of AccuTaq LA DNA polymerase Mix (Sigma, Cat. Nr. D8045) and nuclease free water to total reaction volume of 50 µl. PCR conditions consisted of 5 min initial DNA denaturation at 94°C, followed by 10 cycles of 1 min DNA denaturation at 94°C, 30 s of primer annealing at 62°C, 2.5 min of DNA chain extension at 68°C and 20 cycles of 1 min DNA denaturation at 94°C, 30 s primer annealing at 55°C, 2.5 min of DNA chain extension at 68°C and final extension for 5 min at 68°C. Concentration, proper length and purity of PCR product was checked by electrophoresis on agarose gel. PCR product was cleaned by ExoSAP-IT kit (GE Healthcare, Cat. Nr. 78200).

Sequencing was performed on a Beckmann Coulter CEQ8000 sequencing machine. Sequencing reactions were done by using CEQ DTCS Quick Start kit (Beckman, Cat. Nr. 608120).

After obtaining of DNA sequence similarity searches were carried out against the NCBI database using BLAST program.

***Propagation of Asaia* sp. *bacteria*.** Typical colony was selected from Plate Count Agar and it was brought forward to the 50 ml of liquid medium (5 g of peptone, 3 g yeast extract, 2.5 ml of glucose per 1 l of medium). This formulation was then inoculated 2 days at the temperature of 25°C.

***Determination of effect of preservation agents on Asaia* sp. *bacteria*.** The growth medium (apple nectar with 50% of fruit content) was placed to the 100 ml medicinal bottle. These bottles were sterilised at the temperature of 121°C during 15 min and then inoculated by 0.1 ml suspense with the *Asaia* sp. bacteria. Then it was added solution of preservation agent so that final concentration of this agent was 1.5, resp. 7 mmol/l. The samples were cultivated at the temperature of 25°C and in the time intervals (6, 24, 30, 48, 54, 72, 78, 96 h) the absorbance was measured at the wave length of 530 nm. Former concentrations correspond with the maximal concentrations defined by the Notice 4/2008 Sb.

Technique of microscopy. The suspension of bacterial cells was applied on the microscopic grating coated with the carbon layer. The bacteria were adhered on the grating during five minutes. Then the grating was washed by water and the adhered cells were coloured by the 0.8% solution of sodium silica-tungstate at the pH value of 7.4 during 10 seconds. Then the grating was dried and inserted into the microscope. The transmission electron microscope JEOL JEM-1010 with camera MegaView III and software AnalySIS were used. The observation was carried out at the accelerating voltage 80 kV.

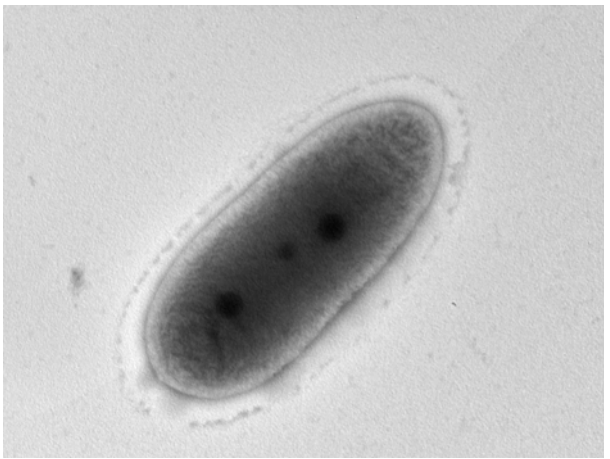

Figure 1. *Asaia* sp. cell with visible

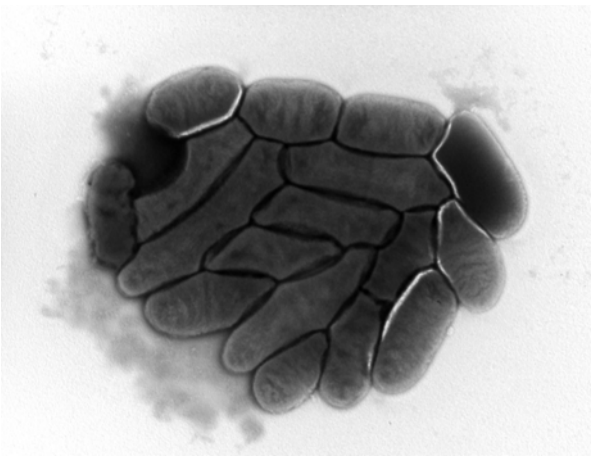

Figure 2. Agglomerate of *Asaia* sp. cells (12 000×) polysaccharide encapsulation (30 000×)

RESULTS AND DISCUSSION

The bacteria causing occurrence of villiform impurities within fruit based still beverages were isolated and then identified as *Asaia* sp. by the PCR technique. The isolated bacteria *Asaia* sp. exhibit the polysaccharide encapsulation (Figure 1). Compact agglomerates of bacterial cells (Figure 2) and formation of very resistant biofilm were observed.

The tested concentration 1.5 mmol/l is near the concentration 250 mg/l used for the stabilisation of similar fruit beverages production. From the results in Figure 3 it is obvious that the presence of preservatives was almost no effect on the *Asaia* sp. growth. It is able to grow in this environment and this concentration of tested preservation agents is ineffective. The minimum inhibition concentration for sorbic and benzoic acid in the conditions of the model fruit drink (pH 3.45; Rf 10 Brix) were between 250 and 500 mg/l, DMDC has almost no effect in all tested concentrations (up to 1000 mg/l). The reasonable inhibition effect was found for the concentrations 7 mmol/l of sorbic or benzoic acids (it corresponds with about 1000 mg/l) (Figure 4). The biggest effect was obtained using benzoate where after 54 h of cultivation the decrease of about 64% against the blank was observed.

CONCLUSION

On the basis of foregoing results it was found that commonly used concentration of preservation agents are insufficient for the growth inhibition of *Asaia* sp. These bacteria are able to grow in the environment at pH 3.45, in the presence of sorbate, benzoate and dimethyldicarbonate in concentrations 1.5 mmol/l and higher. Benzoic acid has slightly higher inhibition effect than sorbic acid, DMDC has no effect within all tested concentrations. The resistance of *Asaia* sp. To common preservatives limits the available possibilities to prevent spoilage of similar drinks to the elimination of potential sources and careful sanitation procedures. The contamination of the technological equipment always brings the serious problem, common sanitation procedures used in the bever-

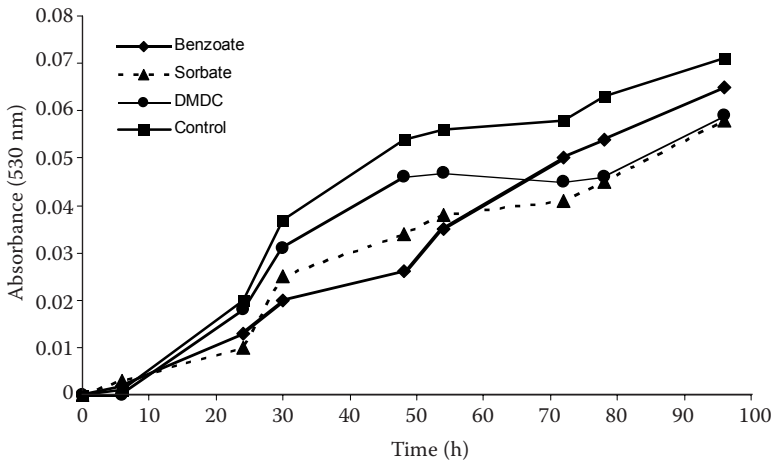

Figure 3. Effect of preservation agents in concentration 1.5 mmol/l (benzoate, sorbate and dimethyldicarbonate) on *Asaia* sp. isolate

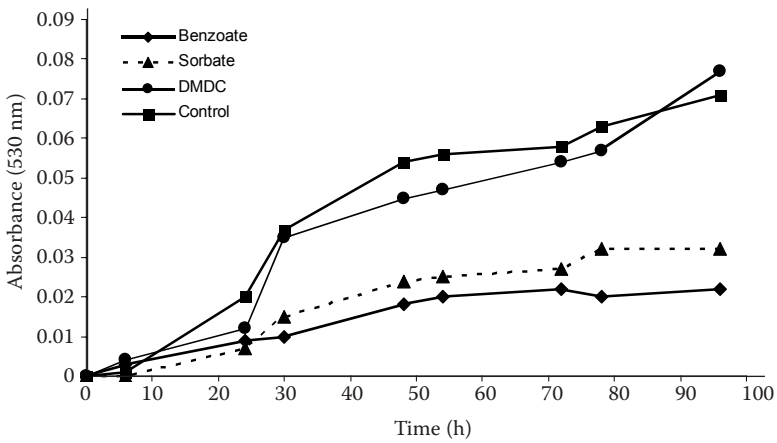

Figure 4. Effect of preservation agents in concentration 7 mmol/l (benzoate, sorbate and dimethyldicarbonate) on *Asaia* sp. isolate

age production may be insufficient to eliminate the very rigid biofilm, which is formed by *Asaia* sp. in the equipment. Its reliable elimination may require more forcing condition (e.g. hot sodium hydroxide and detergents solutions) and in any hardly accessible points (pipe bends, branches, connections, valves) mechanical treatment is the only possibility.

Acknowledgements: The authors are grateful for financial support provided by the Ministry of Education, Youth and Sports of the Czech Republic (Project No. MSM 6046137305).

References

De Vero L., Giudici P. (2008): Genus-specific profile of acetic acid bacteria by 16S rDNA PCR-DGGE. International Journal of Food Microbiology, **125**: 96–101.

Katsura K., Kawasaki H. (2001): *Asaia siamensis* sp. nov., an acetic acid bacterium in the alpha-Proteobacteria. International Journal of Systematic and Evolutionary Microbiology, **51**: 559–563.

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

Tuuminen T., Heinäsmäki T., Kerttula T. (2006): First report of bacteremia by *Asaia bogorensis*, in a patient with a history of intravenous-drug abuse. Journal of Clinical Microbiology, **44**: 3048–3050.

Yamada Y., Katsura K. (2000): *Asaia bogorensis* gen. nov., sp. nov., an unusual acetic acid bacterium in the alpha-Proteobacteria. International Journal of Systematic and Evolutionary Microbiology, **50**: 823–829.

Yamada Y., Yukphan P. (2008): Genera and species in acetic acid bacteria. International Journal of Food Microbiology, **125**: 15–24.