

Preparation of Fungal and Bacterial Spores' formation Slides

Bay Dar¹, May Thin Kyu², KhinShweNyunt³, NangSandarMyint⁴, Khin Thein Soe⁴,
Wut Yee Aye⁴ and Aye AyeMyint⁵

Abstract

Spores' formation slides of microorganisms are useful as teaching aids for upper secondary students, undergraduate student and researchers especially for the Microbiology specialization. In the agriculture and clinical laboratories, studies on spores' formation slides play an important role for identifying their cells or spores structure. In this study, seven bacteria were isolated from litter soil, Botany garden, University of Yangon. Bacterial spores' formations such as Cocci, Diplococci, Staphylococci, Coccobacilli, Bacilli, Diplobacilli and Streptobacilli were studied. And also seven fungi including *Rhizoctonia*, *Trichoderma*, *Paecilomyces*, *Fusarium*, *Penicillium*, *Aspergillus* and *Culvularia* species were purified and cultured on Czapekdox agar medium. The characteristics of spores' formation of fungi were then studied under microscope using Lactophenol Cotton Blue. In addition, spores' formation slides of 2 different yeasts from toddy juice and bean sprout pickle were prepared and studied by Gram staining method. The qualified prepared slides will be beneficial for teaching and learning microbiology in the academic as well as research area.

Keywords: Spores' formation slides, Bacteria, Yeasts, Fungi, Grams positive, Grams negative

¹Associate Professor, (Botany), Universities' Research Center, University of Yangon

²Demonstrator, Department of Botany, University of Yangon

³Lecturer, Department of Botany, University of Yangon

⁴Lecturer, (Zoology), Universities' Research Center, University of Yangon

⁵Professor, (Chemistry), Universities' Research Center, University of Yangon

Introduction

The fungal and bacterial spore formation slides are used for learning the microorganisms which are useful as well as harmful to plants, animals and human beings because it produces many antibiotics, natural products and toxic substances and they are also used in clinical and agriculture field, industrial fermentation processes and food processing.

Bacteria are very small, transparent, and invisible to our naked eyes so it is very difficult to observe these microorganisms. So staining of microorganism in microbiology is a very essential step for bacteriological studies. The Gram stain is fundamental to the phenotypic characterization of bacteria. The staining procedure differentiates organisms of the domain bacteria according to cell wall structure. Gram-positive cells have a thick peptidoglycan layer and stain blue to purple. Gram-negative cells have a thin peptidoglycan layer and stain red to pink. In this study, seven types of bacteria such as cocci, diplococci, staphylococci, coccobacilli, bacilli, diplobacilli and streptobacilli were stained according to Bergey *et al.* (1994).

The branch of science that deals with study of fungal species is called Mycology. Fungi are eukaryotic organisms and they are classified into two main groups yeast and molds. Its cell wall is made up of chitin. Fungal cells have both macroscopic as well as microscopic structure.

It is harmful in the sense as it causes diseases in human and important crops. So it is very important to study the staining of these fungal cells and identify fungal species. In this study, seven fungi which are *Rhizoctonia*, *Trichoderma*, *Paecilomyces*, *Fusarium*, *Penicillium*, *Aspergillus*, *Culvularia* species and yeasts were stained by Lactophenol Cotton Blue method (Sharma *et al.*, 1986) for preparing spores' formation slides, also called mounting of fungus. The method to be described is simpler and yields permanently stained specimens which are ideal for teaching and research purposes.

The objective of this study is to visualize and identify the structural components of fungi (using Lactophenol cotton blue) and the morphological spores' formation of bacteria (using Gram stains) applying spores' formation slides.

Materials and Methods

Litter soil sample, toddy juice and bean sprouts pickle were selected as sources for preparation of bacterial and fungal spore formation slides.

The selective media used for isolation and cultivation of spores were Nutrient Agar Medium (Ronald, 1993) for bacteria and yeasts and Czapekdox agar medium (Raper and Thom, 1945) for fungi. In this work, microscopic examination of bacteria, yeast and molds was carried out by special microscopic slide technique using Olympus (BX 51).

Sample collection

Soil sample was collected from litter soil near the Botanical garden, Department of Botany, University of Yangon. Toddy and bean sprouts were purchased from the retail market.

Isolation of microorganisms

Isolation of fungi and bacteria from litter soil sample was carried out by serial dilution method (Johnson *et al.*, 1957). Two different types of yeasts were also isolated from fermented toddy juice and from bean sprout pickle. The isolated microorganisms from various sources are shown in Table 1.

Table 1. Isolation of Microorganisms from Various Sources

Sr. no.	Source	Medium	Types of Microorganism	No of Each Type
1	Litter soil	Nutrient Agar	Bacteria	7
2	Toddy juice, Pickle	Nutrient Agar	Yeast	2
3	Litter soil	Czapek dox agar	Fungi	7

Soil Dilution Method

Soil dilution method was used according to the method of Johnson *et al.* (1957).

1. One gram sample of soil was placed in a graduated cylinder. Water was added to the soil so that a total volume of 100 mL was reached. The suspension was stirred and poured into a 300 mL Erlenmeyer flask. The flask containing the suspension was shaken for 30 minutes.

2. One mL of sample was then transferred immediately through successive 9 mL sterile water in test tube until the desired final dilution is reached. Each suspension was shaken by hand for a few seconds, and was in motion while being down into the pipette.

3. Use of the preceding method for making soil dilutions yield dilutions of soil in water (10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} and 10^{-7} mL).

4. Firstly, sterilized petridishes were poured with 12 to 15 mL of autoclaved Czapek dox agar medium for culturing fungi and Nutrient agar medium for culturing bacteria, and cooled to just above the solidifying temperature, and then one mL of the desired soil dilution was transferred to each dish.

The dishes were rotated by hand in a broad swirling motion so that the diluted soil was dispersed in the agar medium. These petridishes were incubated at 25-35 °C for 3-7 days.

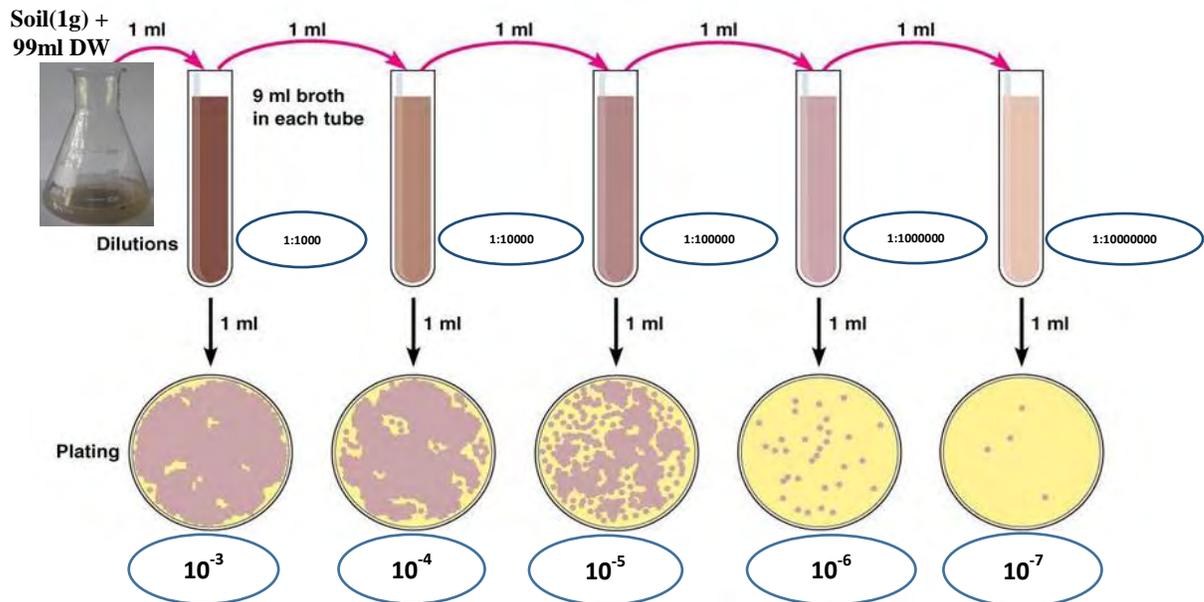


Figure 1 - Soil dilution procedure (Method by Johnson *et. al.*, 1957)

Culture medium for Yeast and Bacteria

The Nutrient agar medium was used according to method of Ronald (1993) as stock culture medium or sub-culture medium when yeasts and bacteria were isolated from the toddy juice, bean sprout pickle and litter soil, respectively.

Nutrient Agar Medium

Agar	-15.0 g
Peptone	-5 g
NaCl	-5 g
Yeast extract	-2 g
Beef extract	-1 g
Distilled water	-1000 mL
pH	-7.4 ± 0.2 at 25 °C.

Preparation of reagents for staining (Gram stain)

1. The primary stain (crystal violet reagents for staining)

Solution A

Crystal violet	- 2 g
Ethanol	-20 mL(95 % v/v)

Solution B

Ammonium oxalate - 0.8 g
Distilled water - 80 mL

Solutions A and B were mixed to obtain crystal violet staining reagent, stored for 24 hours and then filtered through paper prior to use.

2. Mordant (grams iodine) (to fix the primary dye to the cell wall)

Iodine - 1 g
Potassium iodide - 2 g
Distilled water - 300 mL

Using a mortar, iodine and potassium iodide were ground, while slowly adding water with stirring until all the iodine had completely dissolved. The solution was stored in an amber bottle.

3. Decolorizing agent (to remove the primary stain - crystal violet)

Acetone - 50 mL
Ethanol (95%) - 50 mL

4. Counterstained (Safranin)

Stock solution : Safranin O - 2.5 g + Ethanol (95%) - 100 mL
Working solution : 10 mL of the stock solution + 90 mL of distilled water

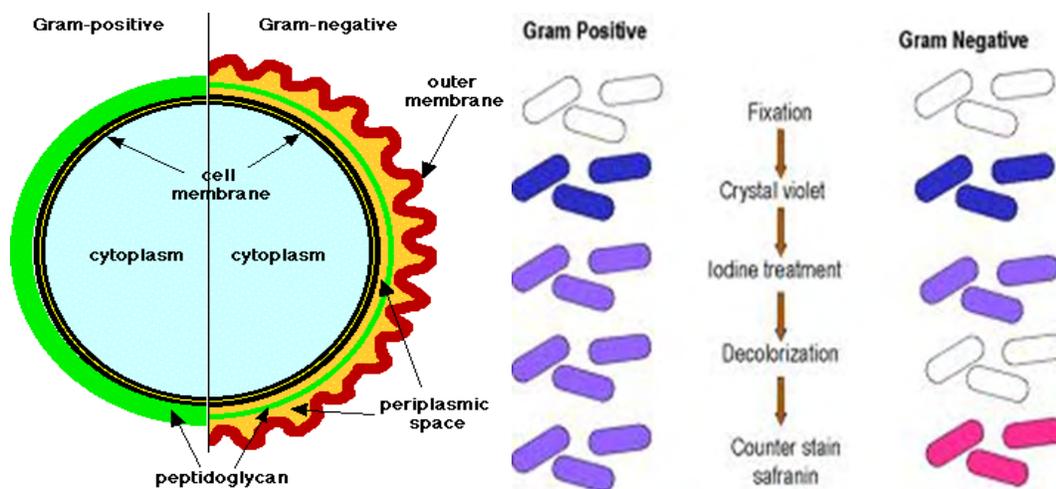


Figure 2 - Structure and Reactivity to Gram Staining

Preparation of bacterial spores' formation slides

A drop of water was added to the slide and a minute amount of a pure colony was aseptically transferred. The culture was spread with a sterilized inoculation loop to form an even thin film over a circle approximately the size of a dime.

Staining procedure was carried out according to Gephardt *et.al.*, 1981.

1. Smear of cells was heat-fixed for 1 minute with crystal violet staining reagent.
2. The slide was washed in a gentle and indirect stream of tap water for 5 seconds.
3. The slide was flooded with the mordant: Gram's iodine for 1 minute.
4. The slide was washed in a gentle and indirect stream of tap water for 5 seconds.
5. The slide was flooded with decolorizing agent for 15 seconds or added drop by drop to slide until decolorizing agent running from the slide runs clear.
6. The slide was flooded with counterstain, Safranin for 30 seconds to 1 minute.
7. The slide was washed in a gentle and indirect stream of tap water until no color appeared in the effluent and then dried with absorbent paper.
8. The result of the staining procedure was observed under oil immersion using a Brightfield microscope. At the completion of the Gram Stain, gram-negative bacteria will stain pink/red and gram-positive bacteria will stain blue/purple.
9. The permanent slides were labeled and kept in slide boxes for microscopic studies.

Culture medium for fungi

When molds were isolated from the litter soil, the Czapek dox agar medium was used as stock culture medium or sub-culture medium according to method of Raper and Thom (1945).

Czapek dox agar medium

Sucrose	-30.00 g
NaNO ₃	-2.00 g
K ₂ HPO ₄	-1.00 g
MgSO ₄	-0.50 g
KCl	-0.50 g
FeSO ₄	-0.10 g
Agar	-20.00 g
Distilled water	-1000 mL
Final pH	-7.0

Preparation of Lactophenol Cotton Blue for staining fungi

Lactophenol Cotton Blue [LCB] staining-mounting medium method was done by Sharma *et. al.*, (1986) and it is commonly used for microscopic identification of fungi.

Lactophenol Cotton Blue

Cotton Blue (Aniline Blue)	-	0.05 g
Phenol Crystals ($C_6H_5O_4$)	-	20 g
Glycerol	-	40 mL
Lactic acid ($CH_3CHOHCOOH$)	-	20 mL
Distilled water	-	20 mL

This stain was prepared over two days.

1. On the first day, the Cotton Blue was dissolved in the distilled water and kept overnight to eliminate insoluble dye.
2. On the second day, the phenol crystals was added with wearing gloves to the lactic acid in a glass beaker and placed on magnetic stirrer until the phenol was dissolved.
3. The glycerol was added.
4. The Cotton Blue solution was filtered into the phenol, lactic acid and glycerol solution and mixed and stored at room temperature.

Preparation of fungal spores' formation slides

The process of transferring hyphae to a slide breaks up the structure of hyphae, sporangiophores or conidiophores and conidia from pure colony.

Procedure

1. A clean grease free slide was prepared.
2. A drop of 95 % alcohol was placed on a clear glass slide and a loop of fungal culture was carefully teased out in the alcohol with needles. When it was satisfactorily spread and most of the alcohol was evaporated, and then a drop of lactophenol cotton blue stain was added for 1-2 min.
3. And rinsed with 95% alcohol and air dried.
4. Finally, the stained fungi were mounted with Canada balsam.
5. A clean cover slip was placed on Canada balsam with the help of a forceps and dried overnight.
6. The permanent slides were labeled and kept in slide boxes for microscopic studies.

Results

Bacterial and Yeasts spores' formation visualized by Gram staining method and fungal spores' formation visualized by Lactophenol Cotton Blue method was studied under microscope.

Microscopic Examination on Bacterial Spores' formation

After staining the bacterial cell using Gram stain based on the physical and chemical structure of their outer surface bacteria were categorized as Gram positive or Gram negative. The different types of cell formation were studied by Olympus - BX 51 and identified by Bergey *et al.* (1994). In this study, seven types of bacterial cell formation which are Cocci (Figures 3 and 4), Diplococci (Figure 5), Staphylococci (Figure 6), Coccobacilli (Figure 7), Bacilli (Figures 8, 9 and 10), Diplobacilli (Figure 11), and Streptobacilli (Figure 12) were isolated from litter soil. The cell formation of isolated bacteria from litter soil is shown in Table 2.

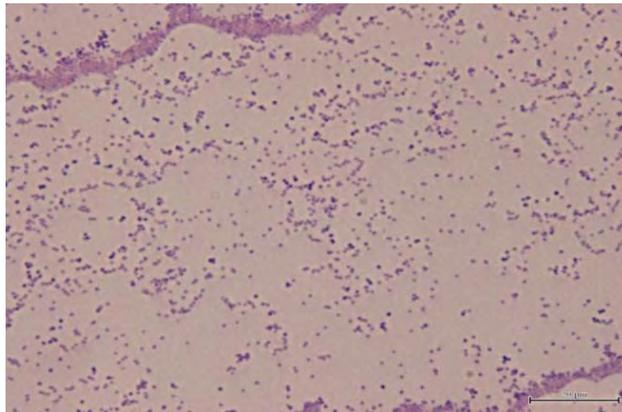


Figure 3 - Cocci (X 1000), Gram Positive

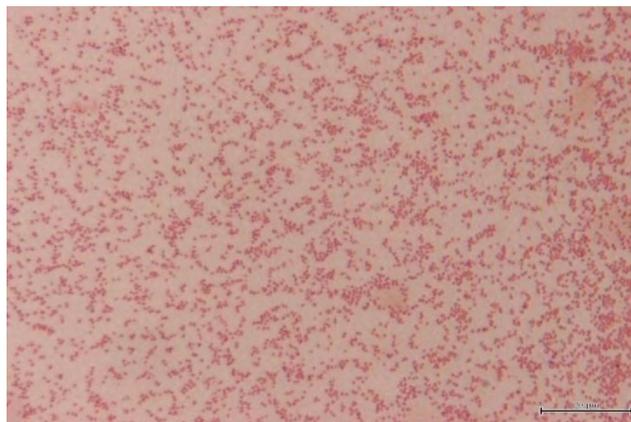


Figure 4 - Cocci (X 1000), Gram Negative

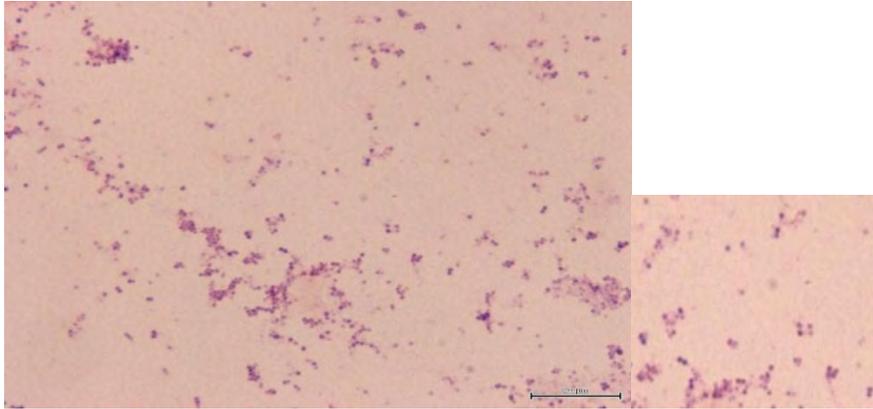


Figure5 -Diploocci (X 1000), Gram Positive

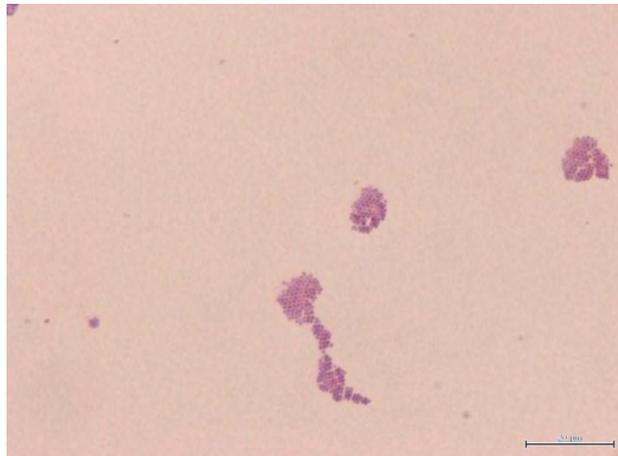


Figure6-Staphylococci(X 1000), Gram Positive

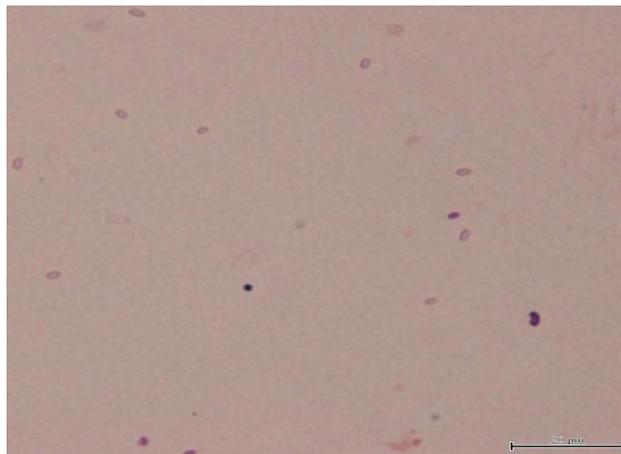


Figure7 -Coccobacilli (X 1000), Gram Negative

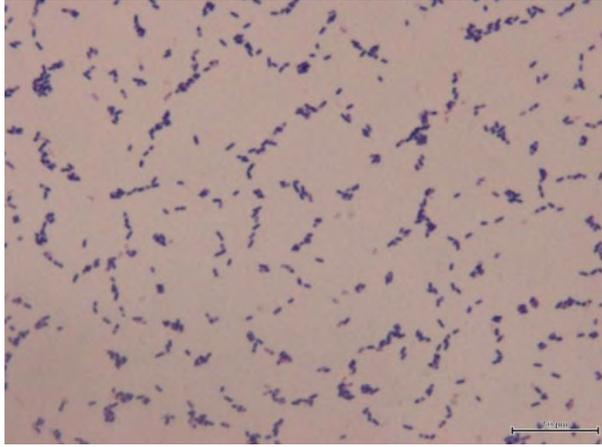


Figure8 -Bacilli (Rod shape) (X 1000), Gram Positive

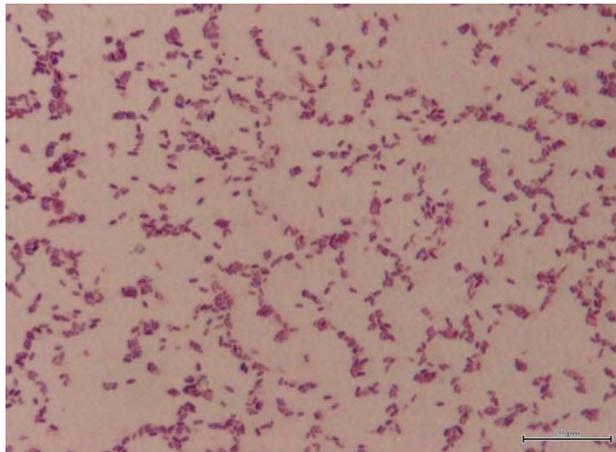


Figure9 – Bacilli(Rod shape) (X 1000), Gram Negative

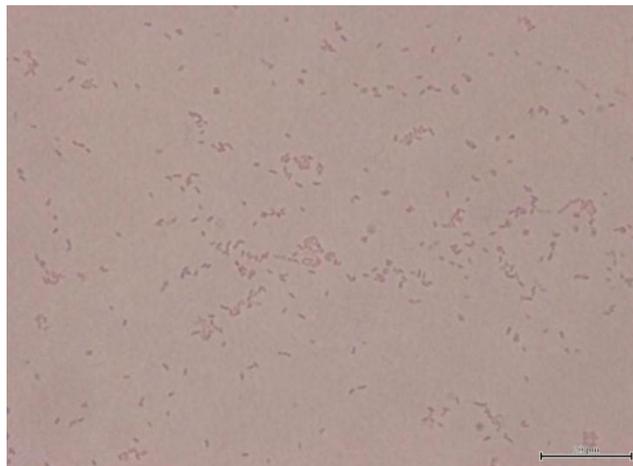


Figure10-Bacilli(Small Rod) (X 1000), Gram Negative

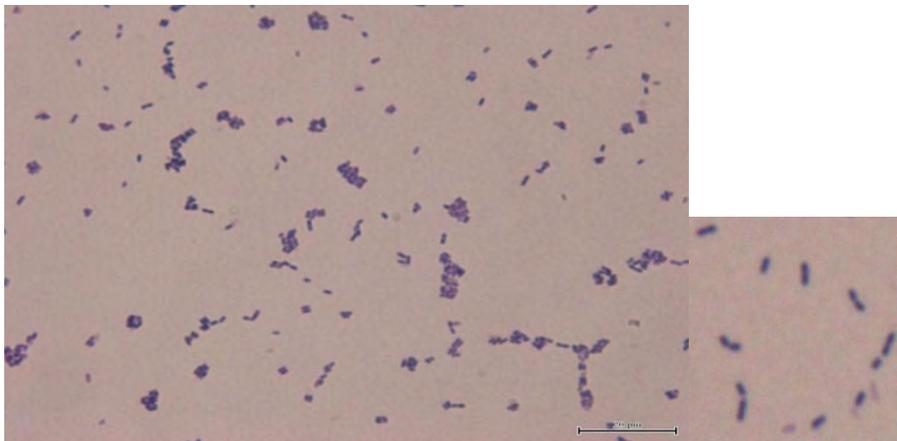


Figure11 -Diplobacilli (X 1000), Gram Positive

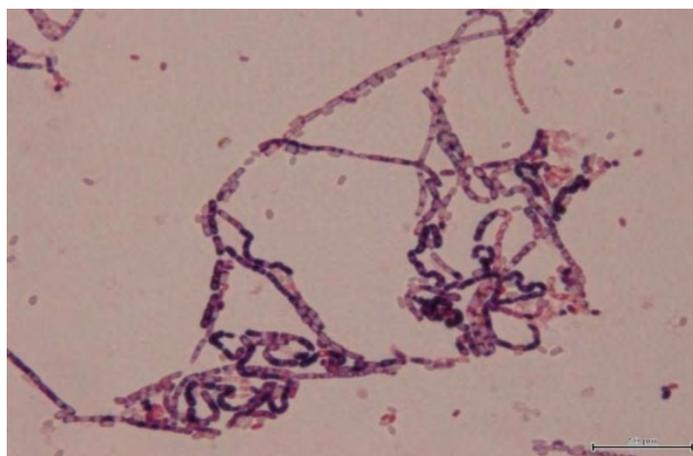


Figure12 – Streptobacilli(Chain) (X 1000), Gram Negative

Table 2.Cell formation of Isolated Bacteria from Litter Soil

Sr. No.	Type	Grams	Shape	Figure
1	Cocci	+	Sphere	3
		-	Sphere	4
2	Diplococci	+	Sphere and fused as in pairs	5
3	Staphylococci	+	Sphere and fused as grape-like clusters	6
4	Coccobacilli	-	Oval	7
5	Baccili	+	Rod	8
		-	Rod	9
		-	Small rod	10
6	Diplobacilli	+	Rod and fused as in pairs	11
7	Streptobacilli	-	Rod and fused as in chains	12

Microscopic Examination on Yeast and Fungal Spores' formation

After staining the yeasts isolated from toddy juice and pickle 2 different types of yeasts were observed (Figures 13 and 14). The spores' formation of fungal cells isolated from litter soil were stained with Lactophenol cotton blue, the different parts of a cell like conidia, conidiophore as well as hyphae were also observed. Based on the observation the type of fungus can be identified according to Barnett (1960) and Tsuneo (2002). In the present study, seven fungi such as *Rhizoctonia* (Figure 15), *Trichoderma* (Figure 16), *Paecilomyces* (Figure 17), *Fusarium* (Figure 18), *Penicillium* (Figure 19), *Aspergillus* (Figure 20) and *Culvularia* species (Figure 21) were observed in spores' formation slides. The characteristics of isolated fungus related to their spores' formation were recorded in Table 3. The overall assessment of fungal and bacterial spores' formation slides from various sources is listed in table 4.

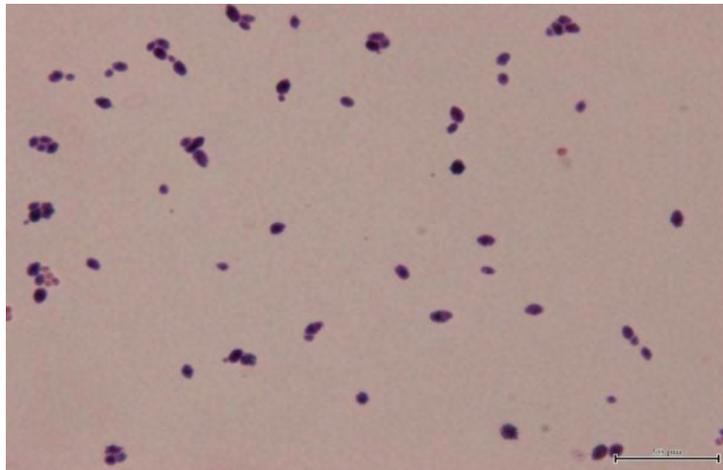


Figure 13 - Yeast (from Toddy juice) (X 1000)

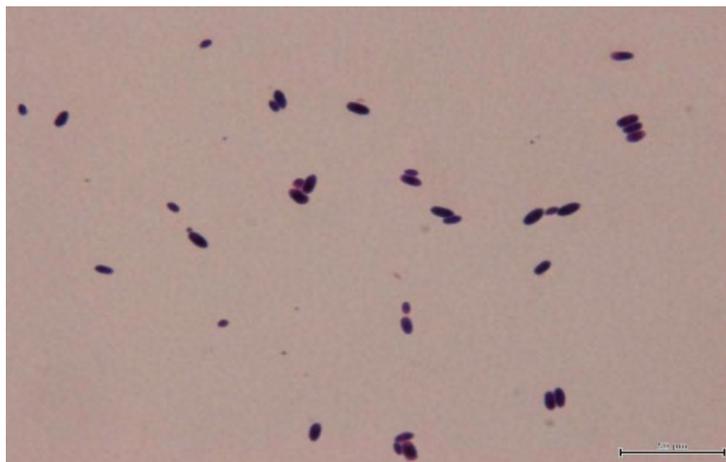


Figure 14 - Yeast (from Pickle) (X 1000)

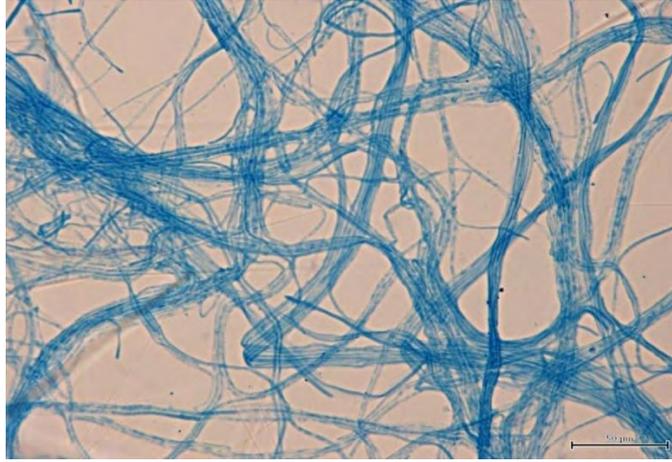


Figure15 -*Rhizoctonia* sp. (X 400)

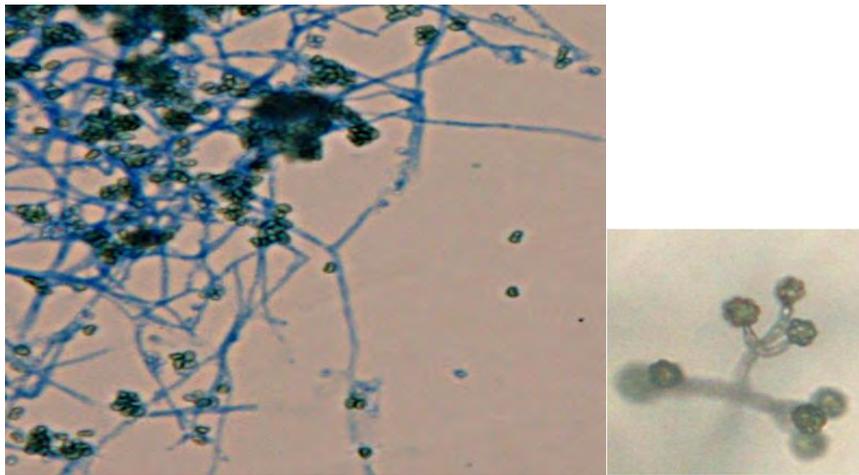


Figure16 -*Trichoderma* sp. (X 400)

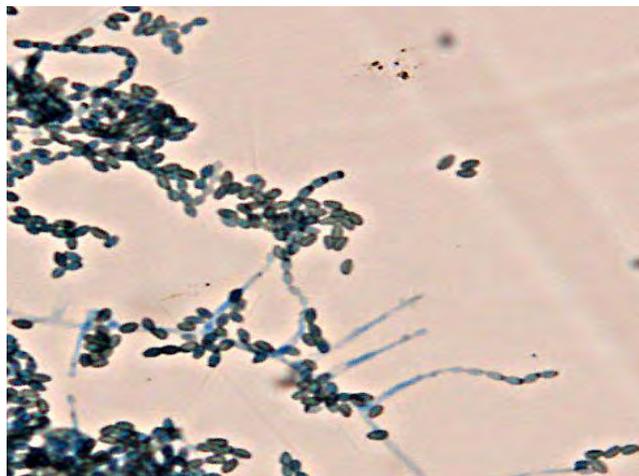


Figure17 -*Paecilomyces* sp. (X 400)

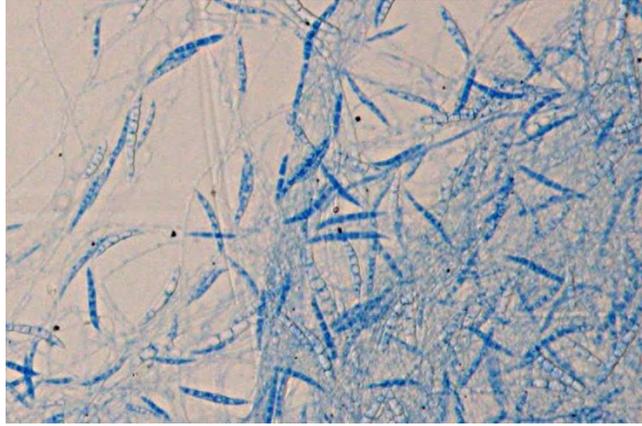


Figure18 -*Fusarium* sp. (X 400)

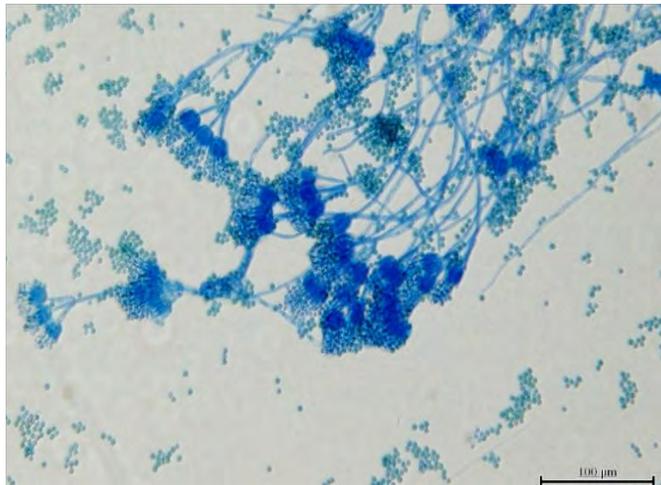


Figure19 -*Penicillium* sp. (X 200)

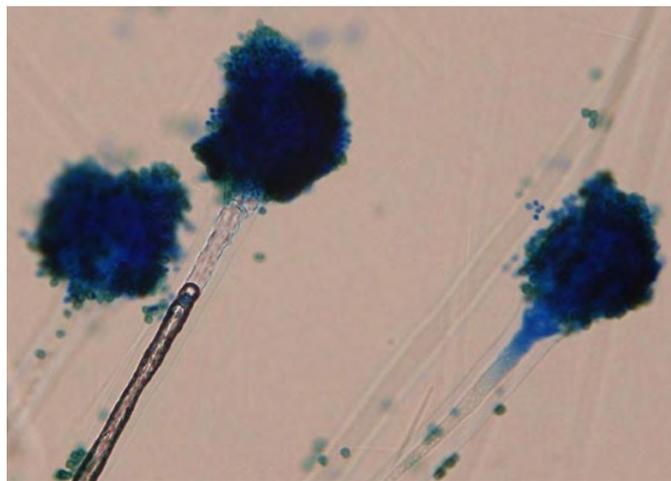


Figure20 -*Aspergillus* sp. (X 400)

Figure21 -*Culvularia* sp. (X 400)

Table 3.Characteristics of Isolated Yeasts from Toddy Juice and Pickle and Fungi from Litter Soil

Sr. No.	Characters of Fungi	Genus	Figure
1	Ovoid cell	Yeast	13
2	Ovoid cell	Yeast	14
3	Mycelium brown color, asexual fruit bodies and variable in form, spores lacking, with long cells, septa of branch set off from main hypha.	<i>Rhizoctonia</i>	15
4	Conidiophores hyaline, upright, much branches; phialides single or in group; conidia hyaline, 1-celled, ovoid, borne in small terminal clusters; usually easily recognized by its rapid growth and green patches or cushions of conidia	<i>Trichoderma</i>	16
5	Conidiophores mostly arising from aerial hyphae. Phialides arising directly from the mycelium. Basal portion of phialide nearly cylindrical, tapering gradually to a long slender tube, conidia produced successively and held together in chains, 1-celled.	<i>Paecilomyces</i>	17
6	Mycelium extensive and cottony in culture, often with some ting of pink, conidiophores variable, slender and simple, branched irregularly, conidia hyaline, variable, principally of two kinds – macroconidia and microconidia, microconidia 1-celled, oblong or slightly curve, borne singly.	<i>Fusarium</i>	18
7	Mycelium yellowish green color inside with green periphery, conidiophores arising from the mycelium singly, branched near the apex to form a brush-like, conidia-bearing apparatus; conidia 1-celled, mostly globose or ovoid, produced basipetally.	<i>Penicillium</i>	19
8	Mycelium dark green color, conidiophores upright, simple, terminating in a globose, radiating from the entire surface; conidia 1-celled, globose, catenulate.	<i>Aspergillus.</i>	20
9	Conidiophores brown, simple or sometimes branched; conidia dark, end cells lighter, 3- to 5- celled, more or less fusiform, typically bent or curved, with one or two of the central cells enlarged.	<i>Culvularia</i>	21

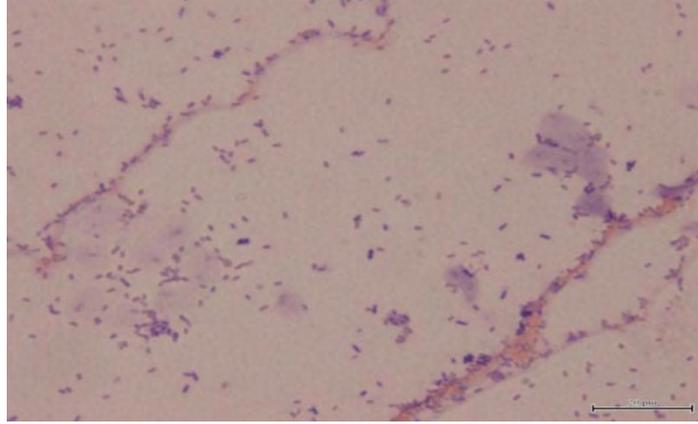


Figure22 - The uneven color specimen

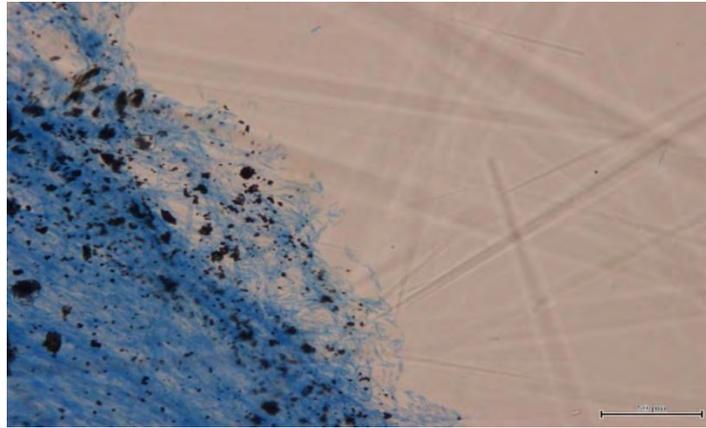


Figure23 - The uneven color specimen

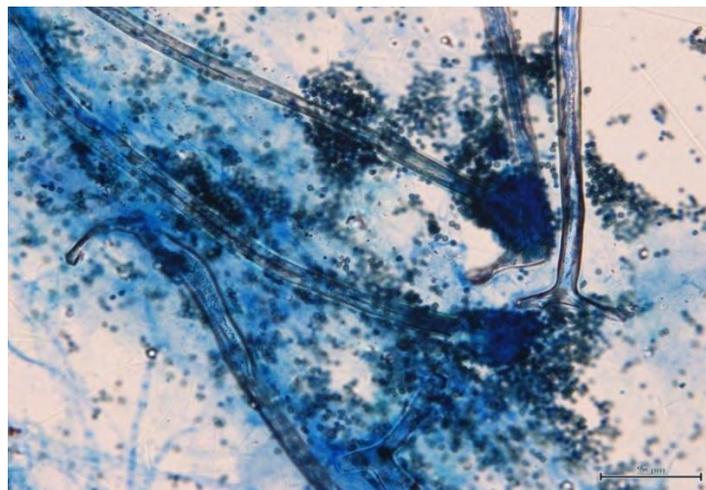


Figure24 - The uneven colorspecimen

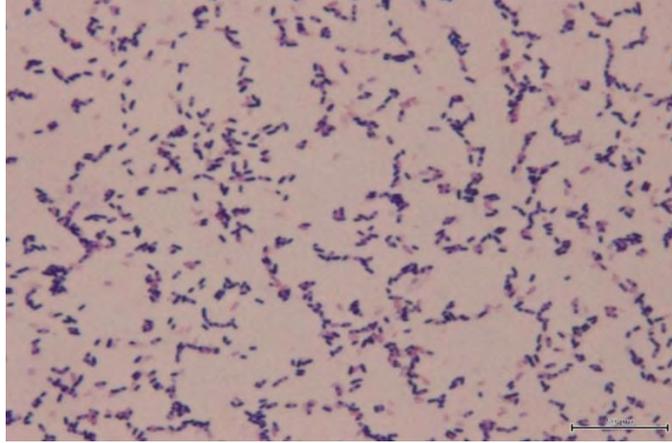


Figure25 - Rod shape (Bacilli) (X 1000), Gram Positive and Gram Negative

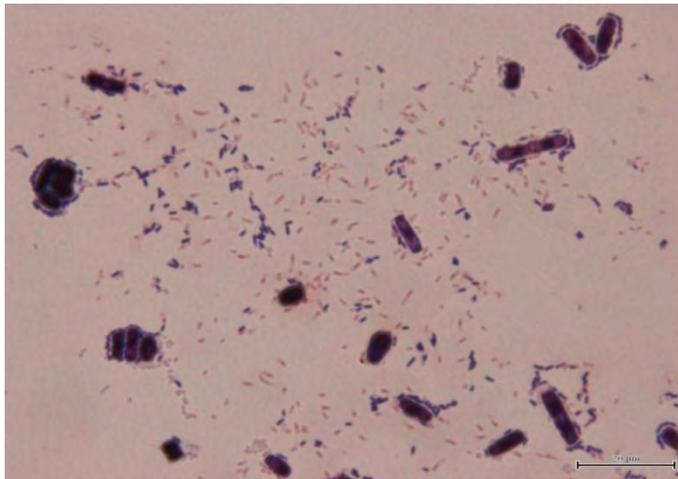


Figure26 - The contaminated colony

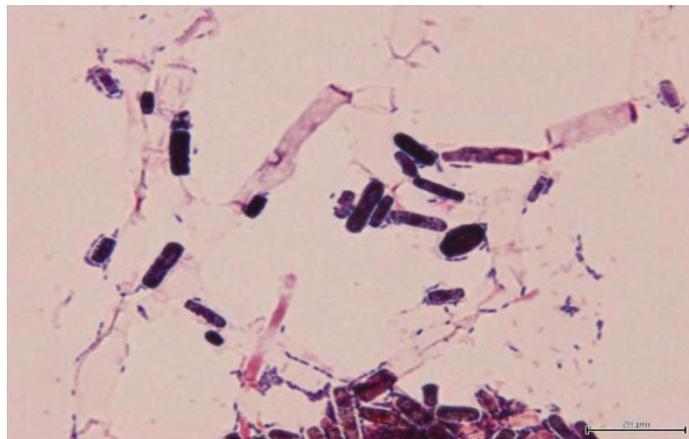


Figure27 - The contaminated colony

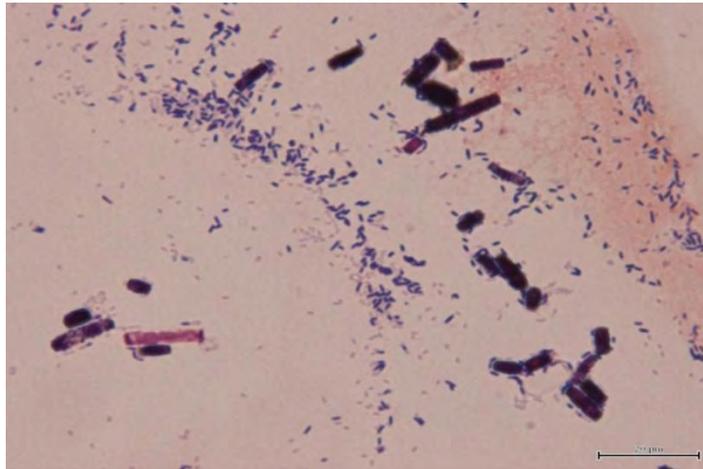


Figure28 - The contaminatedcolony

Table 4. Different Types of Bacteria and Fungi from Various Sources

Sr. no.	Source	Microorganisms	Types of Microorganism
1	Litter soil	Bacteria	Cocci, Diplococci, Staphylococci, Coccobacilli, Bacilli, Diplobacilli, Streptobacilli
2	Toddy juice, Pickle	Yeast	2
3	Litter soil	Fungi	<i>Rhizoctonia</i> sp., <i>Trichoderma</i> sp., <i>Paecilomyces</i> sp., <i>Fusarium</i> sp., <i>Penicillium</i> sp., <i>Aspergillus</i> sp., <i>Culvularia</i> sp.

Discussion

Gram, 1884 reported that bacteria that manage to keep the original purple dye have only got a cell wall called Gram positive. Bacteria that lose the original purple dye can therefore take up the second red dye and they have got both a cell wall and a cell membrane called Gram negative.

The Gram staining procedure does not always give clear-cut results. Anderson, 1983 also examined that some organisms are Gram-variable and may appear either Gram-negative or Gram-positive according to the conditions. With these types of organisms, Gram-positive and Gram-negative cells may be present within the same preparation(Figure25).

Some Gram-positive bacteria appear as Gram-negative when they have reached a certain age, varying from a few hours to a few days. On the other hand, some Gram-negative bacteria may become Gram-positive in older cultures. For this reason Popescuand Doyle(1996) strongly recommended to use very young cultures for the staining procedure, because after growth has become just visible.

When the microorganisms were being stained, some of the specimens were stained as the uneven color as shown in Figures 22, 23 and 24. Gram-positive and Gram-negative cells may be present within the same preparations (Figure 25) and more than one kind of microorganism can be present within the same preparation if the colony were contaminated (Figures 26, 27 and 28).

In the present study, some types of bacterial cell formation such as Cocci (Figures 3 and 4), Diplococci (Figure 5), Staphylococci (Figure 6), Coccobacilli (Figure 7), Bacilli (Figures 8, 9 and 10), Diplobacilli (Figure 11), Streptobacilli (Figure 12) were studied and also some examples of fungi *Rhizoctonia* (Figure 15), *Trichoderma* (Figure 16), *Paecilomyces* (Figure 17), *Fusarium* (Figure 18), *Penicillium* (Figure 19), *Aspergillus* (Figure 20) and *Culvularia* species (Figure 21) were observed as spores' formation slides and isolated them from litter soil.

Conclusion

Seven types of bacteria and seven fungi were isolated from litter soil, botanical garden and two different types of yeasts were isolated from toddy juice and pickle (bean sprout), respectively.

Based on the findings and experience of this study, it can be concluded that storing slides in a jar with 95% ethanol will ensure clean slides and flame slide before use. Freshly isolated cultures of all species yield the best results. Overheating during heat fixation, over decolourization with alcohol, and even too much washing with water between steps may lose the crystal violet-iodine complex formation in gram-positive bacteria.

Bacterial and fungal spore formation slides can be used as teaching aid in biological syllabus of high schools and undergraduate courses, especially in Microbiology and Plant pathology specialization. This study will provide the information involving the characteristics of microorganisms which will support to Applied Microbiology.

ACKNOWLEDGEMENTS

The authors acknowledge the Department of Higher Education (Lower Myanmar), Ministry of Education, Yangon, Myanmar for allowing them to carry out this research programme and feel indebted to Professor Dr Pho Kaung, Acting Rector, University of Yangon for providing us with research facilities. We would like to convey our sincere thanks to all who URC staff that helped in this study.

References

- Anderson, G.K., T.J. Beveridge and H.C. Clark. (1983). **Chemical Mechanism of the Gram Stain and Synthesis of a New Electron-opaque Marker for Electron Microscopy Which Replaces the Iodine Mordant of the Stain.** *J. Bacteriol.* 156 (2) 837-845.

- Barnett, H.L. (1955). **Illustrated Genera of Imperfect Fungi**. 2nd Edition, Burgess Publishing Company, United States of America.
- Bergey, D.H., J.G. Holt, N.R. Krieg, Peter. and H.A. Sneath. (1994). **Bergey's Manual of Determinative Bacteriology**. 9th Edition,
- Gephart, P., R.G.E. Murray, R.N. Costilow, E.W. Nester, W.A. Wood, N.R. Krieg, and G.B. Phillips. (1981). **Manual of Methods for General Bacteriology**, ASM Press, Washington: D.C.
- Gram, H.C.J. (1884). **Über die isolierte Färbung der Schizomyceten in Schnitt- und Trockenpräparate.**, in *Fortschritte der Medizin*, 2:185-189
- Johnson, D.F., E.A. Curl, and J.H. Bond. (1957). **Formulase of Selected Culture Media, Methods for Studying Soil Microflora**. 145.
- Popescu, A. and Doyle, R.J. (1996). **The Gram Stain after More than a Century.** *Biotechnic & Histochemistry*. 71(3)145.
- Raper, K.B. and C. Thon. (1949). **A General purpose Media, Methods for Studying Soil Microflora**. 141.
- Ronald, M.A. (1993). **Handbook of Microbiological Media**. CRC Press. 666.
- Sharma, P., S. Pajin, N. Dhillon, D.V. Vadehra, and D.K. Dube. (1986). **The Staining Solution of Lactophenol Cotton-blue.** *The Practice of Medical Microbiology*, 12th Edition., 2.
- Tsuneo, W. (2002). **Pictorial Atlas of Soil and Seed Fungi; Morphologies of Cultured Fungi and Key to Species**. Second Edition, CRC Press, Printed in the United States of America.