

Original Research Article

<http://dx.doi.org/10.20546/ijcmas.2016.510.115>

Selection of Storage Methods for Maintenance of Different Stock Cultures

Angshumanjana*, Anirbanjana, Dipjitdey, Arijitmajumdar,
Jayantabikashdey and Nikhil Tudu

IIT Campus, Khargapur, West Bengal, India

*Corresponding author

ABSTRACT

Now a day, culture maintenance is a big problem for quality control of microorganisms. The microbiologist needs to have some convenient methods for maintaining microorganisms. There are multiple methods for microbial preservation. Samples were collected from the patients in the department of microbiology at BSMC & H, Bankura, a tertiary care hospital, over a period of four months from March to June 2016. In this study performed four simple methods for preservation of bacteria such as 20% glycerol supplemented broth preserved at -20°C temperature, 0.4% semisolid agar media at 4°C, Nutrient agar at 4°C, Nutrient agar at 8°C. During preservation we performed biochemical test and observed antibiotic sensitivity pattern after each 2 weeks interval. In 20% glycerol broth at -20°C of *Pseudomonas sp* and *Klebsiella sp* were not viable after 7-8 weeks of preservation. In semisolid agar media all of 6 bacteria were alive upto 12 weeks. In nutrient agar medium at 4°C and at 8°C bacteria were alive up to 6-9 weeks but antibiotic sensitivity pattern showed marked change in change zone diameter for some bacteria. This large change of zone diameter was not due to human error so we cannot preserve for more than 6 weeks in Nutrient agar. From this above experiment we can conclude that preservation in 0.4% semisolid agar media at 4°C is the ideal media for the preservation of Gram positive and Gram negative bacteria.

Keywords

Stock culture,
20% glycerol,
semisolid agar,
nutrient agar,
Pseudomonas sp
and *Klebsiella sp*.

Article Info

Accepted:
28 September 2016
Available Online:
10 October 2016

Introduction

Maintenance of microbial culture is a problem common to many areas of microbiology. The microbiologist needs to have some convenient methods for maintaining microorganisms. A number of industries must maintain cultures used in their manufacture of their products, whatever it be beer, wine, antibiotics, bread, or milk products such as cheese or butter milk. It would be desirable to be able to define the conditions for optimal survival of the various

organisms but this is not yet possible (Advances in Applied Microbiology, 1978). The maintenance of a large collection of stock cultures of bacteria for ready availability is a major task in microbiology laboratory as a positive or negative control of quality control. Long and short term preservation of microorganisms for future study has a long tradition in microbiology. Culture collections of microorganisms are valuable resources for scientific research in

microbial diversity and evolution, molecular research, epidemiological investigations and educational purposes. Preserved individual's strains of microorganisms serves as permanent records of microorganism's unique phenotypic profiles and provide the material for further genotypic characterizations (Cathy *et al.*).

The method of frequent transfer on a suitable culture medium, as commonly employed, requires not only a great expenditure of time, materials and effort, but also involves the possible loss of certain biological, immunological and cultural characteristics. The problem is the occasional loss of the strain as contamination, drying of the medium and contact with accumulating metabolites. Some species are poorly viable and their cultures usually lost within a few days (Mackie & McCartney's Practical Medical Microbiology). There are many factors to be considered and there is no single "best" method applicable to all situations, but by comparing the results of the various procedures it is hoped that the task of selecting procedures of preservations of specific cultures can be simplified. To preserve cultures, and at the same time to maintain them in as nearly as possible their original state, various techniques have been advocated.

There are multiple methods for microbial preservation. Effective storage is defined by the ability to maintain an organism in a viable state free of contamination and without changes its genotypic and phenotypic characteristics. Secondly, the organism must be easily restored to its conditions prior to preservation. The underlying principle of these methods is to slow down the metabolic rate to an extreme minimum level. These can be achieved by exclusion of water which is essential for optimal functioning of life process, or by lowering the temperature to a

level where normal enzymes are non-functioning. Exclusion of oxygen in the case of aerobic organisms also helpful in slowing down their metabolic activities (Banerjee *et al.*, 2008).

The objective of preservation methods is to maintain the viability and genetic stability of the culture by reducing the organism's metabolic rate thereby extending the period between subcultures. Success of the preservation depends on the use of the proper medium and cultivation procedure and on the age of the culture at the time of preservation. There are several factors available that increase the time period between subcultures. These include manipulation of growth conditions by limiting carbon, nitrogen and energy sources, lowering the temperature, or preventing dehydration. Frozen storage or cryopreservation is storage at a temperature where the organism is frozen to reduce or completely prevent metabolism and physical change.

Materials and Methods

Bacteria that were used in our study were selected from those bacteria which were routinely isolated in the department of microbiology at BSMC&H, Bankura, a tertiary care hospital from the periods of March to June 2016. The bacterial samples were transferred in a NA plate and incubate at 37°C for 24 h. We only consider those clinical samples that showing growth of the organisms *Staphylococcus sp*; *Klebsiella sp.* and *Pseudomonas species* as the representative samples as they are the most frequently isolated organisms which are very commonly isolated in these hospital. We selected six numbers of organisms that were regularly subcultured in selected stock culture media.

Bacterial culture was stained by Gram staining methods and biochemical test were

done for identification of the cells. The identified bacterial cultures were preserved in 20% glycerol broth, 0.4% semi solid agar and nutrient agar at 4°C and 8°C respectively. After every 15 days over four month periods the stock cultures were maintained in different media and biochemical test and antibiotic susceptibility test were performed. The method of preservation is mainly of two types: short- term preservation and long-term preservation. Short-term methods include mainly the serial transfer of organisms to fresh medium, storage at low temperature, maintenance of spores of spore formers in dry sterile soil etc. long term methods are now widely used and use either freeze drying or ultra-freezing in liquid nitrogen (-196°C). It is important to recognize that there is no universal method of preservation that is successful for all microorganisms.

Regular Subculture

Periodic transfer on fresh, sterile media can maintain microbial culture. The frequency of transfer varies with the organism (Mackie & McCartney's Practical Medical Microbiology; Maintenance and Preservation of Microbial Cultures, 2008). To keep the cultures viable, it is necessary to use an appropriate growth medium and a proper storage temperature. The frequency of subculture can be reduced if growing it on a medium containing minimal nutrition lowers the metabolism of the organism. Solid media should be chosen in preference to liquid as there is a higher chance of contamination in liquid media.

Storage by Freezing

The simplest way to preserve a culture is to add 15 % glycerol to the culture and then to store it at -20°C or -80°C in a freezer. Cultures can be preserved for a number of months in glycerol, at a temperature of -40°C in a freezer (David *et al.*, 1954; Dexter, 1955).

Criteria for preservation: - 1. Ability to reproduce

After preservation the bacteria must be growing in a culture media, this is the first criteria for preservation of microorganism. If the microorganism is not growing in the culture media then the bacteria must be considered as nonviable. Then we can no longer use these type preservatives.

Functional properties

It is very important that the preserve microorganism can no longer loss its own phenotypic and genotypic properties (Advances in Applied Microbiology, 1978).

Results and Discussion

Sample was collected from the patient at BSMC&H in department of microbiology. The collected bacterial sample was transferred in a NA plate and incubates at 37°C for 24 h. this is followed by gram staining and biochemical testing of all six organisms to confirm the purity of the organisms. On next day we perform the antibiotic sensitivity testing as in our antibiotic policy of hospital. We preserve all six organisms in 20%glycerol supplemented broth preserved at -20°C temperature, 0.4% semisolid agar media at 4°C, Nutrient agar at 4°C and Nutrient agar at 8°C.The same is performed in every 2weeks intervals continuing over four months and the results are summarize in table 1 to 4.

From this study some interesting points have been detected which are different from other previously performed studies.In this work the bacterial culture was preserved under Glycerol broth, at -20°C, semisolid agar at 4°C, nutrient agar medium at 4°C and nutrient agar medium at 8°C temperature. After preservation of 120 days it is observed that, in Glycerol broth, at -20°C *Pseudomonas*

sp and *Klebsiella sp* were not viable after 6-7 weeks where as *Staphylococcus sp* was alive. It was also observed that there is no change of biochemical property and antibiotic susceptibility pattern during the preservation. 20% glycerol solutions used to helps lower the water content available for the bacterial cells. When an organism grows in a medium with a low water activity, it can only obtain water from its environment by increasing its internal solute concentration. This increase can be accomplished, for instance, by accumulating compatible solutes, which are not inhibitory to biochemical processes. Glycerol is such a compatible solute. The concentration of compatible solutes in the cell is a function of external solute level.

The larger susceptibility of our *Pseudomonas sp* and *Klebsiella sp* strains at higher glycerol concentrations could be explained by the thinner peptidoglycan layer (10% of the cell wall) of the Gram-negative cell wall compared with the Gram positive peptidoglycan layer (90% of the cell wall). So we cannot tell that Glycerol broth, at -20°C is a good preservative media of all types of bacteria.

Preserve *Pseudomonas sp* and *Klebsiella sp* 7-8 weeks and sub culture the bacteria within 4-5 weeks. But in case of *Staphylococcus sp* it was alive till 90 days of preservation and there are no changes of biochemical and antibiotic susceptibility pattern occur and serial subculture was necessary within 12 weeks of preservation.

For preservation at 4°C in semisolid agar medium, the three bacteria *Pseudomonas sp*, *Klebsiella sp* and *Staphylococcus sp* respectively, bacteria remain alive for 90 days and there is no biochemical change during this periods of preservation. The antibiotic susceptibility profile was same as with control plate. So we should make serial

subculture necessary within 12 weeks of preservation.

In nutrient agar medium at 4°C, *Pseudomonas sp*, *Klebsiella sp* and *Staphylococcus sp* shown in table & fig no 3 respectively, remain alive and there is no biochemical change before 45 days of preservation. But the antibiotic susceptibility profile is significantly changed in some bacteria but zone of inhibition change of 4-5 mm do not fulfill the ideal criteria of preservation. So this is not a proper method for long term preservation but in short term preservation it is a good method where subculture should be done within 3-4 weeks.

In nutrient agar medium at 8°C, *Pseudomonas sp*, *Klebsiella sp* and *Staphylococcus sp* shown in table & fig no.4 respectively, some biochemical property was changed after 30 days of preservation. Antibiotic susceptibility profile was also significantly changed. Serial subculture was necessary after 3 weeks in nutrient agar medium at 8°C.

From the above experiment we can conclude that in glycerol broth at -20°C of *Pseudomonas sp* and *Klebsiella sp* was dead in 7-8 weeks of preservation. So always make subculture within 6 weeks and in *Staphylococcus sp* was alive after 14-16 weeks of preservation and without change of biochemical and antibiotic change. So we can keep the stock for 12 weeks.

In semisolid agar media all of 3 bacteria were alive until 12 weeks without biochemical change but in some bacterial zone of inhibition change of 1-2 mm. But we cannot consider it significant because some human error like the concentration of the bacteria in peptone water which is not ideal to McFarland concentration and thickness of plates containing MHA media.

Table.1 Storage at (20% glycerol, -20°C)

Table 2: Storage at (0.4% semisolid agar, 4°C)

Organisms	CONTROL	2 nd wk	4 th wk	6 th wk	8 th wk	10 th wk	12 th wk	14 th wk	16 th wk	Organisms	CONTROL	2 nd wk	4 th wk	6 th wk	8 th wk	10 th wk	12 th wk	14 th wk	16 th wk	
<i>S.aureus</i>	Cat, Coa	G	G	G	G	G	G	G	NG	<i>S.aureus</i>	Cat, Coa	G	G	G	G	G	G	G	G	NG
<i>S.aureus (MRSA)</i>	Cat, Coa	G	G	G	G	G	G	G	NG	<i>S.aureus (MRSA)</i>	Cat, Coa	G	G	G	G	G	G	G	G	NG
<i>K.pneumoniae</i>	I,C,U,TSI,O,M	G	G	G	G	NG				<i>K.pneumoniae</i>	I,C,U,TSI,O,M	G	G	G	G	NG				
<i>K.oxytoca</i>	I,C,U,TSI,O,M	G	G	G	NG					<i>K.oxytoca</i>	I,C,U,TSI,O,M	G	G	G	NG					
<i>P.aeruginosa (pyocyanin)</i>	I,C,U,TSI,O,M	G	G	G	G	NG				<i>P.aeruginosa (pyocyanin)</i>	I,C,U,TSI,O,M	G	G	G	G	NG				
<i>P.aeruginosa (pyoverdin)</i>	I,C,U,TSI,O,M	G	G	G	NG					<i>P.aeruginosa (pyoverdin)</i>	I,C,U,TSI,O,M	G	G	G	NG					

Cat: catalase, Coa: coagulase, I:indole, C:citrate, U:urease, TSI: triple sugar iron, O: oxidase, M: motility.

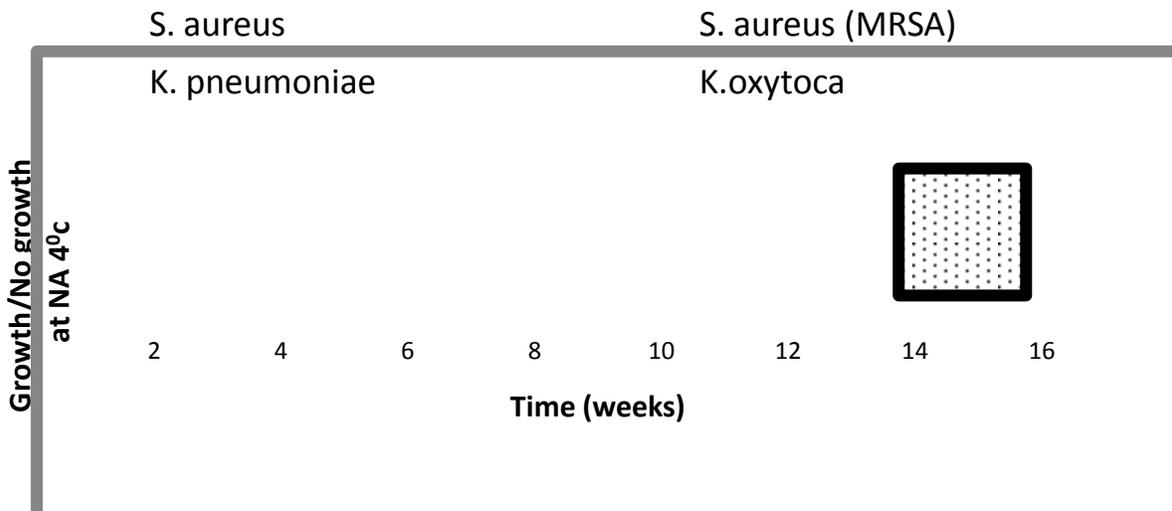
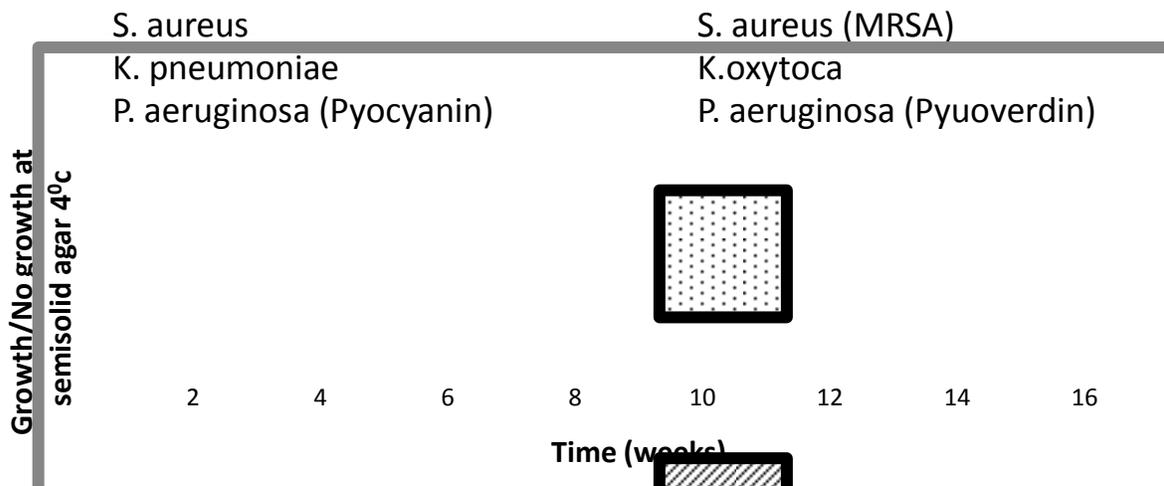
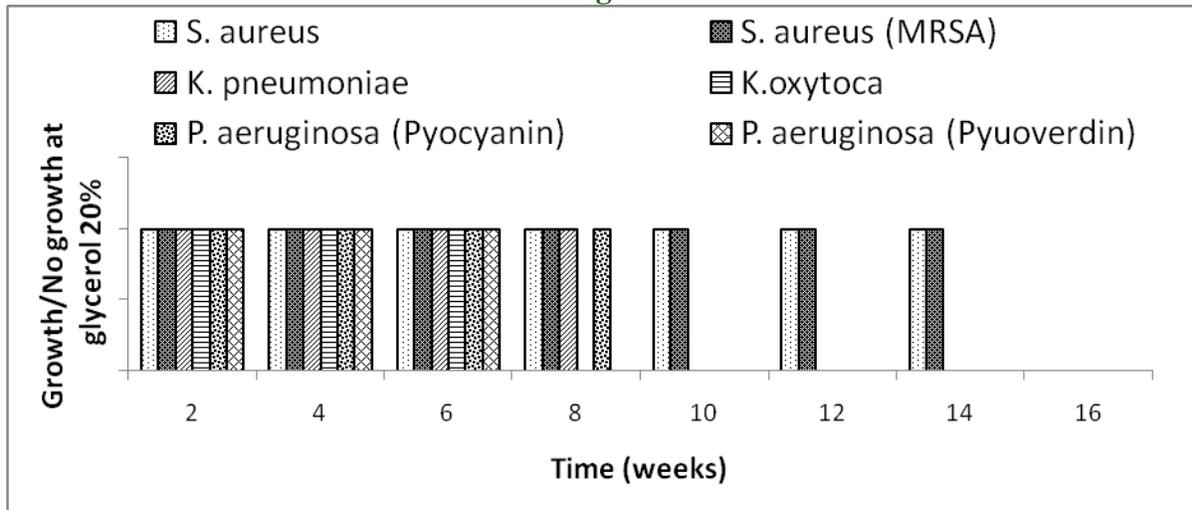
Table 3: Storage in (NA 4°C)

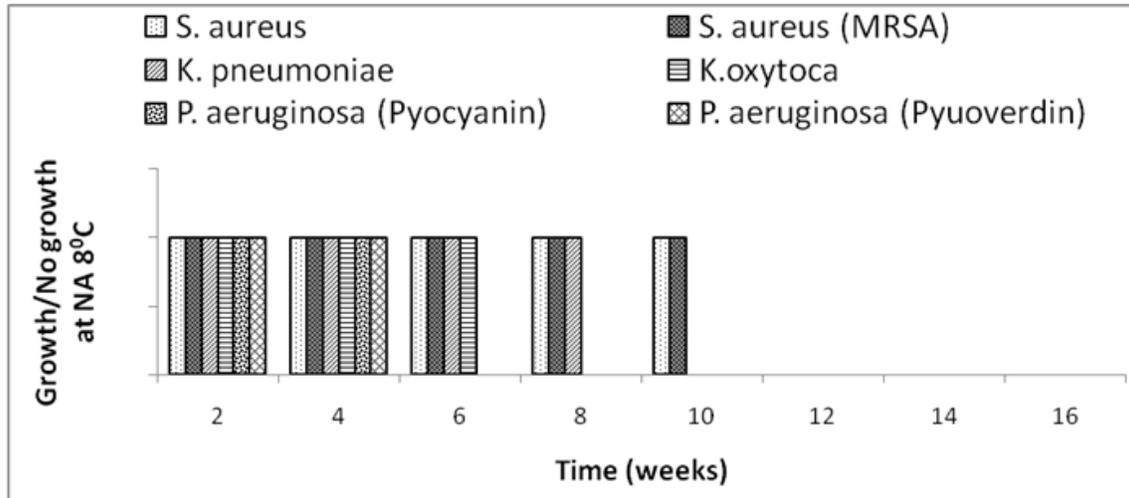
Table 4: Storage in (NA 8°C)

Organisms	CONTROL	2 nd wk	4 th wk	6 th wk	8 th wk	10 th wk	12 th wk	14 th wk	16 th wk	Organisms	CONTROL	2 nd wk	4 th wk	6 th wk	8 th wk	10 th wk	12 th wk	14 th wk	16 th wk
<i>S.aureus</i>	Cat, Coa	G	G	G	G	G	G			<i>S.aureus</i>	Cat, Coa	G	G	G	G	G	NG		
<i>S.aureus (MRSA)</i>	Cat, Coa	G	G	G	G	G	NG	NG		<i>S.aureus (MRSA)</i>	Cat, Coa	G	G	G	G	G	NG		
<i>K.pneumoniae</i>	I,C,U,TSI,O,M	G	G	G	G	NG				<i>K.pneumoniae</i>	I,C,U,TSI,O,M	G	G	G	G	NG			
<i>K.oxytoca</i>	I,C,U,TSI,O,M	G	G	G	G	NG				<i>K.oxytoca</i>	I,C,U,TSI,O,M	G	G	G	NG				
<i>P.aeruginosa (pyocyanin)</i>	I,C,U,TSI,O,M	G	G	NG	NG					<i>P.aeruginosa (pyocyanin)</i>	I,C,U,TSI,O,M	G	G	NG					
<i>P.aeruginosa (pyoverdin)</i>	I,C,U,TSI,O,M	G	G	NG	NG					<i>P.aeruginosa (pyoverdin)</i>	I,C,U,TSI,O,M	G	G	NG					

Cat: catalase, Coa: coagulase, I:indole, C:citrate, U:urease, TSI: triple sugar iron, O: oxidase, M: motility.

Fig.1





So 0.4% semisolid agar media is ideal media for the maintained of both Gram positive and Gram negative bacteria under 4°C temperature for 12 weeks and we make subculture after 3 months interval.

In nutrient agar medium at 4°C of these 3 bacteria were alive up to 6-9 weeks and no biochemical change during the preservation period but for some antibiotics there was marked change of zone of inhibition. This large change is not occurring only due to human error so we cannot preserve for 6 weeks. Serial subculture was done monthly.

And In nutrient agar medium at 8°C, though the 3 bacteria were alive up to 8th weeks but antibiotic susceptibility was change within 3-4 weeks. So we cannot preserve bacteria in this condition for long time. We can preserve the bacteria for 15 days and serial subculture was done within 2 weeks.

So, in our study semisolid agar medium at 4°C is a good medium for culture preservation for up to 3 months. But we can preserve in glycerol broth at -20°C for 7-8 weeks and nutrient agar medium at 4°C for 6 weeks.

Limitation of the study

- Low temperature or ultra-low temperature instrument was absent in this lab.
- High load of culture to be stock.
- Short time span.

References

- Advances in Applied Microbiology, vol 24, 1978.
- Banerjee, A., Banerjee, N. 2008. *Fundamentals of Microbiol. Immunol.*
- Cathy, A., Petti, Karen, C., Carroll. Procedures for the storage of microorganisms.
- Darrell, N., Kotton, Gustavo, Mostoslavsky, George, J., Murphy. 2004. Bacterial storage and glycerol stocks.
- David, H., Hollander, E. Ellen Nell. 1954. Improved Preservation of *Treponemna pallidum* and Other Bacteria by Freezing with Glycerol. The Department of Microbiology, School of Hygiene and Public Health, 7th Johns Hopkins University, Baltimore, Maryland.
- Dexter, H., Howard. 1955. The preservation of bacteria by freezing in glycerol broth. Department of Infectious

Diseases, School of Medicine,
University of California, Los Angeles
24, California.

Mackie & McCartney's Practical Medical
Microbiology, 14th edition, Edited by
J.G. Collee & B.P. Marmion.

Maintenance and Preservation of Microbial
Cultures. 2008. Committed to the

advancement of Clinical & Industrial
Disinfection & Microbiology. *J.*
Hygiene Sci., 1: iv.

Monica Cheesbrough. 2005. District
Laboratory Practice in Tropical
Countries, vol 1, eds 2.

How to cite this article:

Angshumanjana, Anirbanjana, Dipjitdey, Arijitmajumdar, Jayantabikashdey and Nikhil Tudu.
2016. Selection of Storage Methods for Maintenance of Different Stock Cultures.
Int.J.Curr.Microbiol.App.Sci. 5(10): 1097-1104.
doi: <http://dx.doi.org/10.20546/ijcmas.2016.510.115>