International Journal of Scientific Research in Engineering and Management (IJSREM)
Volume: 04 Issue: 05 | May -2020 ISSN: 2582-3930

PREPARATION OF STOCK BACTERIAL SUSPENSIONS AND THEIR STANDARDIZATION

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ABSTRACT:

Stock Cultures of different Bacteria are basic prerequisite for any type of microbiological routine activities, especially for industrial & research operations. Industry, like – Pharma, Food, FMCG follow different regulatory standards, like – United States Pharmacopeia (USP), British Pharmacopoeia (BP), Indian Pharmacopoeia (IP), Bureau of Indian Standards (BIS) etc. where 10-100 Colony Forming Units (CFU)stock cultures are required in Growth Promotion Tests (GPT) of different media as well as for preparingthe Positive Controls. This study provides the easiest way to prepare those 10-100 CFU stock suspensions of different Bacterium, like - *Escherichia coli, Staphylococcus aureusand Salmonella abony* to use in routine microbiological activities based on theiroptical density characteristics. This study helps to reduce the microbiological incubation timeto get standardized 10-100 CFU stock cultures every time, which directly benefits in time reduction & indirectly helps in cost reduction tasks.

Key Words: 10-100 CFU, Bacteria,cultures, regulatory standards, Pharma, FMCG, GPT, etc.

INTRODUCTION:

For doing any microbiological analysis, like - Total Bacterial Count (TBC) or Total Viable Count (TVC) or Microbial Limit Testing (MLT) in any professional industry or research laboratory, a positive control plate is prepared, where stock cultures of different Gram Positive & Gram Negative Bacteria are used in the concentration of 10-100 CFU. It is mandatory to use as per regulatory standards, like – USP[1], BP[2], IP[3] or BIS.Other than microbiological analysis & positive plating, for GPT of media[1], that 10-100 CFU cultures are essentials. General process for preparing those stock cultures are time consuming & tedious task, because to prepare & maintain those cultures, 3 to 5 days incubation period of inoculated cultures with several serial dilutions are required every time. Depending on the size of the Organization or Laboratory & their volume of work, sometimes, it has been found that, one or more dedicated microbiologists are required to maintain those stock cultures.

To reduce such tedious work & time for preparation of such stock cultures to use in regular microbiological testing, a simple & effective method for preparing and maintaining 10-100 CFU cultures has been establish through this study. In this study, following four activities have been performed –

1. Preparation of Bacterial pure cultures.
2. Preparation of Bacterial suspensions for different Gram Positive & Gram Negative Bacteria (*Escherichia coli, Staphylococcus aureusand Salmonella abony*) through traditional microbial serial dilution &culturing method for single time.
3. Standardization for getting 10-100 CFU/ml culture through spectrophotometric (optical density) characteristics of the same (bacterial suspensions, outcome of point no. 2) in subsequent stages, so that repetitive sub-culturing can be avoided& time can be saved.
4. Validation of the Study.

This study helps in establishing a simple ‘get ready & use theory” before starting any microbiological exercise with positive controls by using Bacterial suspensions.

MATERIALS:

- Soybean-Casein Digest Agar (SCDA) HiMedia; Material Code: GMH290
- EMB Agar (Eosin Methylene Blue Agar) HiMedia; Material Code: M317
- MS Agar (Mannitol Salt Agar) HiMedia; Material Code: M118
- Xylose-Lysine Deoxycholate Agar (XLD Agar) HiMedia; Material Code: M031
- Bacterial Cultures:
  - *Escherichia coli* (ATCC 8739)
  - *Staphylococcus aureus* (ATCC 6538)
  - *Salmonella abony* (ATCC 6017)
- 0.9% Saline Water
- Petri Plates (HiMedia 110mm Sterile Petri Plates)
- AutoclavableFalcon Tubes (HiMedia Polypropylene Sterile Falcone Tube – 50ml)
- Autoclavable Micro Tips (Tarsons– 1000µl)
- Micropipette (Mettler Toledo; 100-1000µl)
- Autoclave (Equitron Medica)
- Incubator - Temp: 35±2.5°C (Remi Sales & Engineering Ltd)
- Laminar Air Flow (Klenzaids)
- Inoculating Loop
- Spirit Lamp
- Vortex Machine
- Spectrophotometer (Shimadzu UV-1800)
- Refrigerator (Godrej 185L)

METHOD:

Step-1: Preparation of Pure Cultures:

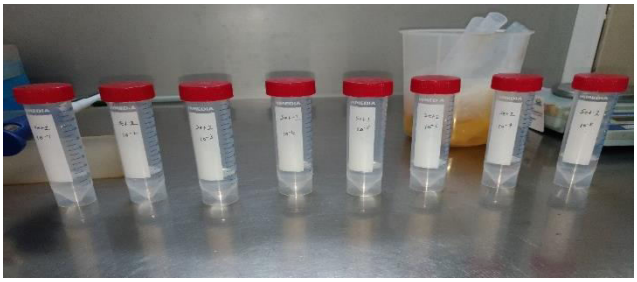
1. From respective subcultures, pure culture for all Bacterium (*Escherichia coli, Staphylococcus aureus, Salmonella abony)*are made using SCDA media plates.
2. To confirm the purity of the cultures, inoculation done over selective media plates as per following matrix & incubated at 35±2.5°C for 24 hours –

Organism	Media	Expected Colony Characteristics
E. coli	EMB Agar	Brick-red colonies
S. aureus	MS Agar	Yellow colonies
S. abony	XLD Agar	Red colonies with or without black center

Step-2: Preparation of Bacterial Suspensions:

From respective subcultures, Bacterial Suspensions are prepared as follows –

1. From respective selective media plates, 2-3 loop-full pure cultures are inoculated in 10 ml 0.9% Saline Water containing falcon tubes. Those tubes shall be treated as 'mother suspensions'.
2. From the 'mother suspensions', now several serial dilutions (10^{-1}-10^{-8}) of respective cultures are prepared & properly labelled.



3. Form each & every dilutions; TBC are performed in SCDA media plates (Pour Plate Method[1,] inoculum volume = 1ml)for all three organisms to identify the concentration of the suspensions in CFU/ml practice.

Step-3: Standardization of Bacterial Suspensions:

All the prepared suspensions (10^{-1}-10^{-8}) of respective cultures are kept in refrigerator (around 4°C) until incubation period of SCDA media plates over. As the suspensions are prepared with only 0.9% Saline Water, no further growth or enrichment of cultures happen in suspension form. Hence, colonies formed in SCDA plates represent (CFU/ml) exact number of organisms present in suspensions.

Now, the suspension tubes, containing 10-100 CFU/ml cultures (for respective organisms) are used to check the OD at λ_{max}. That OD values for respective organisms represent the maximum probability for availability of 10-100 CFU/ml organisms in that suspensions. To prove this method's validity, cross verification or reverse

verification for this relation (CFU vs OD)are also donewith all three organisms.

Step-4: Validation - Verification of OD vs CFU:

In that case, Bacterial suspensions are prepared from 'mother suspension' with 0.9% Saline Water in the OD range, established for 10-100 CFU/ml cultures (Step-3). Then, TBC are performed in SCDA media plates (Pour Plate Method[1,] inoculum volume = 1ml)with that diluted suspensions& are checked for actual Bacterial counts (in CFU/ml form) after incubation. Same exercise have been performed thrice for validation purpose.

OBSERVIATIONS& RESULTS:

Step-1: Identification of Pure Cultures:

After 24 hours incubation of all selective media plates, typical growth of the respective organisms have been observed (Fig 1), which proved that, all three cultures were pure in form.

Fig 1: Growth of E. coli, S. aureus & S. abony in EMB Agar, MS Agar & XLD Agar Plates

Step-2: Colony Counts of Bacterial Suspensions on SCDA Plates:

After incubation of all media plates for all the dilutions (10^{-1}-10^{-8}), in has been observed an acceptable counts in 10^{-6} & 10^{-7}dilutions as per Table 1.

Organism	Dilution	CFU/ml
E. coli	10^{-7}	~ 40
S. aureus	10^{-7}	~ 60
S. abony	10^{-6}	~ 40

Table 1: Dilution vs CFU Counts

Step-3: Standardization of Bacterial Suspensions:

Now, OD at $\lambda_{max}$for those three specific dilutions of suspension (Reference Table 1) have been checked & data have generated as per Table 2. To identify the λ_{max},initially, scanning done for all three organisms separately in UV range & found the λ_{max}at 227 nm.

Organism	Dilutions	CFU/ml	OD (at 227nm)
E. coli	10^{-6}	420	0.090
	10^{-7}	40	0.074
S. aureus	10^{-6}	640	0.089
	10^{-7}	60	0.063
S. abony	10^{-5}	480	0.027
	10^{-6}	40	0.015

Table 2 – CFU vs OD

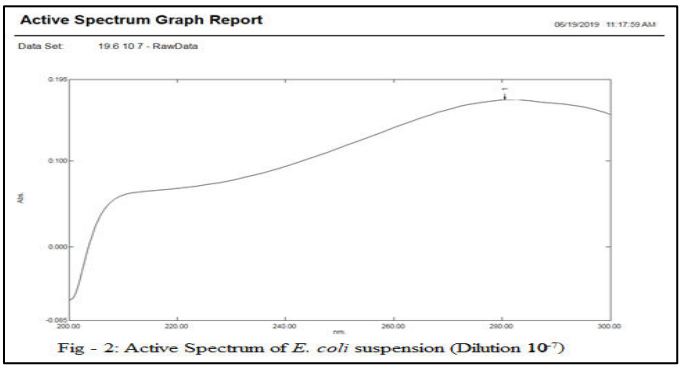


Fig - 2: Active Spectrum of *E. coli* suspension (Dilution 10^{-7})

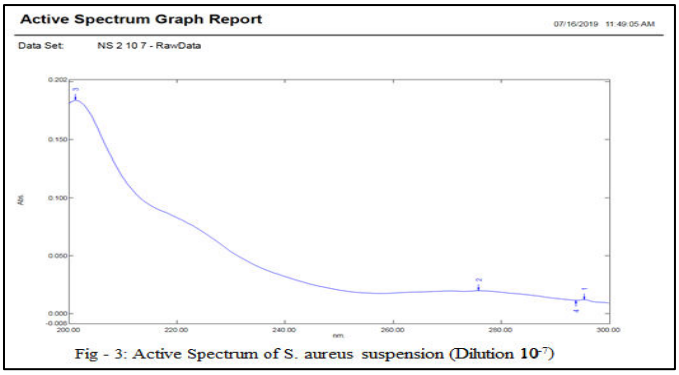


Fig - 3: Active Spectrum of S. aureus suspension (Dilution 10^{-7})

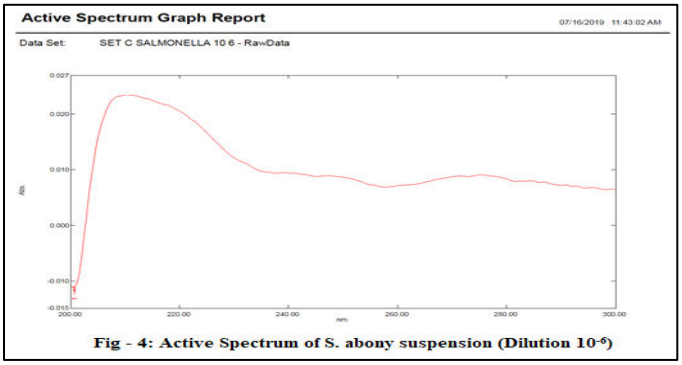


Fig - 4: Active Spectrum of S. abony suspension (Dilution 10^{-6})

Step-4: Validation - Verification of OD vs CFU:

To validate the study, 10^{-6} & 10^{-7} dilutions of 'mother suspension' have been made (three sets) & taken the OD

values at 227 nm wavelength. When TBC have been performed with those suspensions, a satisfactory CFU have been observed in the range of 10-100 CFU/ml (reference, Table 3).

Organism	Set	O.D. (at 227nm)	CFU/ml
E. coli	1	0.067	30
	2	0.058	25
	3	0.064	30
S. aureus	1	0.065	50
	2	0.069	50
	3	0.060	40
S. abony	1	0.012	30
	2	0.018	40
	3	0.017	40

Table 3 – OD vs CFU

CONCLUSIONS:

From this study, it has been established that, 10-100 CFU cultures can easily be prepared by using Spectrophotometric method, instead of plating & waiting for 3 to 5 days incubation period every time. If, the range of OD can be established for any specific Bacterium once, where less than 100 CFU is coming, henceforth that range of OD should give less than 100 CFU always. In this way, we can reduce the repetitive actions for plating & incubation; can reduce the total time as well as tedious activitiesfrom microbiologists end. Just single time validation can help to prepare SOP for preparing 10-100 CFU culture for any organism as follows –

SOP for preparation of 10-100 CFU *E. coli* culture:

1. Fresh *E. coli* culture need to be prepared (incubation period 24 hours) from any subculture in SCDA plate.
2. Inoculation of this fresh culture (2-3 loop-full) need to be done in 10 ml of 0.9% Saline Water. This suspension can be stored for long time (preferably 2 week) at refrigerator (around 4°C). This will treated as 'mother stock'.
3. Whenever required, 'mother stock' need to be diluted with 0.9% Saline Water to get the OD at 227nm around 0.05 – 0.07 range. That OD of Bacterial suspension is equivalent to less than 10-100 CFU *E. coli* cells.

In this way, 10-100 CFU culture for any microorganism can be prepared, stored& used.

ABBREVIATIONS:

E. coli - *Escherichia coli*
S. aureus - *Staphylococcus aureus*
S. abony - Salmonella abony
ATCC - American Type Culture Collection
TBC – Total bacterial Counts
OD – Optical Density
SOP – Standard Operating Procedure

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After completion of M.Sc. in Microbiology from University of Calcutta, in the year 2004, has joined as Lecturer at J. K. College, Purulia,West Bengal. Working there until 2006, joined in industry &working in Pharma & FMCG sectortill date in Quality functions. Major courtesy is in industrial hygiene practice setup&assessment.