3.3.1 Microbial enumeration tests

This text is based on the internationally-harmonized texts developed by the Pharmacopoeial Discussion Group (PDG). Some editorial modifications have been made in order to be in line with the style used in The International Pharmacopoeia.

The tests described hereafter will allow quantitative enumeration of mesophilic bacteria and fungi which may grow under aerobic conditions.

The tests are designed primarily to determine whether a substance or preparation complies with an established specification for microbiological quality. When used for such purposes follow the instructions given below, including the number of samples to be taken and interpret the results as stated below.

The methods are not applicable to products containing viable microorganisms as active ingredients.

Alternative microbiological procedures, including automated methods, may be used, provided that their equivalence to the pharmacopoeial method has been demonstrated.

The recommended test solutions and media are described in 3.3.2 Tests for specified microorganisms.

GENERAL PROCEDURES

Carry out the determination under conditions designed to avoid extrinsic microbial contamination of the product to be examined. The precautions taken to avoid contamination must be such that they do not affect any microorganisms which are to be revealed in the test.

If the product to be examined has antimicrobial activity, this is insofar as possible removed or neutralized. If inactivators are used for this purpose their efficacy and their absence of toxicity for microorganisms must be demonstrated.

If surface-active substances are used for sample preparation, their absence of toxicity for microorganisms and their compatibility with inactivators used must be demonstrated.

ENUMERATION METHODS

Use the membrane filtration method or the plate-count methods, as prescribed. The most probable number (MPN) method is generally the least accurate method for microbial counts; however, for certain product groups with very low bioburden, it may be the most appropriate method.

The choice of a method is based on factors such as the nature of the product and the required limit of microorganisms. The method chosen must allow testing of a sufficient sample size to judge compliance with the specification. The suitability of the chosen method must be established.

GROWTH PROMOTION TEST, SUITABILITY OF THE COUNTING METHOD AND NEGATIVE CONTROLS

General considerations

The ability of the test to detect microorganisms in the presence of the product to be tested must be established.

Suitability must be confirmed if a change in testing performance, or the product, which may affect the outcome of the test is introduced.

Preparation of test strains

Use standardized stable suspensions of test strains or prepare as stated below. Seed-lot culture maintenance techniques (seed-lot systems) are used so that the viable microorganisms used for inoculation are not more than 5 passages removed from the original master seed-lot. Grow each of the bacterial and fungal test strains separately as described in Table 1.

Use *buffered sodium chloride-peptone solution pH 7.0* or *phosphate buffer pH 7.2* to make test suspensions; to suspend *A. brasiliensis* spores, 0.05% of polysorbate 80 may be added to the buffer. Use the suspensions within 2 h or within 24 h if stored at 2–8 °C. As an alternative to preparing and then diluting a fresh suspension of vegetative cells of *A. brasiliensis* or *B. subtilis*, a stable spore suspension is prepared and then an appropriate volume of the spore suspension is used for test inoculation. The stable spore suspension may be maintained at 2–8 °C for a validated period of time.

Table 1. Preparation and use of test microorganisms

Microorganism	Preparation of test strain	Growth promotion		Suitability of counting method in the presence of the product	
		Total aerobic microbial count	Total yeasts and moulds count	Total aerobic microbial count	Total yeasts and moulds count
Staphylococcus	*Casein soya bean*	*Casein soya*		*Casein soya bean digest*	

aureus such as ATCC 6538, NCIMB 9518, CIP 4.83 or NBRC 13276	*digest agar or casein soya bean digest broth* 30–35 °C 18–24 h	*bean digest agar and casein soya bean digest broth* ≤ 100 CFU/ 30–35 °C ≤ 3 days		*agar/MPN casein soya bean digest broth* ≤ 100 CFU/ 30–35 °C ≤ 3 days	
Pseudomonas aeruginosa such as ATCC 9027, NCIMB 8626, CIP 82.118 or NBRC 13275	*Casein soya bean digest agar or casein soya bean digest broth* 30–35 °C 18–24 h	*Casein soya bean digest agar and casein soya bean digest broth* ≤ 100 CFU/ 30–35 °C ≤ 3 days		*Casein soya bean digest agar*/MPN *casein soya bean digest broth* ≤ 100 CFU/ 30–35 °C ≤ 3 days	
Bacillus subtilis such as ATCC 6633, NCIMB 8054, CIP 52.62 or NBRC 3134	*Casein soya bean digest agar or casein soya bean digest broth* 30–35 °C 18–24 h	*Casein soya bean digest agar* and *casein soya bean digest broth* ≤ 100 CFU 30–35 °C ≤ 3 days		*Casein soya bean digest agar*/MPN *casein soya bean digest broth* ≤ 100 CFU 30–35 °C ≤ 3 days	
Candida albicans such as ATCC 10231, NCPF 3179, IP 48.72 or NBRC 1594	*Sabouraud-dextrose agar* or *Sabouraud-dextrose broth* 20–25 °C 2–3 days	*Casein soya bean digest agar* ≤ 100 CFU 30–35 °C ≤ 5 days	*Sabouraud-dextrose agar* ≤ 100 CFU 20–25 °C ≤ 5 days	*Casein soya bean digest agar* ≤ 100 CFU 30–35 °C ≤ 5 days MPN: not applicable	*Sabouraud-dextrose agar* ≤ 100 CFU/ 20–25 °C ≤ 5 days
Aspergillus brasiliensis such as ATCC 16404, IMI 149007, IP 1431.83 or NBRC 9455	*Sabouraud-dextrose agar or potato-dextrose agar* 20–25 °C 5–7 days, or until good sporulation is achieved	*Casein soya bean digest agar* ≤ 100 CFU 30–35 °C ≤ 5 days	*Sabouraud-dextrose agar* ≤ 100 CFU 20–25 °C ≤ 5 days	*Casein soya bean digest agar* ≤ 100 CFU 30–35 °C ≤ 5 days MPN: not applicable	*Sabouraud-dextrose agar* ≤ 100 CFU 20–25 °C ≤ 5 days

Negative control

To verify testing conditions a negative control is performed using the chosen diluent in place of the test preparation. There must be no growth of microorganisms. A negative control is also performed when testing the products as described under *Testing of products*. A failed negative control requires an investigation.

Growth promotion of the media

Test each batch of ready-prepared medium and each batch of medium, prepared either from dehydrated medium or from the ingredients described.

Inoculate portions/plates of *casein soya bean digest broth* and *casein soya bean digest agar* with a small number (not more than 100 CFU) of the microorganisms indicated in Table 1, using a separate portion/plate of medium for each. Inoculate plates of *Sabouraud-dextrose agar* with a small number (not more than 100 CFU) of the microorganisms indicated in Table 1, using a separate plate of medium for each. Incubate in the conditions described in Table 1.

For solid media growth obtained must not differ by a factor greater than 2 from the calculated value for a standardized inoculum. For a freshly prepared inoculum growth of the microorganisms comparable to that previously obtained with a previously tested and approved batch of medium occurs. Liquid media are suitable if clearly visible growth of the microorganisms comparable to that previously obtained with a previously tested and approved batch of medium occurs.

Suitability of the counting method in the presence of product

Preparation of the sample

The method for sample preparation depends on the physical characteristics of the product to be tested. If none of the procedures described below can be demonstrated to be satisfactory, an alternative procedure must be developed.

Water-soluble products. Dissolve or dilute (usually a 1 in 10 dilution is prepared) the product to be examined in *buffered sodium chloride-peptone solution pH 7.0, phosphate buffer sterile pH 7.2* or *casein soya bean digest broth*. If necessary adjust to pH 6–8. Further dilutions where necessary are prepared with the same diluent.

Non-fatty products insoluble in water. Suspend the product to be examined (usually a 1 in 10 dilution is prepared) in *buffered sodium chloride-peptone solution pH 7.0, phosphate buffer pH 7.2* or *casein soya bean digest broth*. A surface-active agent such as 1 g/l of polysorbate 80 may be added to assist the suspension of poorly wettable substances. If necessary adjust to pH 6–8. Further dilutions where necessary are prepared with the same diluent.

Fatty products. Dissolve in isopropyl myristate R, sterilized by filtration, or mix the product to be examined with the minimum necessary quantity of sterile polysorbate 80 or another non-inhibitory sterile surface-active reagent, heated if necessary to not more than 40 °C, or in exceptional cases to not more than 45 °C. Mix carefully and if necessary maintain the temperature in a water-bath. Add sufficient of the prewarmed chosen diluent to make a 1 in 10 dilution of the original product. Mix carefully whilst maintaining the temperature for the shortest time necessary for the formation of an emulsion. Further serial 10-fold dilutions may be prepared using the chosen diluent containing a suitable concentration of sterile polysorbate 80 or another non-inhibitory sterile surface-active reagent.

Fluids or solids in aerosol form. Aseptically transfer the product into a membrane filter apparatus or a sterile container for further sampling. Use either the total contents or a defined number of metered doses from each of the containers tested.

Transdermal patches. Remove the protective cover sheets ("release liner") of the transdermal patches and place them, adhesive side upwards, on sterile glass or plastic trays. Cover the adhesive surface with sterile porous material, for example, sterile gauze, to prevent the patches from sticking together, and transfer the patches to a suitable volume of the chosen diluent containing inactivators such as polysorbate 80 and/or lecithin. Shake the preparation vigorously for at least 30 min.

Inoculation and dilution

Add to the sample prepared as described above under *Preparation of the sample* and to a control (with no test material included) a sufficient volume of the microbial suspension to obtain an inoculum of not more than 100 CFU. The volume of the suspension of the inoculum should not exceed 1% of the volume of diluted product.

To demonstrate acceptable microbial recovery from the product the lowest possible dilution factor of the prepared sample must be used for the test. Where this is not possible due to antimicrobial activity or poor solubility further appropriate protocols must be developed. If inhibition of growth by the sample cannot otherwise be avoided the aliquot of the microbial suspension may be added after neutralization, dilution or filtration.

Neutralization/removal of antimicrobial activity

The number of microorganisms recovered from the prepared sample diluted as described above under *Inoculation and dilution* and incubated following the procedure described below under *Recovery of microorganism*, is compared to the number of microorganisms recovered from the control preparation.

If growth is inhibited (reduction by a factor greater than 2) then modify the procedure for the particular enumeration test to ensure the validity of the results. Modification of the procedure may include, for example (1) an increase in the volume of the diluent or culture medium, (2) incorporation of a specific or general neutralizing agents into the diluent, (3) membrane filtration or (4) a combination of the above measures.

Neutralizing agents. Neutralizing agents may be used to neutralize the activity of antimicrobial agents (Table 2). They may be added to the chosen diluent or the medium preferably before sterilization. If used their efficacy and their absence of toxicity for microorganisms must be demonstrated by carrying out a blank with neutralizer and without product.

Table 2. Common neutralizing agents for interfering substances

Interfering substance	Potential neutralizing method
Glutaraldehyde, mercurials	Sodium hydrogensulfite (sodium bisulfite)

Phenolics, alcohol, aldehydes, sorbate	Dilution
Aldehydes	Glycine
Quaternary Ammonium Compounds (QACs), parahydroxybenzoates (parabens), bis-biguanide	Lecithin
QACs, iodine, parabens	Polysorbate
Mercurials	Thioglycollate
Mercurials, halogens, aldehydes	Thiosulfate
EDTA (edetate)	Mg^{2+} or Ca^{2+} ions

If no suitable neutralizing method can be found, it can be assumed that the failure to isolate the inoculated organism is attributable to the microbial activity of the product. This information serves to indicate that the article is not likely to be contaminated with the given species of the microorganism. However, it is possible that the product only inhibits some of the microorganisms specified herein, but does not inhibit others not included amongst the test strains or for which the latter are not representative.

Then, perform the test with the highest dilution factor compatible with microbial growth and the specific acceptance criterion.

Recovery of microorganism in the presence of product

For each of the microorganisms listed separate tests are performed. Only microorganisms of the added test strain are counted.

Membrane filtration

Use membrane filters having a nominal pore size not greater than 0.45 µm. The type of filter material is chosen in such a way that the bacteria-retaining efficiency is not affected by the components of the sample to be investigated. For each of the microorganisms listed one membrane filter is used.

Transfer a suitable amount of the sample prepared as described above under *Suitability of the counting method in the presence of product* (preferably representing 1 g of the product or less if large numbers of CFU are expected) to the membrane filter, filter immediately and rinse the membrane filter with an appropriate volume of diluent.

For the determination of total aerobic microbial count (TAMC) transfer the membrane filter to the surface of *casein soya bean digest agar*. For the determination of total combined yeasts/moulds count (TYMC) transfer the membrane to the surface of *Sabouraud-dextrose agar*. Incubate the plates as indicated in Table 1. Perform the counting.

Plate-count methods

Perform plate-count methods at least in duplicate for each medium and use the mean count of the result.

Pour-plate method

For Petri dishes 9 cm in diameter add to the dish 1 mL of the sample prepared as described under *Suitability of the counting method in the presence of product* and 15–20 mL of *casein soya bean digest agar* or *Sabouraud-dextrose agar*, both media being at not more than 45 °C. If larger Petri dishes are used the amount of agar medium is increased accordingly. For each of the microorganisms listed in Table 1 at least 2 Petri dishes are used.

Incubate the plates as indicated in Table 1. Take the arithmetic mean of the counts per medium and calculate the number of CFU in the original inoculum.

Surface-spread method

For Petri dishes 9 cm in diameter add 15–20 mL of *casein soya bean digest agar* or *Sabouraud-dextrose agar* at about 45 °C to each Petri dish and allow to solidify. If larger Petri dishes are used the volume of the agar is increased accordingly. Dry the plates, for example, in a laminar airflow cabinet or in an incubator. For each of the microorganisms listed in Table 1 at least 2 Petri dishes are used. Spread a measured volume of not less than 0.1 mL of the sample prepared as described under *Suitability of the counting method in the presence of product* over the surface of the medium. Incubate and count as prescribed under *Pour-plate method*.

Most-probable-number (MPN) method

The precision and accuracy of the MPN method is less than that of the membrane filtration method or the plate-count method. Unreliable results are obtained particularly for the enumeration of moulds. For these reasons the MPN method is reserved for the enumeration of TAMC in situations where no other method is available. If the use of the method is justified proceed as follows.

Prepare a series of at least three serial 10-fold dilutions of the product as described under *Suitability of the counting method in the presence of product*. From each level of dilution 3 aliquots of 1 g or 1 mL are used to inoculate 3 tubes with 9–10 mL of *casein soya bean digest broth*. If necessary a surface-active agent such as polysorbate 80 or an inactivator of antimicrobial

agents may be added to the medium. Thus, if three levels of dilution are prepared nine tubes are inoculated.

Incubate all tubes at 30–35 °C for not more than 3 days If reading of the results is difficult or uncertain owing to the nature of the product to be examined, subculture in the same broth, or *casein soya bean digest agar*, for 1–2 days at the same temperature and use these results. Determine the most probable number of microorganisms per gram or millilitre of the product to be examined from Table 3.

Results and interpretation

When verifying the suitability of the membrane filtration method or the plate-count method a mean count of any of the test organisms not differing by a factor greater than 2 from the value of the control defined above under *Inoculation and dilution* in the absence of the product must be obtained. When verifying the suitability of the MPN method the calculated value from the inoculum must be within 95% confidence limits of the results obtained with the control.

If the above criteria cannot be met for one or more of the organisms tested with any of the described methods the method and test conditions that come closest to the criteria are used to test the product.

TESTING OF PRODUCTS

Amount used for the test

Unless otherwise prescribed use 10 g or 10 mL of the product to be examined taken with the precautions referred to above. For fluids or solids in aerosol form sample 10 containers. For transdermal patches sample 10 patches.

The amount to be tested may be reduced for active substances that will be formulated in the following conditions: the amount per dosage unit (e.g. tablet, capsule, injection) is less than or equal to 1 mg or the amount per gram or millilitre (for preparations not presented in dose units) is less than 1 mg. In these cases the amount of sample to be tested is not less than the amount present in 10 dosage units or 10 g or 10 mL of the product.

For materials used as active substances where sample quantity is limited or batch size is extremely small (i.e. less than 1000 mL or 1000 g) the amount tested shall be 1% of the batch unless a lesser amount is prescribed or justified and authorized.

For products where the total number of entities in a batch is less than 200 (e.g. samples used in clinical trials) the sample size may be reduced to 2 units, or 1 unit if the size is less than 100.

Select the sample(s) at random from the bulk material or from the available containers of the preparation. To obtain the required quantity mix the contents of a sufficient number of containers to provide the sample.

Examination of the product

Membrane filtration

Use a filtration apparatus designed to allow the transfer of the filter to the medium. Prepare the sample using a method that has been shown suitable as described in section 4 and transfer the appropriate amount to each of 2 membrane filters and filter immediately. Wash each filter following the procedure shown to be suitable.

For the determination of TAMC transfer one of the membrane filters to the surface of *casein soya bean digest agar*. For the determination of TYMC transfer the other membrane to the surface of *Sabouraud-dextrose agar*. Incubate the plate of *casein soya bean digest agar* at 30–35 °C for 3–5 days and the plate of *Sabouraud-dextrose agar* at 20–25 °C for 5–7 days. Calculate the number of CFU per gram or per millilitre of product.

When examining transdermal patches filter 10% of the volume of the preparation described under *Preparation of the sample* separately through each of 2 sterile filter membranes. Transfer one membrane to *casein soya bean digest agar* for TAMC and the other membrane to *Sabouraud-dextrose agar* for TYMC.

Plate-count methods

Pour-plate method. Prepare the sample using a method that has been shown to be suitable as described in section 4. Prepare for each medium at least 2 Petri dishes for each level of dilution. Incubate the plates of *casein soya bean digest agar* at 30–35 °C for 3–5 days and the plates of *Sabouraud-dextrose agar* at 20–25 °C for 5–7 days. Select the plates corresponding to a given dilution and showing the highest number of colonies less than 250 for TAMC and 50 for TYMC. Take the arithmetic mean per culture medium of the counts and calculate the number of CFU per gram or per millilitre of product.

Surface-spread method. Prepare the sample using a method that has been shown to be suitable as described in section 4. Prepare at least 2 Petri dishes for each medium and each level of dilution. For incubation and calculation of the number of CFU proceed as described for the pour-plate method.

Most-probable-number method. Prepare and dilute the sample using a method that has been shown to be suitable as described in section 4. Incubate all tubes for 3–5 days at 30–35 °C. Subculture if necessary, using the procedure shown to be suitable.

Record for each level of dilution the number of tubes showing microbial growth. Determine the most probable number of microorganisms per gram or millilitre of the product to be examined from Table 3.

Table 3. Most-probable-number values of microorganisms

Observed combinations of numbers of tubes showing growth in each set			MPN per g or per mL of product	95% confidence limits
Number of g or mL of product per tube				
0.1	0.01	0.001		
0	0	0	Less than 3	0–9.4
0	0	1	3	0.1–9.5
0	1	0	3	0.1–10
0	1	1	6.1	1.2–17
0	2	0	6.2	1.2–17
0	3	0	9.4	3.5–35
1	0	0	3.6	0.2–17
1	0	1	7.2	1.2–17
1	0	2	11	4–35
1	1	0	7.4	1.3–20
1	1	1	11	4–35
1	2	0	11	4–35
1	2	1	15	5–38
1	3	0	16	5–38
2	0	0	9.2	1.5–35
2	0	1	14	4–35
2	0	2	20	5–38
2	1	0	15	4–38
2	1	1	20	5–38
2	1	2	27	9–94
2	2	0	21	5–40
2	2	1	28	9–94
2	2	2	35	9–94
2	3	0	29	9–94
2	3	1	36	9–94
3	0	0	23	5–94
3	0	1	38	9–104
3	0	2	64	16–181
3	1	0	43	9–181
3	1	1	75	17–199
3	1	2	120	30–360
3	1	3	160	30–380
3	2	0	93	18–360
3	2	1	150	30–380
3	2	2	210	30–400
3	2	3	290	90–990
3	3	0	240	40–990
3	3	1	460	90–1980
3	3	2	1100	200–4000
3	3	3	More than 1100	

Interpretation of the results

The total aerobic microbial count (TAMC) is considered to be equal to the number of CFU found using *casein soya bean digest agar*; if colonies of fungi are detected on this medium they are counted as part of TAMC. The total combined yeasts/mould count (TYMC) is considered to be equal to the number of CFU found using *Sabouraud-dextrose agar*; if colonies of bacteria are detected on this medium they are counted as part of TYMC. When the TYMC is expected to exceed the acceptance criterion due to the bacterial growth *Sabouraud-dextrose agar* containing antibiotics may be used. If the count is carried out by the MPN method the calculated value is the TAMC.

When an acceptance criterion for microbiological quality is prescribed it is interpreted as follows:

- 10^1 microorganisms: maximum acceptable count = 20;
- 10^2 microorganisms: maximum acceptable count = 200;
- 10^3 microorganisms : maximum acceptable count = 2000, and so forth.