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Evaluation of the efficiency of Mxxxx as a barrier against microrganisms' crossing

A) composition of Mxxxx filter

The filter of Mxxxx has the following characteristics:

1. An outer layer, which is composed by a medical, non woven tissue as a barrier for protecting the inner layers. It has the capacity for filtering substances larger than 2 microns (Figs. 1,2).
2. An active charcoal layer- 200 g/sq. M. - of which 114 g per sq/m is charcoal, which serves as a filter for smoke, odours, etc. Absorption capability of dust 14.6 g out of 16 g (91,25%) at airflow rate of 10 ft3/min(Figs. 3, 4
3. A layer that filters substances from microns of size 0,3 or more. Air permeability (cfm/sf) 55. 0,3-0,5 micron 38,6%, 0,5-0,7 micron 68,8%m, 0,7-1,0 micron 78,6%, 1,0. -2,0 micron 83,7% (Figs. 5, 6).
4. An inner layer witch has the same characteristics of the outer membrane (Figs. 7, 8). Layers 1 and 4 have antiseptic properties because they are also consisted of 0,3% Cetylpyridinium Chloride (CPC) Two different types of Mxxxx (A and B) have been examined. The layers are the above described, but Mxxxx A filter is thinner than Mxxxx B. Consequently breathing is easier with Mxxxx A.

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B) Evaluation of the antiseptic properties of layers 1 and 4

0,1 ml of an 8 hours broth culture (Tryptone Soya Broth) of the different microrganism species tested ***Staphylococcus cohnii ATCC 35662, Staphylococcus aureus*** ATCC 6538, ***Streptococcus pyogenes*** ATCC 19615, ***Enterococcus hirae*** ATCC 10541, ***Pseudomonas fluorescens*** ATCC 49838, ***Escherichia coli*** ATCC 4157, ***Salmonella enteritidis*** ATCC 13076, ***Serratia marcescens*** ATCC 8100, ***Proteus mirabilis*** ATCC 7002, ***Bacillus subtilis*** spores ATCC 6633, ***Bacillus clausii*** spores ***Sonafi-Syntelabo*** Oto S.p.a.- Milan, ***Bacillus stearothermophilus*** spores ATCC 10149, were spread on the surface of Tryptone Soya Agar Petri plate surface using glass spreaders. A fragment of 1cm2 of layers 1 and 4 was put subsequently on the agar inoculated surface and plates were incubated for 24 hours at 36 ± 1 °C for all the micro organisms tested except for the ***Bacillus stearothermophilus*** that had to be cultured under the temperature of 56 ± 1 °C . The halo of growth inhibition was then evaluated.

C) Evaluation of the efficiency of Mxxxx as a barrier against microrganisms' crossing

A bacterial or spore suspension of 1,5 – 7x108 UFC/ml of each of the following micro organisms (Streptococcus pyogenes ATCC 19615, ***Staphylococcus cohnii*** ATCC 35662, ***Serratia marcescens*** ATCC 8100, ***Pseudomonas fluorescens*** 49838, ***Bacillus clausii*** spores ***Sonafi-Syntelabo*** Oto S.p.a.- Milan, ***Bacillus stearothermophilus*** spores ATCC 10149, Bacillus subtilis spores ATCC 6633) was aerosolised by means of a medical device for aerosol therapy. Fragments of Muski filter, previously sterilized by gamma rays, were put into a stainless steel Seitz filter holder, sterilized in steam and plugged in an Erlenmeyer vacuum flask, connected to a vacuum pump, in order to aspirate the microorganisms' aerosol through the Muski. As a control the same described apparatus was used, without the Muski filter. The distance between the aerosol generator and the Seitz filter was about 25 cm. After ten minutes, aerosolisation and vacuum aspirations (0,4m3) were stopped.

In order to evaluate microorganisms' crossing through the apparatus, in the Erlenmeyer flask, 50 ml of a diluent solution (Tryptone natrium chloride) was then introduced in the Erlenmeyer flask, which was then subjected to a mechanical shaker. Both the content of Erlenmeyer and the diluent solution (50 ml), used two times to rinse the

Erlenmeyer flask, were filtered through a membrane filtration apparatus. The filtration membrane is transferred to the surface of a Petri dish containing a suitable culture medium according to the species of microorganisms tested. The culture mediums used were:

1. Pseudomonas isolation agar (Difco) for ***Pseudomonas fluorescens***
2. Chapman mannitol salt agar (Biomerieux) for ***Staphylococcus cohnii***
3. Streptococcus selective medium (Oxoid) with 5% of defibrinated horse blood for ***Streptococcus pyogenes***
4. Hektoen enteric agar (Biomerieux) for ***Serratia marcescens***
5. Tryptone soya agar (Oxoid) for Bacillus subtilis, Bacillus stearothermophilus and Bacillus clausii. All Petri dishes were incubated at 36±1°C for 24-48 hours and the CFU development was evaluated. For ***Bacillus stearothermophilus*** the temperature of incubation was 56 ± 1°C.

In order to detect low growing microorganisms, the culture time was extended to a week. No significant differences in the number of CFU were observed between the 24-48 hours culture and the one week culture.

In all experiences an identification with the API system of the developed CFU was done. The developed microorganisms were always the same, which have been previously aerosolised.
To evaluate the possible correlation, between the size and the capacity of crossing through the filter of the different tested bacteria and spores, droplets of bacteria or spore suspensions were spread over the surface of a cover glass. The different samples were all fixed for 3 hours at 4°C in a 3% glutaraldehyde solution in a 0.1 M phosphate buffer at pH 7.4 . After several washings in phosphate buffer, the material was dehydrated by passing it through solutions with increasing concentrations of ethyl alcohol, in a propylene oxide solution and finally subjected to critical point drying. The samples were then sputter coated with gold palladium (Edwards sputter coater S150) and examined under a scanning electron microscope (Cambridge Stereoscan S360). The dimensions of 50 bacteria or spores of each species were then measured at SEM.

Results

1. **Antiseptic activity of layers 1 and 4**
   The halos of inhibition growth for the tested microorganisms are shown in Table 1.
   At the concentration of 0,3%, ***Cetylpyridinium chloride*** (which is present in layers 1 and 4) demonstrates a high effectiveness against the growth of Bacillus tested species, ***B. stearothermophilus*** and ***B. clausii*** and of ***Enterococcus hirae***, demonstrates a good inhibition against the growth of ***B. subtilis***, ***Staphylococcus cohnii***, ***Staphylococcus aureus, Streptococcus pyogenes*** and results to have moderate effectiveness against the growth of Proteus mirabilis. On the contrary Cetylpyridinium chloride at the concentrations present in layer 1-4, doesn't seem to have any effects on the growth of ***Escherichia coli, Salmonella enteritidis, Serratia marcescens*** and ***Pseudomonas fluorescens .***
2. **Efficiency of Mxxxx as a barrier against microorganisms crossing**
   Our results are resumed in table 3 - 4. In our experimental aspiration apparatus the presence of Mxxxx filter strongly reduced the crossing of microorganisms and spores aerosolised in the air. In the case of aerosolization of vegetative forms of microorganisms the percentage (%) of reduction varies between 98,30% and 100%, whereas by aerosolising ***Bacillus*** spores results a likely lower percentage of reduction (from 94,25% to 99,12%). No evident correlation seems to exist between microorganisms' or spores' size and the capacity of crossing the filter of Mxxxx (compare Table 2 with Table 3 - 4 and Figs. 9-15).

It also seems to exist no difference between the capacity of penetration through the Mxxxx filter of spherical micro organisms (***Staphylococcus cohnii*** and ***Streptococcus pyogenes***) and rod shaped bacteria like ***Pseudomonas fluorescens*** and ***Serratia marcescens***. It seems also very difficult to correlate the sensibility against the antibiotic (Cetylpyridinium Chloride) of different microorganisms tested with their capacity of crossing alive the different layers of the Mxxxx filter. We consider that the good barrier effect of Mxxxx against microorganisms is correlated to both the structure of different layers and the antibiotic, which is present in the outer and in the inner layers of the Mxxxx filter. In conclusion our results demonstrate that Mxxxx filter is a very efficient barrier against bacteria or spores' crossing.

Table 1

Halos of growth inhibition produced by Mxxxx layers 1 and 4.

Type of microrganism	Layer 1	Layer 4
Escherichia coli	0mm	0mm
Salmonella enteritidis	0mm	0mm
Serratia marcescens	0mm	0mm
Proteus mirabilis	1mm	1mm
Pseudomonas fluorescens	0mm	0mm
Staphylococcus cohnii	2mm	2mm
Staphylococcus aureus	3mm	2.9mm
Streptococcus pyogenes	2mm	2mm
Enterococcus hirae	10mm	11mm
Bacillus subtilis	2mm	2mm
Bacillus clausii	8mm	8mm
Bacillus stearothermophilus	8mm	9mm

TABLE 2

Size (mean and standard deviation) of different microrganisms tested; diameter for spherical bacteria, length and breadth for rod shape bacteria or spores.

Type of microrganism	Size in Micron
Staphylococcus cohnii	0.70 ± 0.08
Streptococcus pyogenes	0.80 ± 0.16
Pseudomonas fluorescens	1.50 ± 0.49 x 0.51 ± 0.03
Serratia marcescens	1.10 ± 0.60 x 0.37 ± 0.04
Bacillus stearothermophilus spores	0.86 ± 0.13 x 0.53 ± 0.04
Bacillus clausii spores	1.01 ± 0.13 x 0.57 ± 0.05
Bacillus subtilis spores	1.25 ± 0.15 x 0.58 ± 0.07

TABLE 3

Number of developed CFU, in the filtration apparatus, and % reduction with or without Mxxxx filters after aerosolization of different bacteria.

Test Microrganism	N° of CFU		Type of Mxxxx	Reduction with Mxxxx
Staphilococcus cohnii	with Mxxxx	0	A	100%
	without Mxxxx	516		
	with Mxxxx	0	B	100%
	without Mxxxx	8000		
	with Mxxxx	15	A	99.770%
	without Mxxxx	6300		
Test Microrganism	**N° of CFU**		**Type of Mxxxx**	**Reduction with Mxxxx**
Streptococcus pyogenes	with Mxxxx	0	B	100%
	without Mxxxx	233		
	with Mxxxx	0	B	100%
	without Mxxxx	840		
	with Mxxxx	2	A	98.88%
	without Mxxxx	1650		

Test Microrganism	N° of CFU		Type of Mxxxx	Reduction with Mxxxx
Serratia marcescens	with Mxxxx	5	A	99.4%
	without Mxxxx	8004		
	with Mxxxx	0	B	100%
	without Mxxxx	304		
	with Mxxxx	2	A	99.95%
	without Mxxxx	4000		

Test Microrganism	N° of CFU		Type of Mxxxx	Reduction with Mxxxx
Pseudomonas fluorescens	with Mxxxx	3	A	98.3%
	without Mxxxx	176		
	with Mxxxx	9	B	98.33%
	without Mxxxx	538		
	with Mxxxx	3	A	99,65 %
	without Mxxxx	850		

TABLE 4

Number of developed CFU, in the filtration apparatus, and % reduction with or without Mxxxx filters after aerosolization of different bacteria

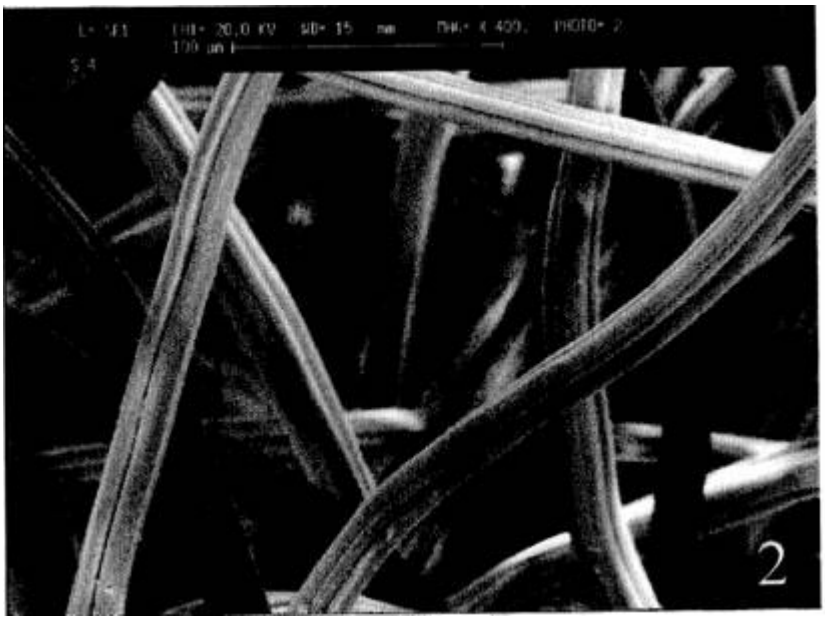
Test Microrganism	N° of CFU		Type of Mxxxx	Reduction with Mxxxx
Bacillus clausii **(spores)**	with Mxxxx	95	A	97.93%
	without Mxxxx	4440		
	with Mxxxx	95	B	97.64%
	without Mxxxx	4025		
	with Mxxxx	17	B	97,50 %
	without Mxxxx	680		
	with Mxxxx	46	B	96,00 %
	without Mxxxx	1148		
Test Microrganism	**N° of CFU**		**Type of Mxxxx**	**Reduction with Mxxxx**
Bacillus stearothermophilus **(spores)**	with Mxxxx	3	A	99.12%
	without Mxxxx	340		
	with Mxxxx	9	B	97.84%
	without Mxxxx	411		

Test Microrganism	N° of CFU		Type of Mxxxx	Reduction with Mxxxx
Bacillus subtilis **(spores)**	with Mxxxx	30	B	94.25%
	without Mxxxx	523		
	with Mxxxx	4	B	97.45%
	without Mxxxx	157		
	with Mxxxx	18	A	96.37 %
	without Mxxxx	496		

Pictures

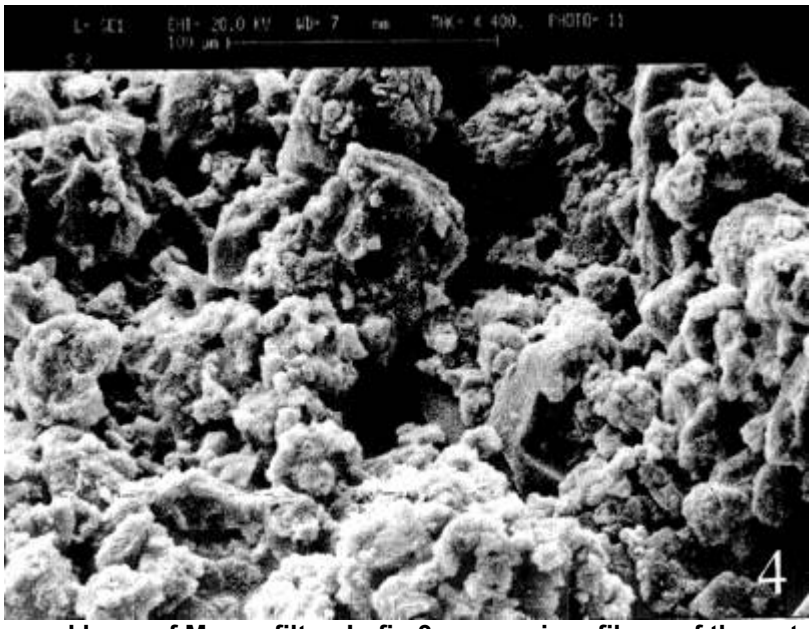
Figs 1-2 - Outer layer of the Mxxxx filter.
Figs 3-4 - Active charcoal layer of Mxxxx filter. In fig 3 some micro fibres of the outer layer are sticking to the charcoal layer.
Figs 5-6 - Intermediate layer of Mxxxx filter.
Figs 7-8 - Inner layer of Mxxxx filter.
Fig 9 - Scanning electron micrograph of ***Staphilococcus cohni***.
Fig 10 - Scanning electron micrograph of ***Streptococcus pyogenes***.
Fig 11 - Scanning electron micrograph of ***Serratia marcescens***.
Fig 12 - Scanning electron micrograph of ***Pseudomonas fluorescens***.
Fig 13 - Scanning electron micrograph of ***Bacillus stearothermophilus*** spores.
Fig 14 - Scanning electron micrograph of ***Bacillus clausii*** spores.
Fig 15 - Scanning electron micrograph of ***Bacillus subtilis*** spores

Outer layer of the Mxxxx filter.



Outer layer of the Mxxxx filter.

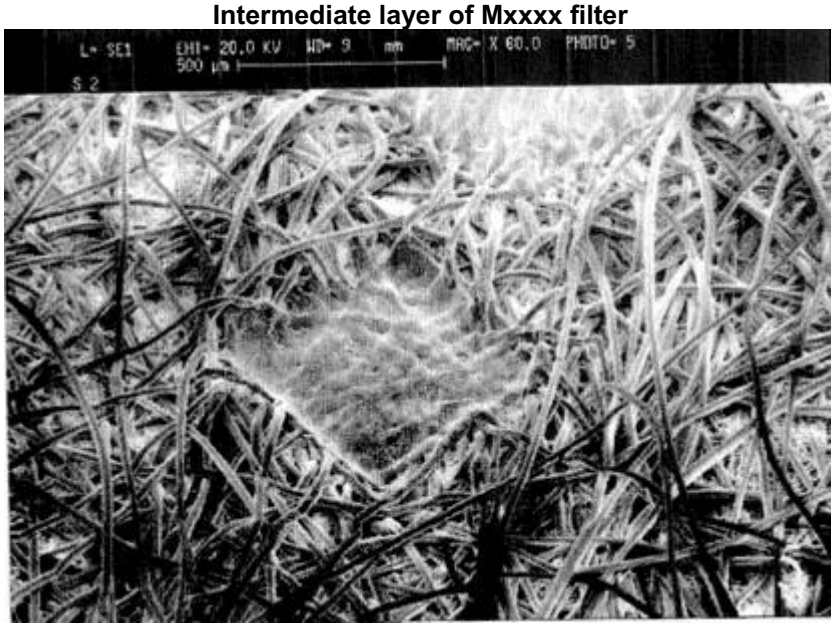
Active charcoal layer of Mxxxx filter. In fig 3 some micro fibres of the outer layer are sticking to the charcoal layer



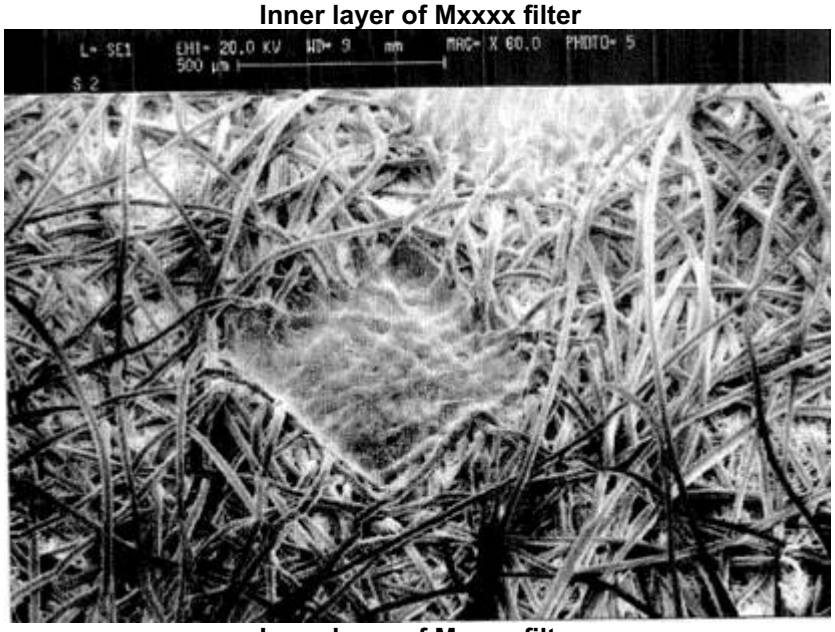
Active charcoal layer of Mxxxx filter. In fig 3 some micro fibres of the outer layer are sticking to the charcoal layer

Intermediate layer of Mxxxx filter

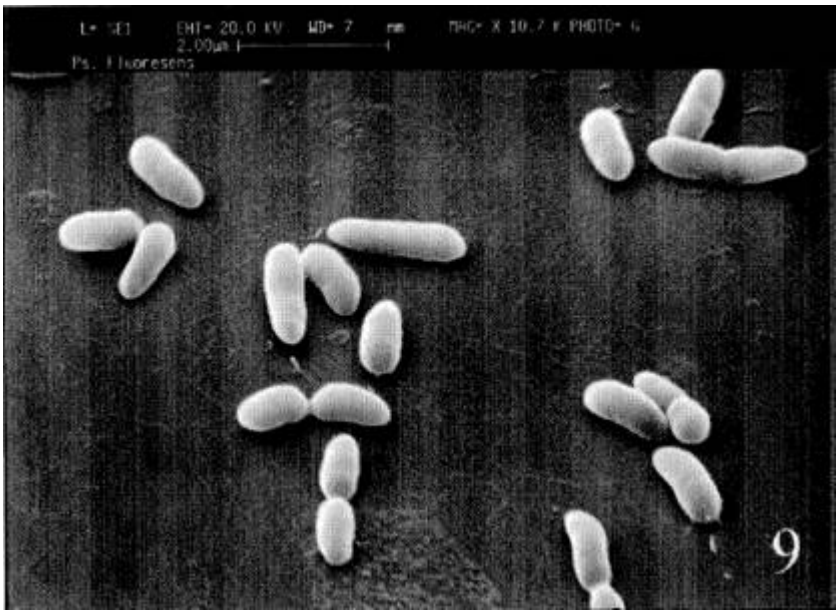
Intermediate layer of Mxxxx filter



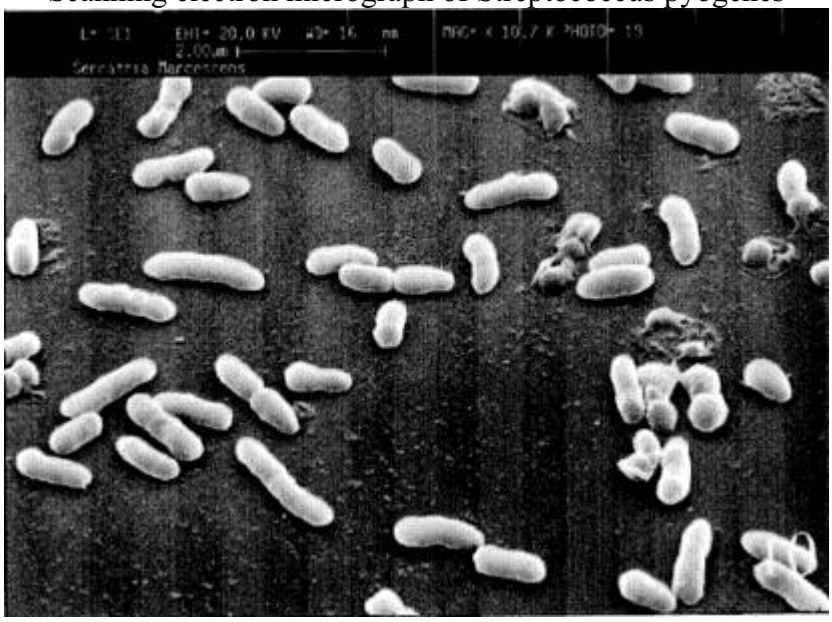
Inner layer of Mxxxx filter



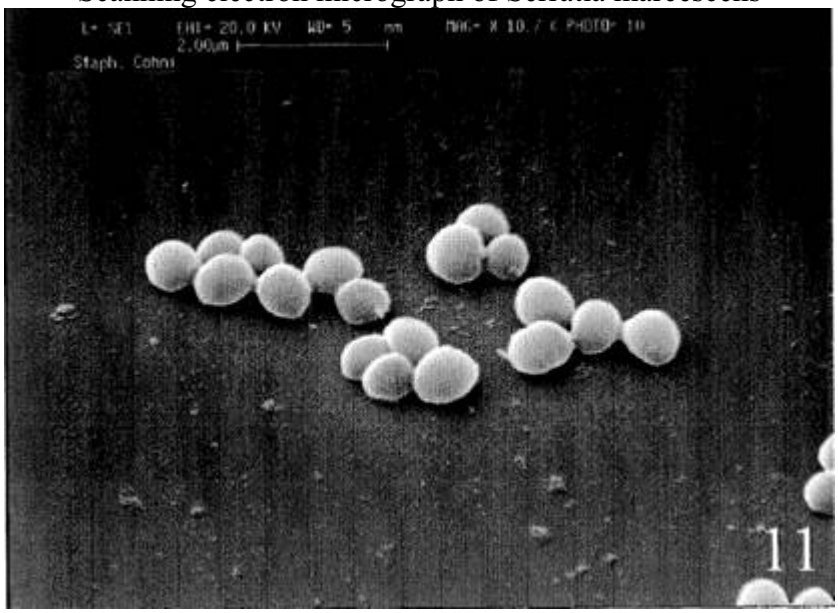
Inner layer of Mxxxx filter



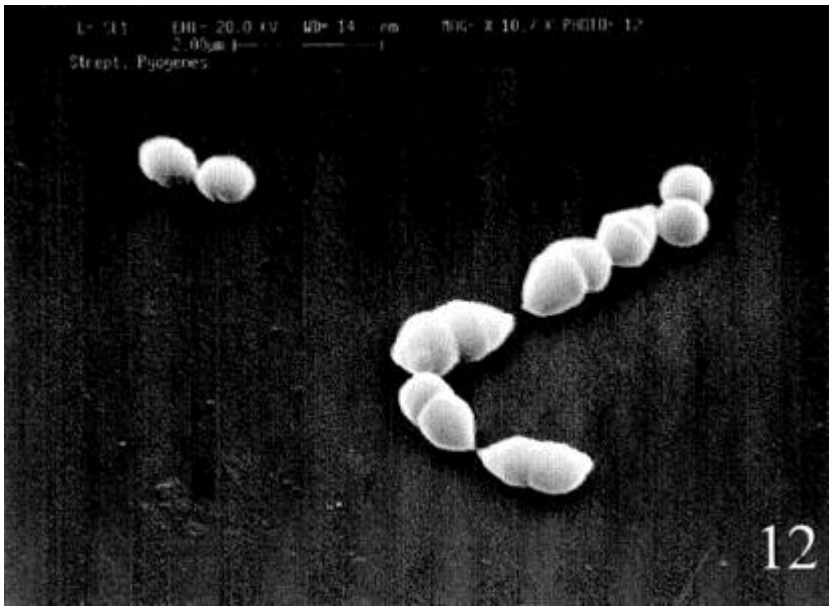
Scanning electron micrograph of Streptococcus pyogenes



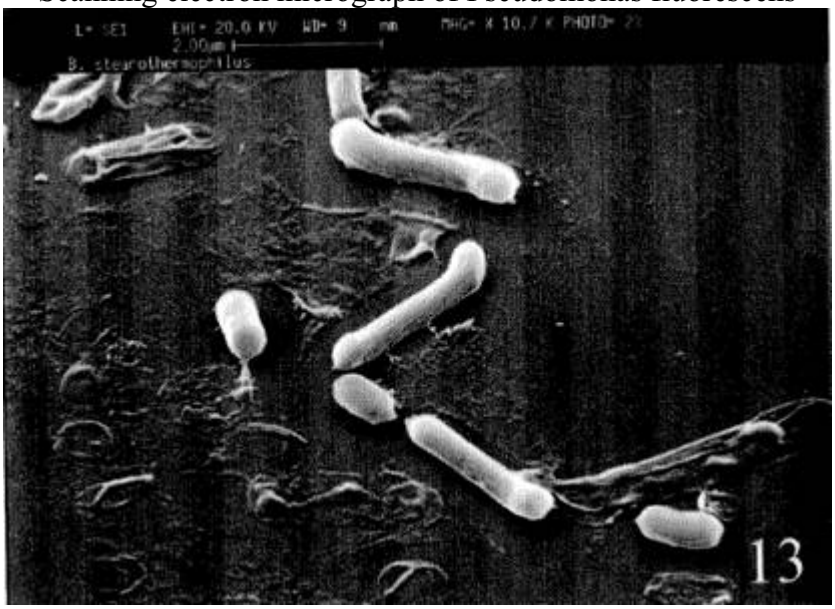
Scanning electron micrograph of Serratia marcescens



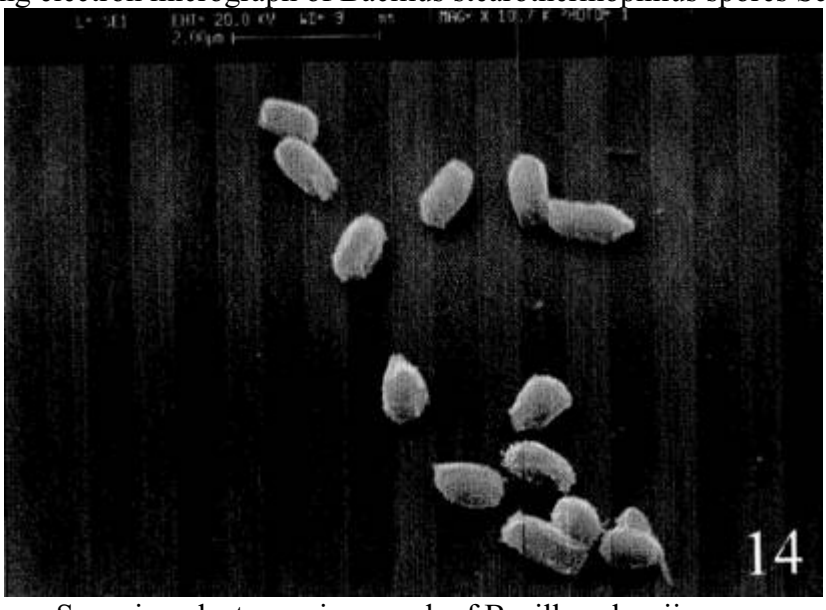
Scanning electron micrograph of Pseudomonas fluorescens



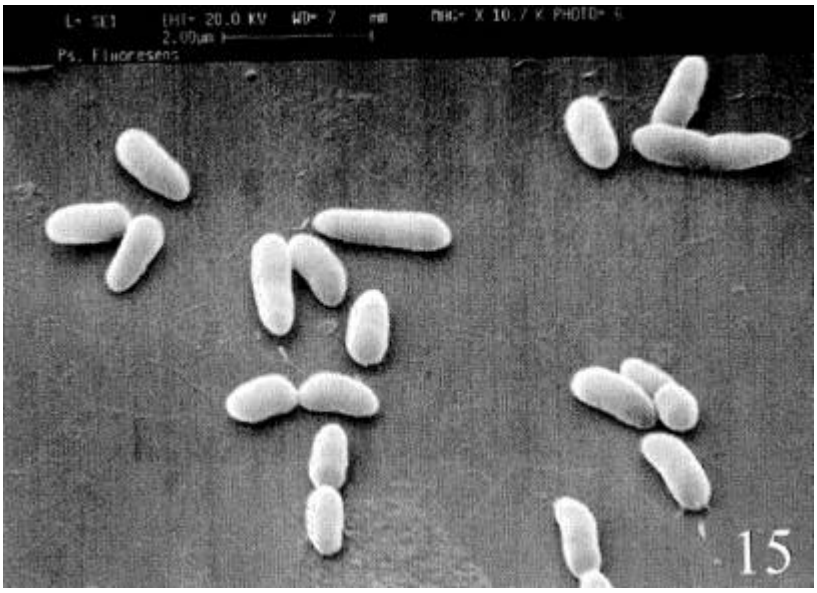
Scanning electron micrograph of Pseudomonas fluorescens



Scanning electron micrograph of Bacillus stearothermophilus spores Scanning



Scanning electron micrograph of Bacillus clausii spores



Scanning electron micrograph of Bacillus subtilis spores

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