USE OF UV-C TREATMENT FOR THE INACTIVATION OF MICROORGANISMS

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Beverages can be microbiologically stabilised using filtration, application of preservatives or by heat treatment (pasteurisation) (Dittrich, 1993). Despite this, fruit industry products mostly spoil as a result of growth of microorganisms. Most of the non-spore-forming microorganisms are killed by pasteurisation (Pontius et al., 1998). However, secondary contaminations during the production and the filling process by fruit spoilage microorganisms like yeasts, moulds and bacteria often occur. During the last decades, a new fruit juice spoilage microorganism emerged, which is able to survive heat treatments (Chang and Kang, 2004; Walker and Phillips, 2007).

Alicyclobacillus acidoterrestris is a spore-forming bacterium, which is more resistant to heat and acid as common fruit spoilage organisms (Wisotzkey et al., 1992; Walls and Chuyate, 2000). Originally, alicyclobacilli were thought to belong to the genus *Bacillus*, but later they were assigned to a new genus on the basis of 16S rRNA gene sequence analyses and the occurrence of ω-alicyclic fatty acids in the cell membrane (Wisotzkey et al., 1992).

Due to their spore formation, alicyclobacilli are able to survive the pasteurisation process, or can be even stimulated to grow as a result of the heat treatment. The spoilage of fruit juices caused by *Alicyclobacillus* is visually almost not detectable (Cerny et al., 1984; Walker and Phillips, 2008). The main spoilage characteristic is the occurrence of `medical` odours caused by the production of guaiacol (2-methoxyphenol). Guaiacol is detectable in apple and orange juices as soon as alicyclobacilli reach a cell count of ca. log 5 CFU/ml (Pettipher et al., 1997; Orr et al., 2000). This is dependent on the shelf life temperature, the oxygen content and the presence and concentrations of substances, e.g. vanillin, from which guaiacol can be formed.

Alternative preservation methods to conventional thermal conservation of food products, e.g. UV-C treatment, may be interesting especially when microorganisms are heat tolerant or when certain food constituents are heat sensitive. In Germany, UV-C treatment of food products is only allowed for surfaces of fruits and vegetables, hard cheeses and water. However, this spectrum of applications could be broadened to other foods, if a process is properly applied for and approved by the authorities. UV-C treatment of fruit juices as an alternative for heat treatment is already permitted in the USA if a minimal 5-log inactivation of relevant pathogenic microorganisms in the juice is achieved by the process (Anonymus, 2000; Basaran et al., 2004).

In this study, a new UV-C laboratory device, provided by Bayer Technology Services (BTS), was used for the inactivation of microorganisms in naturally cloudy apple juice. This UVivatec® Lab System developed and so far used for the inactivation of viruses in the pharmaceutical industry. The penetration depth of UV-C energy in juices is generally relatively low. In the UV-C device used in this study, the liquid flows in a tube which is helically coiled wound around a mercury source. This special liquid flow leads to the formation of `Dean vortices`, which allow that the entire liquid is mixed and gets into contact with the UV-C source. Therefore, the disadvantage of the relatively low penetration depth of the UV-C energy is compensated.

The aim of the study was to investigate the inhibition of alicyclobacilli in apple juice using this new device and to develop a molecular biological method, which allows a quick and accurate identification and quantification of *Alicyclobacillus*.

MATERIALS AND METHODS

MICROORGANISMS AND CULTURE CONDITIONS

The microorganisms used in this study were obtained from the German collection of microorganisms and cell cultures (Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig) or from the culture collection of our laboratory and consisted of species of the genera *Alicyclobacillus, Bacillus* and genera which

are closely related to the genus *Bacillus (Geobacillus* and *Paenibacillus*) (Table 1). All alicyclobacilli were inoculated into Potato Dextrose Broth (Merck, Darmstadt, Germany, the pH was adjusted to pH 3.5 using tartaric acid after autoclaving) and were incubated at 50 °C using a shaking incubator at 180 rpm/min over night. Strains of *Geobacillus*, *Bacillus* and *Paenibacillus* were inoculated into Trypticase-Soy-Broth (Becton Dickinson, Heidelberg, Germany) and incubated at 55 °C (G. stearothermophilus DSM 279) or at 37 °C (*Bacillus- and Paenibacillus*-strains) using a shaking incubator at 180 rpm/min over night.

PRODUCTION OF A SPORE SUSPENSION OF *ALICYCLOBACILLUS ACIDOTERRESTRIS* DSM 2498

A spore suspension was made to isolate DNA of spores of *A. acidoterrestris* DSM 2498 for use in a genus-specific PCR (see below). For this, 3 l of a 60 % Potato Dextrose Broth were inoculated with 200 ml of an overnight culture of *A. acidoterrestris* DSM 2498 and incubated for ten days at 50° C and 110 rpm/min at the shaking incubator. The number of spores was checked using a phase contrast microscope. The mixture of cells and spores was centrifuged at 16.911 xg and 4 °C for 20 min. The supernatant was discarded and the pellet was resuspended in 70 % ethanol to kill vegetative cells. The suspension was again centrifuged after 60 min at 16.911 xg and 4 °C for 20 min. The pellet was washed twice with 20 ml sterile distilled water. After that, the pellet was resuspended in 25 ml sterile distilled water and stored at 4 °C.

UV-C-INACTIVATION OF *ALICYCLOBACILLUS ACIDOTERRESTRIS* DSM 2498 IN NATURALLY CLOUDY APPLE JUICE

The UV-C-device UVivatec® (Bayer Technology Services) was used (Fig. 1a). The essential part of the device is a helically wound Teflon®-tubing which is wrapped around a quartz glass tube containing a UV lamp (Fig. 1b). The 9 Watt UV-C low pressure mercury lamp (254 nm) is specified to have an energy density of 60 W/m^2 and a durability of more than 1000 hours. Flow rates of liquids from 2 to 20 l per hour can be adjusted by a pump (Schmidt and Kauling, 2007). The dose of the operating UV-C energy is regulated by the flow rate (l/h). Regarding the electric installed power of the UV-C lamp (9 W), the used flow rates of 8.4 l/h and 16.8 l/h result in energy input of 3857 J/l or 1929 J/l per flow cycle. Naturally cloudy apple juice bought in a supermarket was inoculated with ca. 6 x 10^3 to 2x 10^5 CFU/ml *Alicyclobacillus* cells from a fresh overnight culture. The inoculated apple juice was treated with UV-C in three different assays. For each

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Tab. 1: Microorganisms used in this study		
Bacterial strains		**Source**
Alicyclo-bacilli	*Alicyclobacillus (A.) acidiphilus* DSM 14558^T	DSMZ
	A. acidocaldarius ssp. acidocaldarius DSM 446^T	DSMZ
	A. acidoterrestris DSM 3922^T	DSMZ
	A. acidoterrestris DSM 2498	DSMZ
Bacilli	*Bacillus (B.) amyloliquifaciens* BFE 5359	MRI/Oguntoyinbo et al. (2010)
	B. cereus BFE 5400	MRI/Oguntoyinbo et al. (2010)
	B. coagulans DSM 1^T	DSMZ
	B. firmus DSM 12^T	DSMZ
	B. licheniformis DSM 13^T	DSMZ
	B. licheniformis BFE 5370	MRI/Oguntoyinbo et al. (2010)
	B. megaterium DSM 32^T	DSMZ
	B. mycoides DSM 2048^T	DSMZ
	B. pumilus DSM 27^T	DSMZ
	B. subtilis ssp. subtilis DSM 10^T	DSMZ
	B. subtilis BFE 5301	MRI/Oguntoyinbo et al. (2010)
Other spore-forming bacteria	*Geobacillus stearothermophilus* DSM 297	DSMZ
	Paenibacillus larvae DSM 3615	DSMZ
	Paenibacillus macerans DSM 24^T	DSMZ
	Paenibacillus polymyxa DSM 36^T	DSMZ

Fig.1a: UV-C reactor UVivatec® Lab

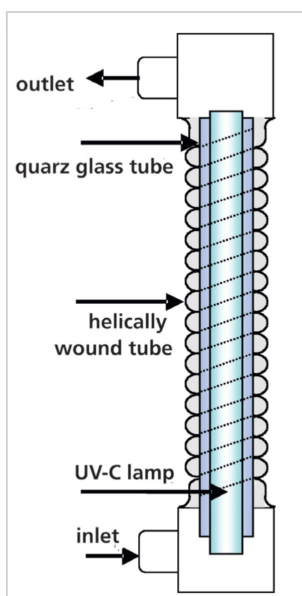

Fig.1b: helically wound Teflon® -tube and UV-C lamp Lab

assay, the inoculated apple juice was totally pumped through the reactor (one cycle). Therefore, each cycle resulted in an increase of the dose. The cell counts of the beginning were determined and samples were taken after the 1st 2nd 4th and 5th cycle. The cell counts were determined by plating on Potato Dextrose agar. All assays were done in triplicate.

DETECTION OF ALICYCLOBACILLI USING GENUS-SPECIFIC PCR

The total genomic DNA of *Paenibacillus*-, *Geobacillus*- and *Alicyclobacillus*-strains was isolated after overnight incubation using the method of Pitcher et al. (1989). The DNA concentration was measured using a spectrophotometer at 260 nm (Genequant, Biorad, Munich).

To obtain DNA from spores, the spores were pre-treated. For this, 1 ml of a spore suspension of *A. acidoterrestris* DSM 2498 was heated in a water bath at 70 °C for 25 min to kill any surviving vegetative cells. To digest the DNA of the vegetative cells, 10 µl of a DNAse stock solution was added and incubated for 30 min at room temperature to prevent further germination of the spores. After that, the spores were centrifuged at 17.860 xg and 4 °C for 10 min and the supernatant was discarded. The pellet was washed with 1.5 ml 1 x TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8) followed by DNA isolation according to the method of Pitcher et al. (1989).

Two specific primer pairs were designed for the differentiation of *Alicyclobacillus*-species from the genera *Paenibacillus, Bacillus and Geobacillus*. One primer pair was specific for the amplification of a part of the 16S rRNA gene of alicyclobacilli and the other primer pair was specific for the amplification of the squalene-hopene-cyclase gene of alicyclobacilli. The corresponding gene sequences of *Bacillus*, *Alicyclobacillus* and *Paenibacillus*-species were (if available) obtained from the Genbank-database and compared and showed a considerable heterogeneity between the genera. The genus-specific primer targeting the 16S rRNA gene and the squalene-hopene-cyclase gene, and the PCR conditions are shown in Table 2. The PCR reactions contained 100 ng template DNA, 200 µM dNTP's, 25 pMol of each primer, 1.5 U Taq-polymerase (GE Healthcare, Freiburg, Germany) and 1 x Taq-polymerase buffer. The PCR products were separated on a 2.0 % agarose gel with 1 x TBE buffer at 100V for 2 h.

QUANTIFICATION OF *ALICYCLOBACILLUS ACIDOTERRESTRIS* DSM 2498 IN APPLE JUICE USING QUANTITATIVE REAL TIME PCR

An *A. acidoterrestris* DSM 2498 overnight culture was diluted in a ten-fold dilution series up to 10-5 in either clear, pasteurised apple juice, or in a quarter-strength Ringer`s solution (Merck). A volume of 1 ml was taken from each dilution of either clear apple juice or Ringer`s solution, and centrifuged at 17.860 xg for 5 min. The pellets were washed once with 1x TE buffer and this was followed by isolation of the bacterial DNA according to the method of Pitcher et al. (1989). These samples were used for a real time PCR using iQ™ SYBR®-Green Supermix-Kit (Bio-Rad, Munich). The volume of the PCR assay was 25 µl and contained 12.5 µl iQ™ SYBR®-Green Supermix, 5 pMol of both Ali16SqRT primers (Table 2) and 3 µl template DNA. The real time PCR was performed using the iCycler iQ5 (Bio-Rad) (see Table 2).

RESULTS

UV-C-INACTIVATION OF *ALICYCLOBACILLUS ACIDOTERRESTRIS* DSM 2498 IN NATURALLY CLOUDY APPLE JUICE

The initial cell counts varied between the different assays, therefore the naturally cloudy apple juice was inoculated with 5x 10^3 to 2x 10^5 CFU/ml in the three assays (Fig. 2). The UV-C treatment of *A. acidoterrestris* DSM 2498 in all three assays resulted in a ca. 3.5 log10 CFU/ml decrease in cell count. The treatment of *A. acidoterrestris* with an initial cell count of approx. 5.5 x 10^3 CFU/ml with the highest energy dose (5x 3857 J/l, lowest flow rate, i.e. 8.4 l/h) killed all cells, whereas the assay with the middle dosage setting (5x 1929 J/l; 16.8 l/h) reduced the cells from ca. 2x 10^5 CFU/ml to 1x 10^2 CFU/ml (Fig. 2). A treatment with the lowest energy dose (5x 946 J/l; flow rate 16.8 l/h and half of the lamp was covered with aluminium foil) still reduced the cell counts from 3x 104 CFU/ml to 8x 100 CFU/ml.

DETECTION OF ALICYCLOBACILLI USING GENUS-SPECIFIC AND QUANTITATIVE REAL TIME PCR

Fig. 3a shows the results of the genus-specific PCR with the Ali16SqRT primers. All Alicyclobacillus-strains showed a strong signal at the size of the expected 16S rRNA gene fragment (134 bp). The signal was weaker for strain *A. acidocaldarius* DSM 446T but still visible. Strains belonging to the *Bacillus*, *Geobacillus* and *Paenibacillus* showed none, or only a very weak signal. Fig. 3b shows the results of the genus-specific PCR with the AliSqual primers. All *Alicyclobacillus*-strains except *A. acidiphilus* DSM 14558T showed the expected PCR-product with a size of 275 bp, whereas the *Bacillus*-, *Geobacillus*- and *Paenibacillus*-strains, as expected, did not show any PCR-product. The quantitative real time PCR was performed using the 16S gene primer pair. *A. acidoterrestris* could be detected using real time PCR both in Ringer`s solution as well as in apple juice up to a germination number of ca. 5x 10^2 CFU/ml.

DISCUSSION

UV-C-INACTIVATION OF *ALICYCLOBACILLUS ACIDOTERRESTRIS* DSM 2498 IN NATURALLY CLOUDY APPLE JUICE

The results of this study show that the UV-C-inactivation method using the UVivatec® device is suitable to reduce

Tab. 2: Primers and conditions for the *Alicyclobacillus* genus specific PCR

Target gene	Primer	Sequence	Product size	PCR reactions
Alicyclobacillus 16S rRNA Gen	Ali16-SqRTfw	5'-CTC GGG GAG AGC GRY AAG GAG A-3'	134 bp	The same PCR reaction was performed for both target genes: One denaturation step at 94°C for 2 min; 32 cycles with one denaturation step at 94°C for 1 min, primer annealing at 67°C and polymerisation at 72°C for 30s.
	Ali16-SqRTrev	5'-CTT TAC GCC CAG TGA TTC CG-3'		
squalene-hopene-cyclase-gene	Ali-Squalfw	5'-TAC TGG TGG GGG CCG CTW YTG-3'	275 bp	
	Ali-Squalrev	5'-CCG CCC TSG YTC TGA ATG AA-3'		

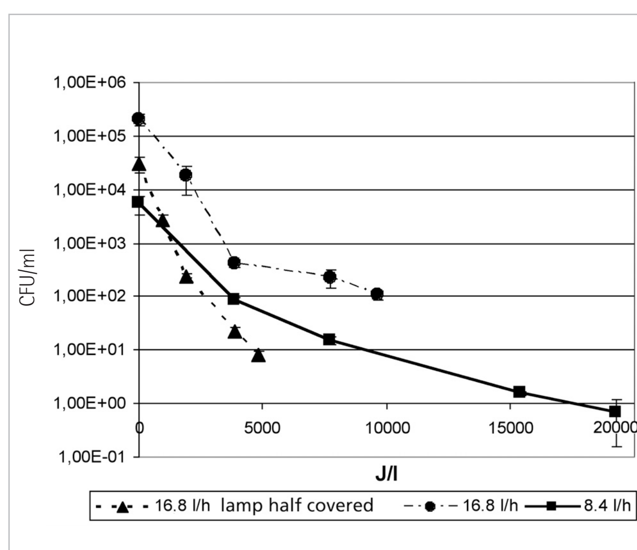

Abb. 2: Inactivation of Alicyclobacillus acidoterrestris DSM 2498 using UV-C treatment at three different dosages

the numbers of vegetative cells of *Alicyclobacillus acidoterrestris* DSM 2498 in naturally cloudy apple juice. It is well known that UV-C energy has only a low penetration depth, but this disadvantage is circumvented by a specific liquid flow in the UVivatec® reactor and the emergence of Dean vortices, so that the bacteria in the juices get into close contact with the UV-C source and can therefore be effectively killed. In this study, vegetative cells of *Alicyclobacillus* were used. In further studies it will be investigated how suitable this method is to inactivate spores of *Alicyclobacillus acidoterrestris*. The use of a UV-C treatment would be advantageous, for example, if juices are stored for a period of time after pasteurisation and spores could germinate. In this case, UV-C treatment could guarantee that germinated bacterial cells would be effectively reduced and spoilage of the fruit juice would be prevented.

DETECTION OF ALICYCLOBACILLI USING GENUS-SPECIFIC AND QUANTITATIVE REAL TIME PCR

A quick and accurate detection of alicyclobacilli in fruit juices is difficult. Firstly, the bacteria grow slowly during the cultivation and secondly, they can not be easily differentiated from other *Bacillus*-species which could also be heat and acid resistant. The polymerase chain reaction (PCR) is a very sensitive and specific molecular biological method which also can be used for the quantitative detection of specific bacteria. The reaction is based on the use of specific DNA-oligomers (primers) which bind to a certain target gene leading to a specific amplification of this gene. The primers used in this study were designed to bind either to the 16S rRNA gene or the squalene-hopene-cyclase gene, and specifically only to these genes of alicyclobacilli. Therefore, *Alicyclobacillus* species can be differentiated specifically from other *Bacillus*, *Paenibacillus* or *Geobacillus* species. Moreover, using these primers in a quantitative, real time PCR, it could be shown that *A. acidoterrestris* can be quantitatively detected in apple juice up to a cell count of 5x 10^2 CFU/ml. This method of analysis represents an accurate and sensitive possibility for the detection of alicyclobacilli in apple juice and could be developed to a routine method useful for the fruit industry.

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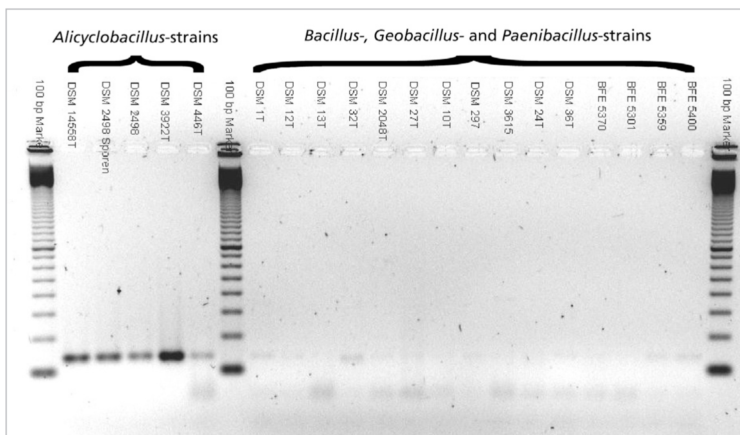

Fig. 3a: Agarose gel with PCR products of different alicyclobacilli and related genera performed using the Ali16SqRT primers for the specific detection of Alicyclobacillus.

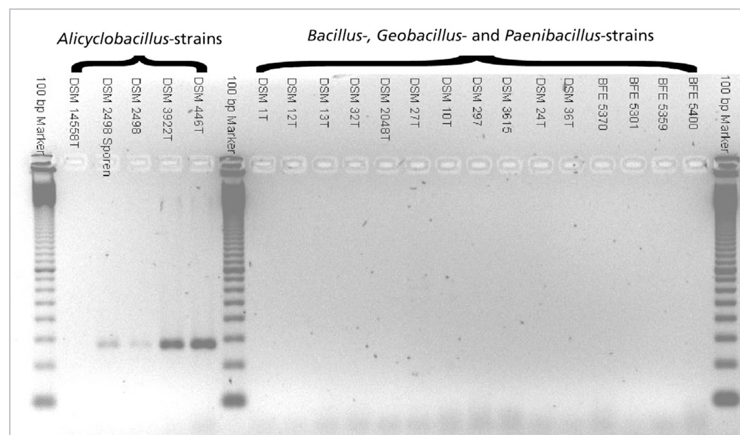

Fig. 3b: Agarose gel with PCR products of different alicyclobacilli and related genera performed using the AliSqual primers for the specific detection of Alicyclobacillus.

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