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18

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Detection of Microorganisms in Compressed Gases

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Abstract

The International Standard Norm ISO 14698-1/2[1] demands the monitoring of bio contamination in compressed gas systems which are used in pharmaceutical production processes. The norm describes the detection of bio contamination by sampling and enumerating viable units with appropriate methods.

Two main questions were investigated:

Do microorganisms survive in compressed gas systems?
If they survive, what appropriate method has to be applied in order to sample and enumerate them?

13 different medical relevant species of microorganisms where grown and put under pressure from 10 to 170 bars. Their growth and behaviour under pressure and the effect of decompression has been analysed. Based on the MAS-100 CG Ex (Microbiological Air Sampler for Compressed Gas) this presentation shows that microorganisms can be measured and what trapdoors are to be avoided when dealing with colony forming units under pressure.

Key words: Microorganisms under pressure, detection of pressurised microorganisms,

Do microorganisms survive in compressed gas systems?

1. Introduction

Pressurised gases are commonly used in the pharmaceutical and food industry for various purposes. In the food industry some gases are used to prevent products to oxidise after the production process and increase their shelf live. In the production of pharmaceutical products Nitrogen, Argon, Carbondioxyde gases are used during filling of ampoules, or to ad gases to fermenters.

To answer the first questions about the survival of microorganisms in pressurised systems 13 random selected microorganisms have been selected and cultivated under different pressures.

For the second question an automatic gas collection system MAS-100 CG Ex[2] was used to collect microorganisms in pressurised gases and compared with a membrane filtration method.

2. Method

The test organisms were cultivated on Tryptone Soy Agar or Sabouraud Dextrose agar respectively. The microorganisms were suspended in NaCl solution and a serial dilution was prepared. 20µl of the suspensions where inoculated into 1.5ml of TSB (Cryptic Soy Broth) in sterile ampoules. The same quantity of sample was used for positive and negative control under normal atmospheric conditions. The ampoules where transferred in a stainless steel cylinder and filled with compressed air with 10 bar and 170 bar respectively. They have been incubated under pressure for 4 days at 30°C. To avoid damage of the organisms during the decompression of the gas cylinder the time of decompression was chosen to be very slowly. (2 h at 170 bar).

2.1 Table of tested organisms

Tests at 10 bar

No.	Test organisms	Class	Microbial count	Positive control	Negative control	**growth at 10 bar**	growth at 1 bar
A	Bacillus atrophaeus spores	ATTCC 9327 (1)	34	+	-	+	
B	Aspergillus niger spores	ATCC 16404	70	+	-	-	+
C	Micrococcus luteus	BKL 564 (2)	95	+	-	+	
D	Escherichia coli	ATCC 8739	250	+	-	+	
E	Enterobacter cloacae	BKL 211	170	+	-	+	
F	Streptococcus faecalis	ATCC 4083	150	+	-	+	
G	Klebsiella pneumoniae	BKL 459	70	+	-	+	
H	Salmonella newport	BKL 848	160	+	-	+	
I	Yersinia enterocolitica	BKL 955	26	+	-	+	
K	Proteus morganii	BKL 1053	20	+	-	+	
L	Staphylococcus aureus	BKL 1266	126	+	-	+	
M	Serratita marcescens	ATCC 8100	17	+	-	+	
N	Saccharomyces cerevisiae	DSM 426 (3)	116	+	-	-	+

Tests at 170 bar

No.	Test organisms	Class	Microbial count	Positive control	Negative control	**growth at 10 bar**	growth at 1 bar
A	Bacillus atrophaeus spores	ATTCC 9327 (1)	45	+	-	-	-
B	Aspergillus niger spores	ATCC 16404	24	+	-	-	-
C	Micrococcus luteus	BKL 564 (2)	35	+	-	-	-
D	Escherichia coli	ATCC 8739	250	+	-	-	-
E	Enterobacter cloacae	BKL 211	226	+	-	-	-
F	Streptococcus faecalis	ATCC 4083	205	+	-	-	-
G	Klebsiella pneumoniae	BKL 459	74	+	-	-	-
H	Salmonella newport	BKL 848	126	+	-	-	-
I	Yersinia enterocolitica	BKL 955	106	+	-	-	-
K	Proteus morganii	BKL 1053	220	+	-	-	-
L	Staphylococcus aureus	BKL 1266	44	+	-	-	-
M	Serratita marcescens	ATCC 8100	50	+	-	-	-
N	Saccharomyces cerevisiae	DSM 426 (3)	180	+	-	-	-

(1) American Type culture Collection

(2) Biological Control-Lab, Novartis Pharma AG

(3) German Collection of Microorganisms

Test performed by R. Meier, Microbiological Consultant

3. Interpretation of results

The tests at 10 bar shows, that 11 out of 13 microorganisms grew under 10 bar pressure and the Aspergillus Niger spores and Saccharomyces Cerevisiae (yeast) grew again after incubation at atmospheric pressure.

The tests at 170 bar showed that no microorganisms grew under this pressure applied neither recovered at atmospheric pressure.

4. Conclusions

Microorganisms do survive in pressurised systems up to 10 bar and some are able to recover after being pressurised.

If they survive, what appropriate method has to be applied in order to sample and enumerate them?

5. Introduction

A few methods are known to collect microorganisms from presurised gas distribution systems. The filtration method combined with a rota meter and a stop watch to measure the collected volume is one of them. The Impinger is another method where the gas is expanded and the gas flow directed into a bottle (impinger) which is filled with a broth. Other systems consists of a kind of an inverted funnel (see picture Fig1) where the gas is expanded into the funnel and aspirated to a microbiological air sampler.

The MAS-100 CG Ex is a fully automatic system which passes the compressed gas under pressure onto an agar Petri dish (impaction principle) and expands after the collection process. (see picture Fig2) The release of pressurised gas under pressure into atmospheric conditions leads to an explosive expansion of the compressed gas with in a fraction of a second. (Example: 10 Liter at 10 bars will result in 100 liters at 1 bar meaning that the volume will increase with a factor of 10) Due to the fact that the microorganisms are transported under pressure and then suddenly are released into atmospheric conditions, they may be damaged through this enormous pressure drop the immediate expansion of the gas and the resulting shearing forces. Observations from previous tests have shown evidence to this theory.

Fig1: Diffusor funnel (expansion takes place **before** collection, risk to damage microorganisms)

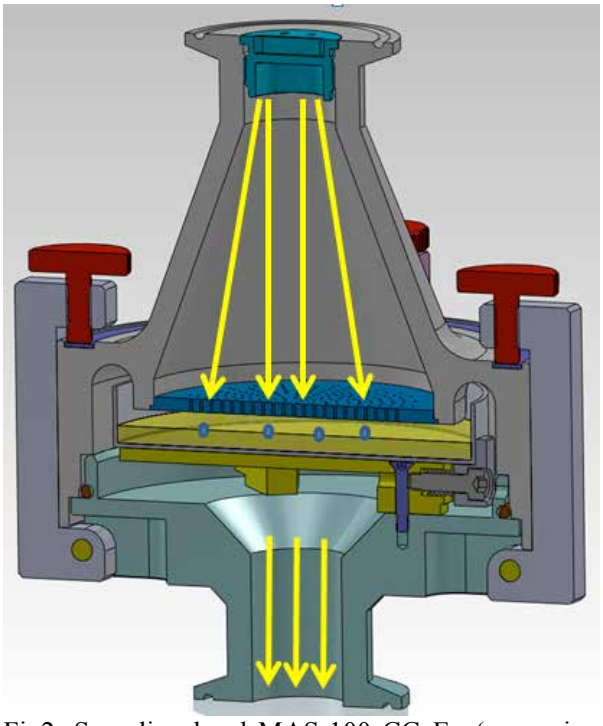

Fig2: Sampling head MAS-100 CG Ex (expansion takes place **after** collection. The sampling head is used up to 10 bar of pressure.

5.1 Test: Microbial behaviour during decompression

20µl of the suspensions used in the previous tests where inoculated on a 2 TSA and 2 SDA agar Petri-dishes and spread by means of a spatula. 1 of each Petri Dish was used for the positive control whereas the other plate was put under 10 bar of pressure for one hour, then decompressed for 5 seconds and after incubated at 30°C for 3 days. (The decompression time corresponds to the default decompression cycle in the MAS-100 CG Ex).

5.2 Interpretation of results

The average microbial yield of the decompressed samples amounts of 94% to the yield of the control plates (paired t-test= 1.41, 2p 0.05= 2.05)
The decompression cycle was not influencing the viability of the microorganisms tested in contrary it was increasing the viability of the microorganisms due to mild conditions of decompression.

6. Collection of microorganisms with the MAS-100 CG Ex

Fig3: MAS-100 CG Ex

The MAS-100 CG Ex is an impaction principle based instrument built with the experience of the MAS-100 Microbiological Air Sampler. It is designed to collect microorganisms in pressurised gases. The major advantage of this instrument is that the sample is taken under pressure and the automatic decompression cycle after the sampling will prohibit damages to the collected micro-organisms. The system is preprogramed for the most used gases in the pharmaceutical industries: Compressed Air, Nitrogen, Carbon-Dioxide, Argon, and Oxygen. It may be used in explosion proof areas.

6.1 Met ode

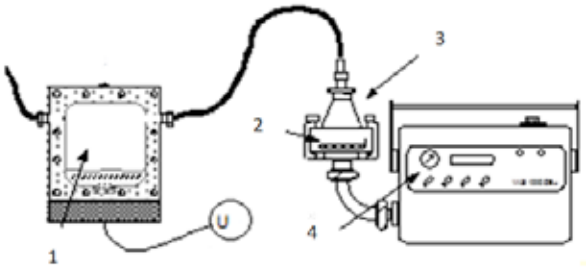

Fig4: Test set up: 1: nebulizer, 2 Agar petri dish, 3 Sampling head, MAS-100 CG Ex

The system was set up as sown in figure 4. The nebulizer (1) was filled with 100 ML of test spore suspension (10^6) and a test pressure of 2.6 bar was applied. 250 liters of the spore containing aerosol was then automatically aspirated with the MAS-100 CG Ex sampler onto a TSA Agar Petri Dish and incubated for 2 days at 30°C.
The same procedure was done with a 0,45 µm membrane filter and holder.
The system was tested according Iso 14698 with a suspension of bacillus atropaeus (ATCC 9372 Raven Biological Laboratory Inc.) in 80% ethanol.

A test series of 3 times 10 samples were performed with both the impaction and the filtration method. As required by the Iso 14698 the microbial yield of the impaction method was calculated in per cent of the membrane filtration method. In addition a statistical evaluation using paired t-test was performed to evaluate the significance of the variations of the two methods.

Test Series of 3 x 10samples with filtration and impaction principle (under pressure)

Test 1			Test 2			Test 3		
MF	MAS-100 CG Ex		MF	MAS-100 CG Ex		MF	MAS-100 CG Ex	
	r	Pr		r	Pr		r	Pr
12	18	19	15	21	22	22	24	25
18	20	21	11	23	24	24	20	21
21	15	15	20	9	9	11	26	27
20	10	10	22	19	20	31	19	20
22	13	13	22	24	25	23	20	21
23	20	21	27	15	15	29	16	16
29	29	30	9	17	17	23	21	22
28	18	19	12	50	21	21	16	16
19	19	20	22	17	17	25	20	21
16	15	15	35	25	26	27	24	25
m= 20.8		m= 18.3	m= 20.5		m= 19.6	m= 23.6		m= 21.4
t= 1.05			**t= 0.32**			**t= 1.06**		

MF= Membrane Filter r= CFU Pr= corrected CFU (according Feller[3]) t= paired t-test

Summary of the collection Efficiency (Performance) of the MAS-100 CG Ex

No.	Membrane Filtraion (MF)	MAS-100 CG Ex	**Performance**
Test 1	m= 20.8	m= 18.3	**88%**
Test 2	m=20.5	m=19.6	**96%**
Test 3	m= 23.6	m=21.4	**91%**

6.1 Interpretation of results

Compared to the membrane filtration method, the MAS.-100 CG Ex has an average efficiency of 92%. The result of the paired t-test shows no significant variation between the two methods.

The MAS-100 CG Ex is a very high performance, very easy to use automatic instrument. The collection of microorganisms under pressure and the decompression after the collection cycle show very high recovery of the microorganisms.

The author recommends using air monitoring systems for pressurised gases which work und pressure during the collection cycle. Systems collecting microorganisms after decompression should not be used due to probable damage of the organisms during expansion. It is although very important to know the impaction speed of the microorgansinms during the sampling cycle. The MAS-100 CG Ex has a constant impaction speed (speed of the microorganisms hitting the Agar surface) of 19.6 m/sec. It is very important to keep this speed constant to have equal speed over the whole sampling periode. Due to big pressure variations in gas distribution systems the MAS-100 CG Ex has an integrated mass flow meter wich regulates and guarantees a constant flow and as a result of this a constant impaction speed.

[1] ISO 14698 ISO 14698-1 Cleanrooms and associated controlled environment – Bioconta- mination control-Part 1: General principles and methods (first edition 2003-09-01)
[2] MAS-100 CG Ex is the trade name of an autmatic system especially made for the collection of microorgansims in compressed gase systems.
[3] W.Feller, An introduction to the probability theory and its applications, Vol.1, p.175.
John Wiley & Sons, Inc., New York,1950