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PAKISTAN STANDARD

SPECIFICATION FOR CARBONATED BEVERAGES (2ND REVISION)

PAKISTAN STANDARDS AND QUALITY CONTROL AUTHORITY
Standards Development Centre,
39 – Garden Road, Saddar,
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PAKISTAN STANDARD SPECIFICATION FOR CARBONATED BEVERAGES (2ND REVISION)

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PAKISTAN STANDARD SPECIFICATION FOR

CARBONATED BEVERAGES (2ND REVISION)

0. FOREWORD

0.1 This Pakistan Standard was adopted by the Pakistan Standards & Quality Control Authority, Standards Development Centre on 13-07-2002 after the draft finalized by the Soft Drinks Sectional Committee has been approved by the Agricultural and Food Products Divisional Council.

0.2 In tropical country like Pakistan, the carbonated beverage industry has an important place. The quality of a carbonated beverage depends on the quality of the various ingredients that go in its manufacture – water, acidulants, sweetening agents, emulsifiers, stabilizers, flavors, colors, and carbon dioxide being the most important ones. Thed hygienic conditions of the units producing carbonated beverages also need vigilant control to safeguard public health, PS:1825-1987 for Good Manufacturing Practice in Manufacuturing, Processing, Packing or Holding Human Food, are necessary adjuct to this standard.

0.3 In view of the different varieties of carbonated beverages produced in the country, the standard must encompass different ingredients that are use, possible contaminants, a variety of packages and tests to ensure the quality and safety of the beverages.

0.4 This Pakistan Standard Specification was originally adopted in 1984 and revised in 1999. keeping in view the latest development the Soft Drinks Sectional Committee has felt it necessary to revise it again

0.5 For the purpose of deciding whether a particular requirement of this Pakistan standard is complied with the final value, observed or calculated expressing the result of a test or analysis shall be rounded off in accordance with PS:103-1991 ®. Methods of Rounding for Numerical Values, the number of significant places retained in the rounded off value shall be same as that of the specified value in this standard.

1. SCOPE

1.1 This Pakistan Standard prescribes the requirement and the methods of test for carbonated beverages, including chilled carbonated beverages.

2. TERMINOLOGY

2.1 Carbonated beverages prepared from drinking water (PS:1932-2002) (2nd Rev.) shall mean non-alcoholic beverages, containing dissolved carbon dioxide in properly sealed containers or from dispensing units in amanner which ensures freedom from contamination and spoilage.

3. TYPES

3.1 Carbonated beverages shall be of following types :

3.1.1 Carbonated water or soda water – water with carbon dioxide dissolved under specific pressure (ranging from 0.5 to 5.0 gas volumes) with or without flavours. The optional addition of mineral salts (e.g. sodium carbonate, sodium bicarbonate, potassium sulfate, sodium chloride) are allowed and acidulants acids and their salts (e.g. citric, tartaric and malic acids and/or their salts) arwe allowed at GMP levels.

3.1.2 Diet, Low Calorie or Artificial-Nutritive Carbonated Beverages, water with carbon dioxide dissolved under adequate pressure (ranging from 0.5 to 5.0 gas volumes), with non artificial nutritive sweeteners, flavors, colors and other permitted additives. It shall not have calorific value more than 2 Calorie per 100 ml.

3.1.3 Sweetened Carbonated Beverage – water with carbon dioxide dissolved under adequate pressure (ranging from 0.5 to 5.0 gas volumes) with sugar and/or other nutritive sweeteners, flavors, colors and other functional additives.

4.0 INGREDIENTS

4.1 The carbonated beverages shall be prepared from drinking water (PS:1932-2002) (2^{nd} Rev.) carbon dioxide and any of ther other ingredients listed below. All ingredients used in the preparation of carbonated beverages shall be clean, pure, and fit for human consumption. The ingredients shall meet the applicable Pakistan Standards of identity and purity.

4.2 Carbon dioxide – The beveratges shall be carbonated to a pressure in accordance with their character, typically between 0.5 and 5.0 gas volumes, Carbon dioxide used must be free of odor and flavor and be in compliance with the Appendix – D.

NOTE – One gas volume is the volume of carbon dioxide an equal volume of water will absorb at 1,0332 kg cm^{-2} (1 Atmosphere) and 15.56 ^{o}C (60 ^{o}F).

4.2.1 A recommended method for the measurement of gas volume is given in Appendix-C & D.

4.3 The following Nutritive sweeteners are allowed Sucrose, Fructose, Liquid Glucose Dextrose Monohydrate, Invert Sugar, High Lactose Glucose Syrup, Honey; Fruit Juices and Fructose Corn Syrup.

4.4 Low calorie non-nutritive sweeteners :- The following artificial sweeteners of GMP level singly or incombination may be used.

Sweetener	**At GMP Level**
Acesulfame potassium	GMP
Aspartame	GMP
Saccharin and its Na, K, and Ca salts	GMP
Sucralose	GMP

4.5 Flavoring materials and flavor enhancers : - shall be from an intermationalloy recognized list of flavoring materials such as the list prepared by FAO/WHO Joined Expert committee on food additives (FECFA), the codex Alimentarius (under the auspices of the United National WHO/FAO and Council of Europe).

4.5.1 Flavoring materials include :

4.5.1.1 Natural, concentrated or powdered fruit juices.

4.5.1.2 Natural flavors derved exclusively by physical processes from fruit, vegetables, buds, barks, rrots, leaves and other botanical sources, either in their natural state or processed.

4.5.1.3 Nature-identical flavors that are chemically isolated from aromatic raw materials or obtained synthetically which are chemically identical to substances present in natural products intended for human consumption, either processed or not.

4.5.1.4 Artifical flavors that are obtained synthetically.

4.5.1.5 Flavors and flavor enhancers can be used at GMP levels necessary to achieve their desired effects, as specified in appendix – A.

4.5.2 Flavors and flavor enhancers can be used at GMP levels necessary to achieve their desired effects, as specified in appendix – A.

4.6 Acidualants / Acidity Regulators / Salts – Those permitted under the Copdex Alimentarius at the levels specified in Appendix – A may be used.

4.7 Preservatives / Sequestrants : - Those permitted under the Codex Alimentarius at the levels specified in Appendix – A may be used.

4.7.1 In the case of combinations of benzoic acid and sorbic acid, the sum of the qauantity of each preservative added, when expressed as a percentage of the maximum quantity allowed when used alone, shall not exceed one hundred percent.

4.8 Food Colours : - Those permitted under the codex Alimentarius at the levels specified in appendix – A may be used.

4.9 Emulsifiers / Stabilizers / Clouding Agents / Thickeners : Those permitted under the Codex Alimentarius at the levels specified under appendix – A may be used.

4.10 Foaming Agents : - Albumin, carboxymethyl cellulose, edible gums, gelatin, saponin, licorice and its derivatives may be used as foaming agents as specified in Appendix – A.

4.11 Antifoaming Agents – Those listed in appendix A may be used at the levels specified.

4.12 Vitamins and Minerals – If added to carbonated beverages in accordance with clause 7.3 and PS:1485-1994 for Labelling of Prepackaged Foods shall apply.

4.13 Carriers and Solvents – Those listed in Appendix A may be used at the levels specified.

4.14 Antioxidants : Those permitted under the Codex Alimentarius as level specified in Appendix – A may be used.

5. REQUIREMENTS

5.1 Hygienic Condition - The carbonated beverages shall be manufactured in premises maintained under proper hygienic conditions according to PS:1825-1987 for Good Manufacturing Practices in Manufacturing, Processing, Packing or Holding Human Foods so as to assure the safety of the product and to prevent contamination or other deterioration.

5.2 Flavor and Appearance :

5.2.1 Flavor – The carbonated beverage shall be free of flavors, which are inconsistent with those expected for the particular product type. It shall be free of putrid, decay or damage material and of Halal derivatives. All flavors shall be non alocoholic.

5.2.2 Appearance – The carbonated beverages shall be free of precipitated impurities.

5.3 In the case of sweetened carbonated beverages except for ginger ale and spiced beverages, the sugar content after removal of carbon dioxide shall record a Brix value of not less than 8 degree brix at 20 oC.

5.4 Carbonated beverages shall be free from pathogenic microorganisms.

5.4.1 The product may also confirm total bacteria (total plate count) not to exceed 100 / 100 ml at the time of bottling, and 30 / 100 ml for beverages after one week from date of production (incubation for 48 +/- 3 hours at 35 ^{o}C +/- 1 ^{o}C) (See Appendix – E). PS:4729-2001 for water quality Enumeration of culturable micro organisms-colony count by inoculation in a nutrient agar cultural medium.

5.4.2 Coliform count not to exceed 0/100 ml when using the most probable number method (M.P.N.) see Appendix-K of PS:4639-2002 for Bottled Drinking Water (2nd Revision)/PS:4455/1-1999 for water quality detection and enumeration of coliform organisms thermotolerant coliform organisms and presumptive escherchia coli Part-1.

5.4.3 Total yeast and moulds not to exceed 25/100 ml at the time of bottling and 10/100 ml after one week from date of production (incubation at 25 ^{o}C +/- 1 ^{o}C for 72 hours +/- 3 hours, with final confirmatory results reported after 120 hours +/- 3 hours) See appendix-F/Appendix-G.

5.5 Mineral elements in the final beverage may not exceed the limits listed in Table-1.

TABLE – 1

CHEMICALS	MAXIMUM ALLOWABLE LEVEL (mg/L)
Barium	0.7
Fluoride	1.5
METALS & HEAVY METALS	
Mercury	0.01
Cadmium	0.01
Arsenic	0.01
Copper	0.01
Selenium	0.01
Chromium	0.05
Manganese	0.05

6. PACKAGING, TRANSPORT AND STORAGE

6.1 Carbonated beverages and water may be filled in food grade glass bottles, plastic bottles, cans or any other suitable packages. Packages shall comply with all applicable Pakistan Standards. Metal containers shall have a suitable lacquered liner material, which will not react with the beverage contents. Metal crowns to be used with glass bottles will be designed and produced so as to minimize rusting. All closures, whether metal or plastic, shall have a liner material (lacquer and or plastic liner) suitable for food contact.

6.1.1 All containers should be hygienically clean and free of defects prior to filling. Packages intended for repeated fillings must be designed to be washable in order to achieve acceptable cleanliness, and to be effectively refilled.

6.2 Filled carbonated beverages and carbonated waters should be stored and transported in plastic crates or by any other method which protects from breakage or spoilage. Carbonated beverages and carbonated waters shall be stored and transported in a manner to avoid contamination, and which prevents the acquisition of undesirable odors, flavors or tastes. During storage and transport, exposure to heat and direct sunlight should minimized.

7. LABELLING REQUIREMENTS

7.1 All carbonated beverages and carbonated waters shall be clearly labeled and must comply with the requirements as prescribed in PS:1485-1994 (1st Rev.) for Labelling of Pre-packaged foods and PS:4639-2002 for Bottled Drinking Water (2nd Rev.).

7.2 The following shall appear legibly on each package or crown or closure or label.

7.2.1 Name of the product.

7.2.2 Name and address of the manufacturer.

7.2.3 Net volume of the contents in milliliters (ml) or litres.

7.3 If a claim is made about the content of protein, fat, or carbohydrate, then the amounts of these nutrients must be listed in grams per 100 ml. If a claim is made about the content of vitamins or minerals, then the amounts of these vitamins and minerals must be made in items of the appropriate units per 100 ml.

7.4 In the case of beverages containing artificial sweeteners the label must also contain :

7.4.1 The statement "sweetened with [name of non-nutritive sweetener(s)]" placed immediately after the common or usual name of the product, e.g. Low calorie soft drink sweetened with "x".

7.4.2 If aspartame is used the statement "Contains a source of phenylalanine" shall appear immediately after the list of ingredients.

7.4.3 The caloric content of the product as Keal or Kjoules per 100 ml (optional).

7.4.4 Beverages shall be labeled as per the requirements of PS:4449-1999 for Expiration Periods for Food Products.

8. SAMPLING

8.1 A representative number of samples shall be drawn as prescribed in Appendix – B.

9. TESTS

9.1 Tests shall be carried out as prescribed in appendix C and D.

9.2 Quality of Reagents – Unless other specified analytical grade chemicals that do not contain impurities which affect the results of analysis shall be employed in tests, and distilled water shall be used whenever the use of water as a reagent is intended.

APPENDIX A

CARBONATED BEVERAGE INGREDIENTS

APPROVED LIST OF ADDITIVES AND THEIR LIMITS OF USAGE

Non Nutritive Artificial Low Calorie Sweeteners. (4.4)	**GMP**
Acesulfame potassium	350
Aspartame	1000
Saccharin and its Na, K, and Ca salts	100
Sucralose	300

Flavors. (4.5)	GMP
Caffeine	200
Glutamic acid (and its Na, K, Ca, NH_4 and Mg salts)	10,000
Guanylic acid (and its Na, K, and Ca salts)	500
Iosinic acid (and its Na, K, and Ca salts)	500
Quinine (as quinine sulfate)	100
Saponin	80

APPENDIX A continued

CARBONATED BEVERAGE INGREDIENTS

APPROVED LIST OF ADDITIVES AND THEIR LIMITS OF USAGE

Acidulants / Acidity Regulators / Salts. (4.6)	GMP
Citric acid and its Na, K, and Ca and NH_4 salts	GMP
Malic acid and its Na, K, and Ca salts	GMP
Fumaric acid	1000
Tartaric acid and its Na, K, and Ca salts	GMP
Ascorbic acid and its Na and Ca salts	GMP
Succinic acid	30,000
Acetic acid and its Na, K, and Ca salts	GMP
Adipic acid and its Na, and K salts (as adipic acid)	10,000
Phosphoric acid and its mono-di- and tri- Na Ca and K salts (as P_2O_5)	700
Lactic acid (L-, D-, DL-) and its Na, K, and Ca salts	GMP
Gluconic acid its Na, K, and Ca and its salts	GMP
Glucono-delta-lactone	GMP
Carbonates and bicarbonate (Na, K, NH_4, and Mg salts)	GMP
Hydrochlooric acid	GMP
Sulfuric acid	GMP
Chlorides (K, Ca, Mg salts)	GMP
Hydroxides (Na, K, Ca, NH_4 and Mg salts)	GMP
Sulfates (Na, K, Ca salts)	GMP
Preservatives / Sequestrants. (4.7)	GMP
Benzonic acids and its Na, K, Ca salts	1000
Sorbic acid and its K and Ca salts	1000
Dimethyl dicarbonate	250 (residue not detectable in final product)
Sulfur dioxide and Na, Ca, can K sulfites and Na and K metabisulfites (as sulfur dioxide gas)	50
Calcium disodium EDTA	35
Colors. (4.8)	
The following may be used alone or in combination with other colors	**GMP**
Riboflavin and Riboflavin-5-phosphate	GMP
Chlorophylls and chlorophyllins	GMP
Copper complexes of chlorophylls and chlorophyllins	GMP
Caramel Color (Class I, II, III and IV)	GMP
Carotenes	GMP
Paprika extract (capsanthin, capsorubin)	GMP
Beetroot red (betanin)	GMP
Grape skin extract	GMP
Blackcurrant extract	GMP

APPENDIX A continued

CARBONATED BEVERAGE INGREDIENTS

APPROVED LIST OF ADDITIVES AND THEIR LIMITS OF USAGE

Anthocyanins	GMP
Calkcium carbonate	GMP
Titanium dioxide	GMP
Iron oxides and hydroxides	GMP

Colors. (4.8)	
The following may be used in combination with other colors; the total amount of color must not be exceed 100 mg/L in the finished beverage	GMP
Curcumin	100
Tartrazine	100
Quinoline yellow	100
Sunset yellow FCF (Orange yellow S)	50
Cochineal (carminic acid, carmines)	100
Azorubine (carmoisine)	50
Amaranth	35
Annato Extracts	100
Ponceau 4R (cochineal red A)	50
Allura red AC	100
Patent Blue V	100
Indigotine (indigo carmine)	100
Brilliant blue FCF	100
Green S	100
Fast Green	100
Brilliant black BN (Black PN)	100
Brown HT	50
Vegetable carbon	100
Cycopene	100
Beta-apo-8-carotenal (C 30)	100
Ethyl ester of beta-apo-8-carotenic acid (C 30)	100
Lutein (Xanthophylls)	100
Erythrosine, (FD&C Red 3)	100

Emulsifiers / Stabilizers / Clouding Agents / Thickeners. (4.9)	GMP
Aliginic acid and its Na, K, Ca, and NH_4 salts	GMP
Propane-1,2-diol-alginate	300
Agar	GMP
Carageenan	GMP
Locust bean gum	GMP
Guar gum	GMP
Tragacanth gum	GMP

APPENDIX A continued

CARBONATED BEVERAGE INGREDIENTS

APPROVED LIST OF ADDITIVES AND THEIR LIMITS OF USAGE

Acacia gum, gum Arabic	GMP
Xanthan Gum	GMP
Tara gum	GMP
Gellan gum	GMP
Glycerol	GMP
Pectins	GMP
Sucrose acetate isobutyrate (SAIB)	300
Glycerol esters of wood rosin (Ester gum)	150
Cellulose	GMP
Methyl and ethyl cellulose	GMP
Hydroxypropyl and Hydroxpropyl methyl cellulose	GMP
Ethyl methyl cellulose	GMP
Carboxymethyl celloulose (and sodium salt)	GMP
Fatty acids and Na, K, Ca, and Mg salts	GMP
Mono- and diglycerides of fatty acvids	GMP
Acetic acid esters, lactic acid esters, citric acid esters, tartaric acid esters, mono and diactyl tartaric acid esters, mixed acetic and tartaric acid esters of mono-and diglycerides of fatty	GMP
Quillaia extract (as anhydrous extract)	200
Modified food starches	GMP
Starch sodium octenyl succinate	GMP
Lecithin	GMP
Polydextrose	GMP
Polysorbate –20, -40, -60, -65, -80	50
Sodium diphosphates (as P205)	2000
Potassium diphosphates (as P205)	2000
Potassium triphosphates (as P205)	500
Sdoium polyphosphate (as P205)	700
Potassium polyphosphate (as P205)	500
Sucroesters of fatty acids	1000
Sucrogycerides	200
Sodium dioctyl sulfosuccinate (DOSS)	10
Calcium stearoyl lactylate	2000
Sorbitan esters of lauric, oleic, palmitic, stearic acids	50
Polyvinylrrolidone	GMP
Antifoaming agents. (4.11)	GMP
Refined vegetable oil such as corn oil	GMP
Silicone (food grade)	GMP
Dimethyl polysiloxane	10

APPENDIX A continued

CARBONATED BEVERAGE INGREDIENTS

APPROVED LIST OF ADDITIVES AND THEIR LIMITS OF USAGE

Carriers and solvents. (4.13)	GMP
Isopropyl alcohol	GMP
Propylene glycol	GMP
Polyethylene glycol	GMP
Maltodextrin	GMP
Antioxidants. (4.14)	GMP
Ascorbic acid and its Na and Ca salts	GMP
Fatty acids of ascorbic acid	GMP
Citric acid and its Na, K, and Ca salts	GMP
Erythoribic acid and sodium erythorbate	GMP
Butylated Hydroxyanisole (BHA) (in essential oils)	200 (aone or in combination with BHT)
Butylated Hydroxytoluene (BHT) (in essential oils)	200 (alone or in combination with BHA)
Rasin qualac	500
Sodium hexametaphosphate	GMP
Tocopherols (alpha, gamma, delta) and tocopherol- rich extracts	GMP
TBHQ	200
Propyl, ethyl, octyl and dodecyl gallate (in essential oils)	100 (total gallates)

APPENDIX B

SAMPLING OF CARBONATED BEVERAGES

B.1 SCALE OF SAMPLING

B.1.1 All beverage containers in a consignment belonging to the same batch of manufacture shall constitute a lot. If the consignment is declared to consist of different batches of manufacture, beverage containers of the same batch shall be grouped together and each group so formed shall constitute a separate lot.

B-1.2 The selection of samples to ascertain conformance with the requirements of this standard shall follow a random sampling procedure, which ensures that every beverage container in a lot has an equal chance of being selected regardless of its quality.

B.1.3 The number of samples to be selected for assessing conformance to requirement 5.2 and 5.5 shall be 24.

B.1.4 The number of samples to be selected for assessing conformance to the requirements of 5.4 shall be 24.

B.1.5 The numnber of samples to be selected for assessing conformance to the requirements of carbonation, and brix content Hydrometer/refrectometer (Appendix-C).

B.2 TEST SAMPLES AND REFEREE SAMPLES

B-2.1 The Samples for Requirement 5.2 and 5.5, shall be divided at random into three equal sets and labeled for Taste, Odor & Appearance and mineral analysis and the particulars of sampling. One of these sets of sample bottles shall be for the purchaser, another for the vendor rand the third for the referee. Referee samples shall bear the seals of the purchaser and the vendor (or their representatives) and shall be kept at a place agreed to between the two.

B-2.2 Sample for Requirement 5.4 Microbiology, shall be divided at random into three equal sets and labeled with all the particulars of sampling and the intended testing according to B 2.2.1. One of these sets of sample bottles shall be for the purchaser, another for the vendor and the third for the referee. Referee samples shall bear the seals of the purchaser and the vendor (or their representatives) and shall be kept at a place agreed to between the two.

B.2.2.1 2 samples for Pathogenic microorganism tests
2 samples for Coliform tests
4 samples for Total Bacteria and Total Yeast & Mold

B.2.3 Samples selected as in B.1.5, shall be divided at random into three equal sets and labeled with all the particulars of sampling and the intended testing.

APPENDIX C

TESTING OF CARBONATED BEVERAGES

C.1. TESTING OF SAMPLES

C.1.1 Test for 5.2 & 5.5 Requirements. – 4 samples obtained as in B.2.1. are inspected for taste odor and appearance and the other 4 samples are assessed for mineral content. Anyu non-conformance should be confirmed by a repeat sampling, prior to final disposition.

C.1.2 Test for 5.4 Requirements. – Samples bottles obtained as in B-2.2.1 shall be tested for Pathogens, Total Coliforms and Total Bacteria and Total Yeast and Mold. Any non-conformance should be confirmed by a repeal sampling, prior to final disposition.

C.1.3 Test for the other requirements of carbonation and brix content --- Samples are tested for Carbon dioxide and Brix. Criteria of Conformity are determined by Single sampling plan for Normal Inspection (MIL-STD-105E) at an Acceptable Quality Level of 0.1.

The Table below is a copy of a sample size code letter table from MIL-STD-105E. The first column lists lot size ranges. The next seven columns list sample size code letters for each of the inspection levels. As the letters go from A to R, the sample sizes increase. The actual size of the sample will depend on the specific sampling plan one wishes to use.

	Special Inspection levels				**General inspection level**		
Lot or batch size	**S-1**	**S-2**	**S-3**	**S-4**	**I**	**II**	**III**
35,001-150,000	D	E	G	J	L	N	P
150,001-500,000	D	E	G	K	M	P	Q
500,001 and over	D	E	H	K	N	Q	R

Sample Size Code Letters (Table 1 in MIL-STD-105E)

APPENDIX D (Continued)

TEST METHOD OF NON-ALCOHOLIC CARBONATED BEVERAGES DETERMINATION OF CARBON DIOXIDE CONTENT

3. AOAC AND ASTM METHOD FOR REQUIREMENT 5.5

List of AOAC and ASTM methods recognized by JECFA/WHO are as under :

i. AOAC Chapt.9 Metals and Other Elements at Trace Levels in Foods.
ii. AOAC Chapt.11 Waters; and Salt(s).
iii. ASTM D 858-90 Standard Test Methods for Manganese.
iv. ASTM D 3223-86 Standard Test Method for Total Mercury.
v. ASTM D3557-90 Standard Test Methods for Cadmium.
vi. ASTM D 511-88 Standard Test Methods for Calcium and Magnesium.
vii. ASTM D 1691-90 Standard Test Methods for Zinc.
viii. ASTM D 1687-86 Standard Test Methods for Chromium.
ix. ASTM D 4382-84 (R.88) Standard Test Method for Barium, AAS Graphite Furnace.
x. ASTM D2972-88 Standard Test Methods for Arsenic.
xi. ASTM D 1068-90 Standard Test Method for Iron.
xii. ASTM D 3559-85 Standard Test Methods for Lead.
xiii. ASTM D 3859-88 Standard Test Methods for Selenium.
xiv. ASTM D 2036-89 Standard Test Methods for Cyanides
xv. ASTM D 1179-88 Standard Test Methods for Fluoride Ion.
xvi. ASTM D 1688-90 Standard Test Method for Copper.

APPENDIX – E

MICROBIOLOGICAL TEST METHODS – GENERAL GUIDANCE FOR ENUMERATION OF MICRO-ORGANISMS COLONY COUNT TECHNIQUE AT 30 ^{O}C.

1. SCOPE

1.1 This standard gives general guidelines for enumeration of micro-organisms present in products intended for human consumption of feeding of an imals, by counting the colonies growing on a solid medium after incubating aerobically at 30 ^{o}C.

2. DEFINITIONS

For the purpose of this standard, the following definition shall apply :

Micro-organisms : Organisms growing aerobically at 30 ^{o}C under the conditions specified.

3. SAMPLING

Sampling shall be carried out in conformity with the relevant Pakistan Standard for the product concerned and PS:3946-1997 for Code of Practice for Handling of Food Samples for Microbiological Analysis.

4. APPARATUS AND GLASWARE

usual laboratory equipment and in particular ;

4.1 Instruments for preparation of samples, sterile.
4.2 Balanace, capacity 2500 g. accuracy 0.1 g.
4.3 Apparatus for homogenization.

4.3.1 Rotary blender, operating at not less than 8000 r.p.m. and not more than 45,000 r.p.m. with glass or metal jars fitted with lids and resistant to the conditions of sterilization. Alternatively a stomacher laboratory blender with sterile bags may be used.

4.3.2 Mixer, capable of mixing 1 ml or 2 ml of the sample or of a dilution, in a tube of adequate dimensions with 9 ml or 18 ml of diluent, to obtained a homogeneous suspension and working on the principle of eccentric rotation of the contents of the test tube (Vortex mixer).

4.4 Apparatus for dry sterilization (oven) or wet sterilization (autoclave).

Other thanb the equipment which is supplied sterile, particularly that made of plastic material, glassware shall be sterilized either

- in an oven at 170 ^{o}C to 175 ^{o}C for not less than 1 hour or
- in an autoclave at 121 ± 1 ^{o}C for not less than 20 minutes.

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4.5 Water bath, controlled at 45 ± 0.5 ^{o}C.

4.6 Incubator, controlled at 30 ± 1 ^{o}C.

4.7 Colony counting equipment, consisting of an illuminated base with a dark background and a mechanical or electronic digital counter.

4.8 Tally register.

4.9 Petri dishes, glass or plastic, of diameter 90 mm to 100 mm.

4.10 Total delivery pipettes.

4.10.1 of nominal capacity 1 ml and having an autlet diameter of 2 mm to 3 mm.

4.10.2 Of nominal capacity 10 ml graduated in 1 ml divisions having an outlet diamter of 2 mm to 3 mm.

4.11 Dropping pipettes, that will deliver approximately 50 drops/ml.

Can be prepared from a fine Pasteur pipette by nicking and then breaking the capillary so that the end has an external diamter of 1.0 mm (stubbs wire gauge No.57).

4.12 pH meter, accurate to ± 0.1 pH unit at 25 ^{o}C.

4.13 Test tubes, 18 mm x 180 mm, or flasks or bottles of suitable capacity.

5. CULTURE MEDIA AND DILUTENT

In order to improve the reproducibility of the results, it is recommended that, for the preparation of culture media, dehydrated basic components or complete dehydrated media be used, the manufacutrer's instructions shall be rigorously followed.

The chemicals used shall be of analytical quality.

The water used shall be distilled or deionized and shall be free of substances which would inhibit the growth of micro-organisms under the test conditions.

If the media and diluent are not used immediately, they shall be kept in dark at a temperature between 0 ^{o}C to ± 5 ^{o}C for not longer than one month in conditions that prevent any change in their composition.

5.1 Diluent

Composition

Peptone	1.0 g
Sodium chloride	8.5 g
Water	1000 ml

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Preparation

Dissolve the components in the wate,r by heating if necessary. Adjust the pH so that after sterilization it is 7.0 at 25 ^{o}C.

Dispense the medium into test tubes in quantities of 4 ml per tube, or into 150 ml flasks or bottles, in quantities of 100 ml per container.

Sterilize in an autoclave at 121 ± 1 ^{o}C for 20 minutes

1. This term is only used at present by certain producers of media. Any other casein digest giving comparable results may be used.
2. According to the gel strength of agar.

5.2 Milk plate count agar medium.

This medium shall be used when testing for the microbiological quality of milk and milk products.

Composition

Tryptone	5.0 g
Yeast extract	2.5 g
Anhydrous D-glucose	1.0 g
Skimmed milk powder (see Note)	1.0 g
Agar	12 g to 18 g
Water	1000 ml

NOTE :- Skimmed milk powder shall be free from inhibitory substances. This should be proved by comparative tests using skimmed milk powder known to be free from such substances.

Any standardized brand of readily soluble spray dried skimmed milk powder free from antibiotics may also be used.

Preparation

Dissolve and disperse the components in the water adding the skimmed milk powder last. Heating water will assist the procedure.

Add agar and heat to boil or steam for about 30 minutes, stiring frequently until the agar is completely dissolved. Filter if necessary.

If necessary, adjust the pH so that after sterilization it is 7.0 ± 0.2 at 25 ^{o}C.

Dispense the medium in to test tubes in quantities of 12 ml to 15 ml per tube or into flasks or bottles in quantities of 100 ml to 150 ml.

Sterilize in an autoclave at 121 ± 1 ^{o}C for 15 minutes.

6. PREPARATION OF THE TEST SAMPLE AND INITIAL SUSPENSION.

See the Pakistan Standard appropriate to the product concerned for preparation of the sample.

6.1 Liquid samples

For non-viscous liquid samples (water, milk, soft drinks etc.), shake the sample manually performing 25 up-and-down movements of amplitude about 30 cm in 7 seconds or use a mechanical device to ensure uniform distribution of micro-organisms.

Add 1 ml of this test sample to 9 ml of diluent avoiding contact between the pipette and the diluent to make up 10^{-1} dilution. Carefully mix the test portion and diluent by aspirating ten times with a fresh pipette or in a mechanical mixer for 5 seconds to 10 seconds.

6.2 Other samples

In the sample is frozen and must be thawed, hold in a refrigerator at 2 ^{0}C to 5 ^{o}C for not more than 18 hours prior to analysis. If the frozen sample can be easily comminuted (for example ice cream) proceed without thawing.

Weigh 10 ± 0.1 g of regular or thawed food sample aseptically into a sterile blender jar or plastic bag in the case of a stomacher. Add 90 ml of diluent (5.1) to make up to 10^{-1} dilution.

Operator the rotary blender for a sufficient time to give a total number of 15 000 to 20 000 revolutions. Even with the slowest blender this time shall not exceed 2 ½minutes. Operate the stomacher for 1 minute to 2 minutes according to ther nature of the product (see Note).

Allow the large particles to settle, if necessary up to 15 minutes. Transfer a certain quantity from the top layer to a culture tube, flask or bottle using a 10 ml pipette (4.10). If there is a fat layer, take the sample from the aqueous part.

NOTE :- Attention is drawn to the fact that for certain products, in particular cereals, the time given are not appropriate for microorganisms such as yeasts and moulds. In this case, stomacher allows greater recovery rates than the rotary blender. Operate the stomacher for 10 minutes and avoid separation, as some veasts and moulds could be lost from the supernant liquid.

7. PREPARATION OF FURTHER DECIMAL DILUTIONS

Transfer 1 ml of the initial suspension (10^{-1} dilution) into a tube containing 9 ml of the sterile diluent. Mix well either by shaking 25 times in a 30 cm are or in a mechanical mixer to obtain 10^{-2} dilution.

If necessary repoeat the operations given above using the 10^{-2} and further dilutions to obtain as many dilutions as are necessary to produce acceptable counts (see 9) at two successive dilutions.

In general, dilutions should be prepared from the test sample immediately prior to the analysis; they should be used to inoculate culture media within 30 minutes of preparation.

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8. PROCEDURE

8.1 Pour plate method (Aerobic plate count) – Reference method -

8.1.1 Take two sterile petri dishes (4.9). Using a sterile pipette (4.10) transfer to each dish 1 ml of the test sample. If the product is liquid, or 1 ml of the initial suspension in the case of other products.

8.1.2 Take two other sterile petri dishes. Using a fresh sterile pipette, transfer to each dish 1 ml of the first decimal dilution (10^{-1}) if the product is liquid, or 1 ml of the first decimal dilution (10^{-2}) of the initial suspension in the case of other products.

Repeat the procedure with the other dilutions, using a fresh sterile pipette for each dilution.

8.1.3 Pour about 15 ml of the plate count medium (5.2) at 45 ± 0.5 ^{o}C into each petri dish. The time elapsing between the end of the preparation of initial suspension (or of the 10^{-1} dilution if the product is liquid) and the moment when the medium (5.2) is poured into the dishes shall not exceed 30 minutes.

Carefully mix the inoculum with the medium and allow to solidify placing the petri dishes on a clean horizontal surfance.

8.1.4 After complete solidification, and only in the case where it is suspected that the product being analysed contains micro-organisms whose colonies will over grow the surfance of the medium pour about 4 ml of water agar medium (5.3) at 45 ± 0.5 ^{o}C into the surface of the inoculated medium. Allow to solidify.

This operation, if carried out, shall be mentioned in the test report.

8.1.5 Invert the dishes and incubate at 30 ± 1 ^{o}C for 72 ± 3 hours.

8.2 Drop plate method (modified Miles and Misra) – Routine method -

This method cannot be used for counts less than 3000 per gram.

8.2.1 Prepare plates of plate count medium by pouring 15 ml of the molten agar medium at 45 ± 0.5 ^{o}C into each of the required number of plates. Allow to set and then dry the plates inverted at 37 ^{o}C for 16 hours or open and inverted at 35 ^{o}C for 30 minutes.

8.2.2 Divide the bottom of the plates into 3 equal segments and indicate for each segment the dilution to be used.

8.2.3 Prepare decimal dilutions of the sample as in 6 and 7.

8.2.4 Using the dropping pipette (4.11) calibrated as in the Note, deliver two separate drops (.0.2 ml each) of each dilution starting from the highest dilution, on the surface of the relevant segment. Thus whith only two plates six dilutions can be plated in duplicate.

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8.2.5 Allow the drops to dry at room temperature for about 15 minutes.

8.2.6 Incubate the plates inverted at 30 ± 1 ^{o}C for 72 ± 3 hours.

NOTE :- To calibrate the dropping pipette, determine the number of drops/ml deliverdd by the pipettes as follows :

a) Draw diluent into pipette with the aid of a rubber teat;
b) Keepint pipette vertically discharge the diluent into a 10-ml measuring cylinder at a rate of 30 drops/minute. Count the number of drops required to fill the cylinder to the 3 ml graduation line;
c) Calculate the number of drops/ml at a dropping rate of 30 drops per minute.

9. COUNTING OF THE COLONIES

9.1 Pour plate method

After the specified period of incubation, count the colonies in each dish containing nopt more than 300 colonies using the colony counting equipment (4.7).

Sreject any plate in which more than half of the surface is overgrown. Otherwise count the colonies in half of the plate that is clear and multiply by two.

9.2 Drop plate method

After the specified period of incubation, count the colonies in drop areas containing the greatest number of colonies without confluence.

10. CALCULATION AND EXPRESSION OF RESULTS

10.1 Pour plate method.

10.1.1 Between 15 to 3000 colonies – general case

Retain dishes containing less than 300 colonies at two consecutive dilution. It is necessary that one of these dishes contains at least 15 colonies.

Calculate the number oif micro-organisms (N) per milliliter or per gram of the product, using the following equation.

$$N = \frac{C}{(n_1 + 0.1\, n_2)^d}$$

C is the sum of colonies counted on all the dishes retained;
n_1 is the number of dishes relained in the first dilution;
n_2 is the number of dishes retained in the second dilution; and
d is the dilution factor corresponding to the first dilution.

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Round the result caculated to two significant figures. When the number to be rounded is 5 with no further significant figures, round the number to give an even figure immediately to the left. For example 28,500 is rounded to 28,000; 11,500 is rounded to 12,000.

Report the result as the number of micro-organisms per milliliter or per gram of product, expressed as a number between 1.0 and 9.9 multiplied by 10^x. where x is the appropriate power of 10.

Example :-

A micro-organisms count at 30 ^oC gave the following results :

- at the first dilution retained (10^{-2}); 168 and 215 colonies
- at the second dilution retained (10^{-3}); 14 and 25 colonies.

$$N = \frac{C}{(n_1 + 0.1\, n_2)^d} = \frac{168 + 215 + 14 + 25}{(2 + (0.1 \times 2) \times 10^2} = \frac{422}{0.022} = 19182$$

Rounding the result as specified above gives 19 000 or 1.0×10^x micro-organisms per milliliter or per gram of product.

NOTE :- The method of calculation using a weighted mean value improves the precision of the result compared with calculating from a single dilution.

10.1.2 No colonies

If the two dishes corresponding to the test sample (liquid products) or the initial suspension (other products) contain no colonies, report the result as follows :

- less than 1 micro-organisms per milliliter (liquid products)
- less than 1 x 1/d micro-organisms per gram (other products).
  Where d is the dilution factor of the initial suspension.

10.1.3 less than 15 colonies

If the two dishes, corresponding to the test sample (liquid products) or the initial suspension (other products), contain less than 15 colonies, calculate the arithmetic mean 'm' of the colonies counted on both dishes.

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Report the result as follows :

- estimated number of micro-organisms per milliliter liquid products = m
- estimated number of micro-organisms per gram:
  other products = m x 1/d.

where d is the dilution factor of the initial suspension.

10.1.4 more than 300 colonies.

If no plate produces less than 300 colonies, calculate the result from the plate with lowest number of colonies. Report the result as the estimated number of micro-organisms per gram or milliliter.

10.2 Drop plate method

Calculate the colony count per milliliter or per gram from the arithmetic mean colony count per drop of the suspension by multiplying it by 50 times the dilution factor.

11. TEST REPORT

The test report shall give all the information necessary for the identification of the sample, a reference to the method used, the results and the form in which they are expressed.

The test report shall , in addition, mention any particular phenomena observed during the analysis and any operations not specified in the method or considered as optional. For example addition of water agar medium, which may have effected the results.

APPENDIX – F

MICROBIOLOGICAL TEST METHODS

ENUMERATION OF YEASTS AND MOULDS

1. SCOPE

1.1 This standard gives general guidelines for enumeration of viable yeasts and moulds in productrs intended for human consumption or feeding of animals, by means of the colony count technique at 25 ^{o}C.

NOTE :- Owing to the nature of yeasts and moulds, the enumeration is subject to certain imprecisions.

2. DEFINITIONS

For the purpose of this standard, the following definition shall apply :

Yeasts and Moulds : Micro-organisms which at 25 ^{o}C form colonies in a selective medium under

the conditions specified.

3. SAMPLING

Sampling shall be carried out in conformity with the relevant Pakistan Standard for the product concerned and PS:3946-1997*

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*Code of Practice for Handling of Food Samples for Microbiological Analysis.

4. APPARATUS AND GLASSWARE

Usual laboratory equipment and in particular :

4.1 Instruments for preparation of sample, sterile.
4.2 Balance, capacity 2500 g accuracy 0.1 g.
4.3 Apparatus for homogenization.

4.3.1 Rotary blender, operating at not less than 8000 r.p.m. and not more than 45.000 r.p.m. with glass or metal jars fitted with lids and stomacher laboratory blender with sterile bags may be used.

4.3.2 Mixer, capable of mixing 1 ml or 2 ml of the sample or of a dilution, in a tube of adequate dimensions with 9 ml or 18 ml of diluent, to obtain a homogeneous suspension and working on the principle of eccentric rotation of the contents of the test tube (Vortex mixer).

4.4 Apparatus for dry sterilization (oven) or wet sterilization (autoclave).
- in an oven at 170 ^{o}C to 175 ^{o}C for not less than 1 hour or
- in an autoclave at 121 ± 1 ^{o}C for not less than 20 minutes.

4.5 Water bath, controlled at 45 ± 0.5 ^{o}C

4.6 Incubator, controlled at 25 ± 1 ^{o}C.

4.7 Colony counting equipment, consisting of an illuminated base with a dark background and a mechanical or electronic digital counter.

4.8 Tally register

4.9 Petri dishes, glass or plastic, of diameter 90 mm to 100 mm.

4.10 Graduated pipettes, having nominal capacities of 10 ml and 1 ml, graduated in 0.5 ml and 0.1 ml divisions respectively, with an outlet diameter of 2 mm to 3 mm.

4.11 PH meter, accurate to ± 0.1 pH unit at 25 ^{o}C.

4.12 Culture bottles or flasks, with non-toxic metal screw-caps.

5. CULTURE MEDIA AND DILUENT

In order to improve the reproducibility of the results, it is recommended that, for the preparation of culture media, dehydrated basic components or complete dehydrated media be used. The manufacturer's instructions shall be rigorously followed.

The chemicals used shall be of analytical quality.

The water used shall be distilled or deionized and shall be free of substances which would inhibit the growth of micro-organisms under the test conditions.

If the media and diluent are not used immediately, they shall be kept in dark at a temperature between 0 ^{o}C to ± 5^{o}C for not longer than one month in conditions that prevent any change in their composition.

5.1 Diluent

Composition

Peptone	1.0 g
Sodium chloride	8.5 g
Water	1000 ml

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Dissolve the components in the water, by heating if necessary. Adjust the pH so that after sterilization it is 7.0 at 25 ^{o}C.

Dispense the diluent into test tubes, flasks or bottles of appropriate capacities in quantities such that after sterilization each test tube contains 9 ml of diluent or multiple of 9 ml and each flask/bottle contains 90 ml of diluent. Stopper the tubes, flasks or bottles.

Sterilize in an autoclave at 121 ± 1 ^{o}C for 20 minutes.

5.2 YEAST EXTRACT – DEXTROSE – CHLORAMPHENICOL AGAR MEDIUM

Composition

Yeast extract	5	g
Dextorse ($C_6H_{12}O_6$)	20	g
Chloramphenicol 1	0.1 g	g
Agar 2	12 g to 15	g
Water	1000	ml

Preparation

Dissolve the components in water by boiling. If necessary, adjust the pH sop that after ster8ilization it is 6.6.

Dispense the medium in to culture bottles or flasks.

Sterilize at 121 ± 1 ^{o}C for 15 minutes.

6. PREPARATION OF THE TEST SAMPLE, INITIAL SUSPENSION & FURTHER DECIMAL DILUTIONS :

Proceed as given in Appendix-E of this standard.

7. PROCEDURE

7.1 Take two sterile petri dishes (4.9). Using a sterile pipette (4.10) transfer to each dish 1 ml of the test sample, if the product is liquid, or of the initial suspension in the case of other products.

7.2 Take two other sterile petri dishes. Using a fresh sterile pipette, transfer to each dish 1 ml of the first dicimal dilution (10^{-1}) if the product is liquid, or 1 ml of the first dilution (10^{-2}) of the initial suspension in the case of other products.

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1. Final concentration of chloramphenicol 100 ug/ml of the medium is obtained. Chloramphenicol ($C_{11}H_{12}C1_2N_2O_5$) may be replaced by oxytetracycline ($C_{22}H_{30}N_2O_5$). In this case, prepare the basic medium as described omitting chloramphenicol, dispense in quantities of 100 ml and sterilize. Prepare 0.1 per cent (m/m) solution of oxytetracycline hydrochloride in water and sterilize by filteration. Just prior to use, add 10 ml of this olution aseptically to 100 ml of the basic medium previously melted and maintained at 45 ^{o}C.

2. According to the gel strength of agar.

Repeat the procedure with other dilutions using a fresh st5erile pipette for each dilution.

7.3 Pour about 15 ml of the yeast extract-dextrose-chloramphenicol agar medium (5.2) previously melted and maintained at 45 ± 0.5 ^{o}C into each petri dish. The time elapsing between the end of the preparation of initial suspension (or 10^{-1} dilution if liquid) and the moment when the medium is poured into the dishes shall not exceed 30 minutes.

Carefully mix the inoculum with the medium and allow to solidify placing the petri dishes on a clean horizontal surface.

Prepare a control plate, with 15 ml of the medium, to check its sterility.

7.4 Invert the dishes and incubate at 25 ± 1 ^{o}C.

8. counting of the colonies.

8.1 Count the yeasts and moulds colonies on each plate after 3.4 and 5 days of incubation. After 5 days retain the plates containing not more than 150 colonies.

8.2 If counts of each are required, count the yeast and mould colonies separately.

8.3 Investigate the identity of any doubtful or pin point colonies by microscopic examination, in order to distinguish according to their morphology, the colonies of yeasts and moulds from colonies of bacteria.

8.4 If parts of the plates are overgrown with moulds, or if it is difficult to count well isolated colonies, retain the counts obtained after 4 or even 3 days of incubation.

In this event, record the incubation period in the test report.

9. CALCULATION AND EXPRESSION OF RESULTS

9.1 Less than 15 colonies.

Use counts from the plates containing less than 150 colonies at two consecutive dilutions. Calculate the number of yeasts and moulds (N) per milliliter or per gram of the product, using the following equation :

$$N = \frac{C}{(n_1 + 0.1\, n_2)\, d}$$

Where,

c is the sum of colonies counted on all plates:
n_1 is the number of plates retained in the first dilution:
n_2 is the number of plates retained in the second dilution: and
d is the dilution factor corresponding to the first dilution.

Round the result calculated to two significant figures. When the number to be rounded is 5 with no further significant figures, round the number to give an even figure immediately to the left. For example 28.500 is rounded to 28.000: 11.500 is rounded to 12.000.

Report the result as the estimated number of yeasts and moulds per gram or milliliter of the product as a number between 1.0 and 9.9 multiplied by 10^x where x is the appropriate powder of 10.

Express the results either as the total number of yeasts and moulds or if counts of each are required, as yeasts or moulds separately.

Example

A yeast and mould count gave the following results :

10^{-2} dilution : 83 and 97 colonies.
10^{-3} dilution : 33 and 28 colonies.

$$N = \frac{C}{(n_1 \pm 0.1\, n_2)\, d} = \frac{83 + 97 + 33 + 28}{(2 + (0.1 \times 2) \times 10^{-2}} = \frac{241}{0.22} = 10954$$

Rounding the result as specified above gives 11.000.

The estimated number of yeasts and moulds per gram or per milliliter is therefore 1.1×10^{-4}.

9.2 No colonies

9.2.1 If there were no colonies on plates from the initial suspension, (if the product is a solid) the number of yeasts and moulds per gram should be reported as 'less than 10'.

9.2.2 If there were no colonies on plates from the test sample in the case of liquid products, the number of yeats and moulds per milliliter should be reported as 'less than one'.

9.3 More than 150 colonies.

If there are only dishes containing more than 150 colonies, calculate an estimated count from dishes having a count nearest to 150 colonies and multiply this number by the reciprocal of the value corresponding to the highest dilution.

10. TEST REPORT

The test report shall give all the information necessary for the identification of the sample and a reference to the method used, the results and the form in which they are expressed.

The test report shall, in addition, mention any particular phenomena observed during the analysis, and any operations not specified in the method which may have affected the results.

In the event of results relating to incubation period of 3 or 4 days are used, record the incubation period in the test report.

APPENDIX – G

TITLE :	**RESEARCH AND COUNT OF TOTAL YEASTS & MOULDS**

STEPS	**PROCEDURE**	**CRITICAL CONTROL POINTS**
1-Sample	Non-packed samples are taken with sterile recipients (see ch.4 section 1)	Make sure this is done in sterile zone within the heat of blow lamp.
2-Method	Clean the recipient in modified alcohol.	Handle the sample with care so as to avoid all risk of contamination .
3-Filteration	See ch.5 sub. Paragraph 1. Filter a volume of the sample (250 ml) onto a sterile membrance. Place the membrance in the petridish which contains the OGY medium.	Check that no air bubbles are trapped between the membrane and the gelose.
4-Incubation	Invert the plates on a tray and pout them in an incubator set at 20-25 ^{o}C for 5 days.	Do not stack more than 6 plates. Ensure correct incubation temperature
5 Interpretation/count	a)**the yeasts:** Count all the developed typical colonies which have a mat or brilliant aspect a creamy white pigmentation or pinkish-red with a neal or irregular outer edge and either concave or convex. b)**the moulds:** Count all the developed typical colonies that have a downy aspect and which are colored from black to white.	

Reagents and / or mediums

Mediums	**Sanofi**	**Merck**	**Oxoid**
OGA base medium			CM545
Oxetetracycline solution			SR73