Springer Protocols

Dilip Kumar Arora
Surajit Das
Mesapogu Sukumar
Editors

Analyzing Microbes

Manual of Molecular Biology Techniques

Springer

Springer Protocols

Dilip Kumar Arora
Surajit Das
Mesapogu Sukumar
Editors

Analyzing Microbes

Manual of Molecular Biology Techniques

Springer

Editors
Dilip Kumar Arora
National Bureau of Agriculturally
Important Microorganisms
Maunath Bhanjan
Uttar Pradesh
India

Surajit Das
Department of Life Science
National Institute of Technology
Rourkela
Odisha
India

Mesapogu Sukumar
National Bureau of Agriculturally
Important Microorganisms
Maunath Bhanjan
Uttar Pradesh
India

ISSN 1949-2448 ISSN 1949-2456 (electronic)
ISBN 978-3-642-34409-1 ISBN 978-3-642-34410-7 (eBook)
DOI 10.1007/978-3-642-34410-7
Springer Heidelberg New York Dordrecht London

Library of Congress Control Number: 2013931210

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

Preface

The discipline microbiology is researched actively, and the field is advancing continually. It is estimated that only about 1 % of all of the microbe species on earth have been studied. Rapid advances in molecular biology have revolutionized the study of microorganisms in the environment and improved our understanding of the composition, phylogeny, and physiology of microbial communities. The advent of molecular biology has offered a number of revolutionary new insights into the detection and enumeration of soilborne microorganisms. DNA sequences provide information on identifying unknown species from 16S and ITS rRNA sequences of individual bacterial and fungal species. Molecular methods monitor both pathogens and also beneficial organisms in soils for detection and quantification. The in-depth exploitation of PCR potential led to more sophisticated variants of the technique (improved even from the currently expanding real-time PCR) that increases the speed and sensitivity in microbial identification and diagnostics. These molecular techniques provide new insights about their functions and interactions within ecological niches.

Analyzing Microbes—Manual of Molecular Biology Techniques is a practical guide to the application of important molecular biology techniques in microbiological research. The chapters are written by a group of international scientists who are recognized authorities in their research areas from universities/researchers and often the new techniques that are described. These volumes are aimed for graduate, postgraduate, Ph.D. students, and laboratory technicians working in different biotechnology/microbiology laboratories. It is also valuable to the larger community of researchers who have recognized the potential of genomics research and may be beginning to explore the technologies involved. Moreover, the volumes are also targeted as handouts for students, teachers, and researchers world over.

The central parts of the chapters are the experimental protocols which are presented so as to be readily used at the laboratory bench. Although a number of the procedures described represent the tried and trusted, we have striven to include variants on existing technologies that an experiment can be performed. These step-by-step protocols are intended to be concise and easy to follow. Suggestions to successfully apply the procedures are included, along with recommended materials and suppliers. A special feature of the chapters is that, in addition to the protocols, important background information and representative results of applying the methods are given. References are provided to enable the investigator to become better acquainted with

the topic. Researchers in any field that utilizes microbial systems will find this work of value. In addition to microbiology and bacteriology, this book highlights the current state-of-the-art molecular microbiology techniques in biotechnology, microbiology research, and environmental microbiology.

The aim of the book *Analyzing Microbes—Manual of Molecular Biology Techniques* has been to produce a self-contained laboratory manual which will be useful to both experienced practitioners and beginners in the field. We hope that this book stimulates your creativity and wish you success in your experiments.

Maunath Bhanjan, Uttar Pradesh, India — Dilip Kumar Arora
Rourkela, Odisha, India — Surajit Das
Maunath Bhanjan, Uttar Pradesh, India — Mesapogu Sukumar

Contents

Contributors

Bashir A. Akhoon CSIR-Indian Institute of Toxicology Research, Lucknow, India

Dilip K. Arora National Bureau of Agriculturally Important Microorganisms, Kusumaur, Maunath Bhanjan, Uttar Pradesh, India

Sunil G. Babu Department of Biotechnology, School of Bioscience and Biotechnology, Babasaheb Bhimrao Ambedkar University, Lucknow, Uttar Pradesh, India

Bandamaravuri Kishore Babu Cereals Pathology, International Crops Research Institute for the Semi-Arid Tropics, Patancheru, Andhra Pradesh, India

Surajit Das Department of Life Science, National Institute of Technology, Rourkela, Odisha, India

Hirak Ranjan Dash Department of Life Science, National Institute of Technology, Rourkela, Odisha, India

P.P. Dubey Department of Animal Genetics and Breeding, Guru Angad Dev University of Veterinary, Animal Sciences, Ludhiana, Punjab, India

Paramasamy Gunasekaran Department of Genetics, Centre for Excellence in Genomic Sciences, School of Biological Sciences, Madurai Kamaraj University, Madurai, Tamil Nadu, India

Shishir K. Gupta Society for Biological Research and Rural Development, Lucknow, India

Shailendra K. Gupta CSIR-Indian Institute of Toxicology Research, Lucknow, India

Chandra Mouleswararao Jillepalli Department of Chemistry, Kakatiya University, Warangal, Andhra Pradesh, India

Prem L. Kashyap National Bureau of Agriculturally Important Microorganisms, Kusumaur, Maunath Bhanjan, Uttar Pradesh, India

T. Kiran Babu Cereals Pathology, International Crops Research Institute for the Semi-Arid Tropics, Patancheru, Andhra Pradesh, India

Vikas Koundal Division of Plant Pathology, Advanced Center for Plant Virology, Indian Agricultural Research Institute, New Delhi, India

Manish Kumar National Bureau of Agriculturally Important Microorganisms, Maunath Bhanjan, Uttar Pradesh, India

Sudheer Kumar National Bureau of Agriculturally Important Microorganisms, Kusumaur, Maunath Bhanjan, Uttar Pradesh, India

Dinesh Kumar National Bureau of Animal Genetic Resources (NBAGR), Karnal, Haryana, India

Neelam Mangwani Department of Life Science, National Institute of Technology, Rourkela, Odisha, India

Kamlesh K. Meena National Bureau of Agriculturally Important Microorganisms, Maunath Bhanjan, Uttar Pradesh, India

Sukumar Mesapogu National Bureau of Agriculturally Important Microorganisms, Kusumaur, Maunath Bhanjan, Uttar Pradesh, India

Shelly Praveen Division of Plant Pathology, Advanced Center for Plant Virology, Indian Agricultural Research Institute, New Delhi, India

Jeyaprakash Rajendhran Department of Genetics, Centre for Excellence in Genomic Sciences, School of Biological Sciences, Madurai Kamaraj University, Madurai, Tamil Nadu, India

Thangamani Rajesh Department of Genetics, Centre for Excellence in Genomic Sciences, School of Biological Sciences, Madurai Kamaraj University, Madurai, Tamil Nadu, India

Annette Reineke Department of Phytomedicine, Geisenheim Research Centre, Geisenheim, Germany

Ratul Saikia Biotechnology Division, CSIR-North East Institute of Science and Technology, Jorhat, Assam, India

Sangeeta Saxena Department of Biotechnology, School of Bioscience and Biotechnology, Babasaheb Bhimrao Ambedkar University, Lucknow, Uttar Pradesh, India

Anil Kumar Saxena Division of Microbiology, Indian Agriculture Research Institute, New Delhi, India

Anu Sharma National Bureau of Agriculturally Important Microorganisms, Kusumaur, Maunath Bhanjan, Uttar Pradesh, India

Rajan Sharma Cereals Pathology, International Crops Research Institute for the Semi-Arid Tropics, Patancheru, Andhra Pradesh, India

D.P. Singh National Bureau of Agriculturally Important Microorganisms, Maunath Bhanjan, Uttar Pradesh, India

Ruchi Singh National Bureau of Agriculturally Important Microorganisms, Kusumaur, Maunath Bhanjan, Uttar Pradesh, India

Krishna P. Singh CSIR-Indian Institute of Toxicology Research, Lucknow, India

Bhim Pratap Singh Department of Biotechnology, Mizoram University, Mizoram, India

Suchi Smita Society for Biological Research and Rural Development, Lucknow, India

Alok K. Srivastava National Bureau of Agriculturally Important Microorganisms, Kusumaur, Maunath Bhanjan, Uttar Pradesh, India

Hari Kishan Sudini International Crops Research Institute for the Semi-Arid Tropics, Patancheru, India

K. Uma Devi Department of Botany, Andhra University, Visakhapatnam, India

Jyoti Verma Department of Biotechnology, School of Bioscience and Biotechnology, Babasaheb Bhimrao Ambedkar University, Lucknow, Uttar Pradesh, India

Chapter 1

Microbial DNA Extraction, Purification, and Quantitation

Sukumar Mesapogu, Chandra Mouleswararao Jillepalli, and Dilip K. Arora

Abstract

Cell wall of microorganisms is broken by chemical or enzymatic lysis or a combination of both. Generally lysozyme is used to digest the rigid cell wall structure which has high amounts of lipid while detergent like SDS solubilizes the phospholipids in the cell membrane. EDTA destabilizes the cell envelope and deactivates the DNases by chelating with the magnesium ions in the membranes which are essential for integrity of cell envelope. Insoluble cell debris is removed via centrifugation, leaving an upper aqueous suspension containing the DNA, proteins, and RNA. Purification of DNA from proteins can be achieved by various methods, generally by protease treatment, to hydrolyze the proteins resulting in water-soluble amino acids or shaking the aqueous suspension with phenol chloroform. The aqueous phenol emulsion is then separated by centrifugation. Proteins (having both hydrophobic as well as hydrophilic amino acid residues) get collected at the interphase. While RNA can be removed by RNase treatment, DNA can be concentrated by addition of ice-chilled ethanol or isopropanol and precipitated DNA is collected as pellet by centrifugation. This chapter describes the protocol to check the purity and quantify DNA.

1.1 Introduction

Isolation of genomic DNA from microorganisms has become a useful tool to determine the fates of selected microorganisms or recombinant genes and to reveal genotypic diversity and its change in microbial ecosystems. The protocols in this chapter provides a frame work for isolating high quality genomic DNA from a variety of organisms, including bacteria, plasmid DNA [1], actinomycetes, yeast [2, 3], and fungi [4]. All of these protocols yield high molecular weight (HMW) DNA, which remains of high quality (i.e., not degraded in to smaller fragments) for several years when stored as specified below. For each organism a specific procedure is provided for releasing free chromosomal DNA from its cellular or nuclear location. The first task in each of these protocols is the removal of cell wall that is typically lysed in an SDS solution containing sucrose. The released DNA is prevented

D.K. Arora et al. (eds.), *Analyzing Microbes*, Springer Protocols,
DOI 10.1007/978-3-642-34410-7_1,

from degrading DNAses and other proteins by EDTA and proteinase-K respectively the cellular proteins [2].

The chromosomal DNA of the *Escherichia coli* is a large circular molecule of approximately 3.2 kb size. The DNA is attached to the plasma membrane at many points. Being large in size, DNA is prone to mechanical breakage. However, if extraction is performed carefully, large fragments of chromosomal DNA can be obtained with an average length of 1–2 kb. The bacterial cell wall is enclosed in a cytoplasmic membrane and surrounded by a rigid cell wall. With some species, including *E. coli*, the cell wall may itself be enveloped by second outer membrane. All of these barriers have to be disrupted to release the cell components. Techniques for breaking open bacterial cells can be divided in to physical methods, in which cells are disrupted by mechanical forces and chemical methods, where cell lysis is brought about by exposure to chemical agents that affect the integrity of the cell barriers. Chemical methods are most commonly used with bacterial cells when the object is DNA preparation. Chemical lysis generally involves one agent attacking the cell wall and another disrupting the cell membrane. The chemicals that are used depend on the species of bacterium involved, but with *E. coli* and released organisms, weakening of the cell wall is usually brought about by lysozyme, ethylenediamine tetraacetate (EDTA), or combination of both. Lysozyme is an enzyme that is present in egg white and in secretions such as tears and saliva, and which digests the polymeric compounds that give the cell wall its rigidity. On the other hand, EDTA removes magnesium ions that are essential for preserving the overall structure of the cell envelope, and also inhibits cellular enzymes that could degrade DNA. Under some conditions, weakening the cell wall with lysozyme or EDTA is sufficient to cause bacterial cells to burst, but usually a detergent such as sodium dodecyl sulfate (SDS) is also added [5]. Detergents aid the process of lysis by removing lipid molecules and thereby cause disruption of the cell membranes. Having lysed the cells, the final step in preparation of a cell extract is removal of insoluble cell debris. Component such as partially digested cell wall fractions can be pelleted by centrifugation, leaving the cell extract as a reasonably clear supernatant. Most protocols for the preparation of genomic DNA consist of lysis, followed by incubation with a nonspecific protease and a series of extractions prior to precipitation of the nucleic acids. Such procedures effectively remove contaminating proteins, but are not effective in removing exopolysaccharides which can interfere with the activity of enzymes such as restriction endonucleases and ligases. In this unit, however, the protease incubation is followed by a CTAB extraction whereby CTAB complexes with both polysaccharides and residual protein, effectively removing both in the subsequent emulsification and extraction. This procedure is effective in producing digestible chromosomal DNA from a variety of gram-negative bacteria, all

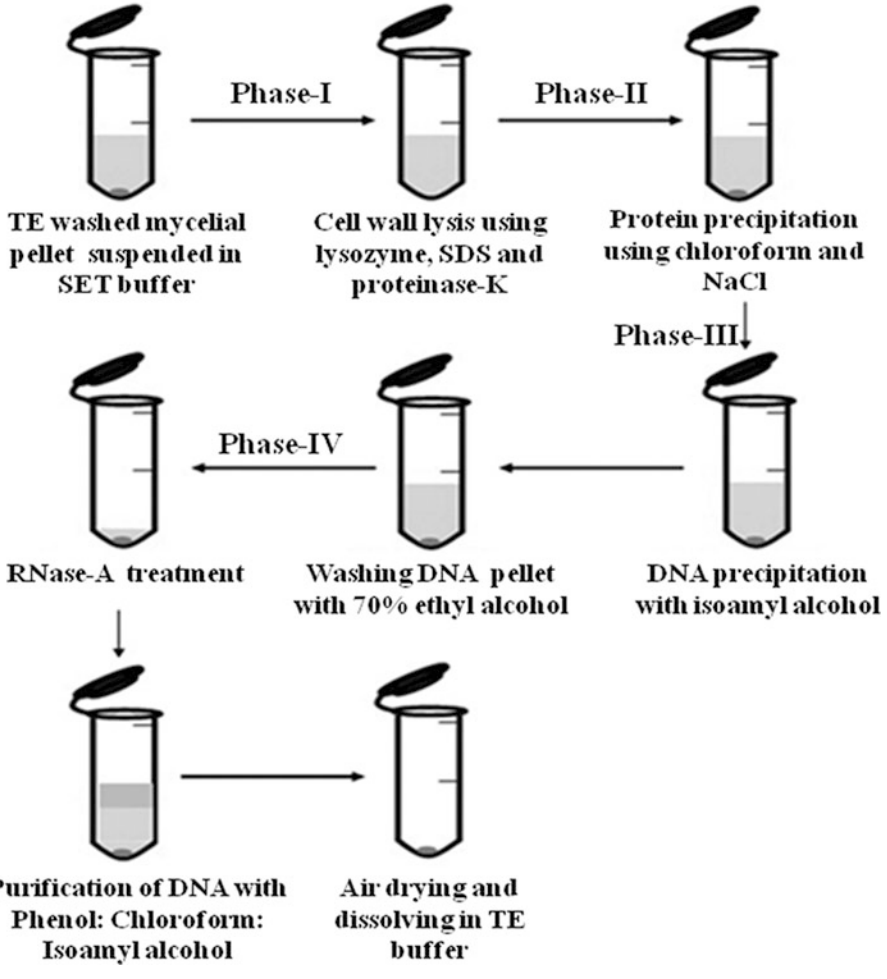

Fig. 1.1 General steps involved in genomic DNA isolation (Phase I: Cell lysis, Phase II: Protein degradation and precipitation, Phase III: DNA precipitation, Phase IV: RNA degradation and precipitation)

of which normally produce large amounts of polysaccharides. The actinomycetes are gram-positive bacteria which have a characteristically high G + C content in their DNA (>55 %). Many species produce a wide variety of secondary metabolites, including antihelminthic compounds, antitumor agents, and the majority of known antibiotics, which have been exploited by their use in medicine and agriculture. The actinomycetes were originally considered to be an intermediate group between bacteria and fungi but now recognized as prokaryotic. The detailed protocol is represented as four phases as shown in Fig. 1.1

It is based on the conformational difference between plasmid and chromosomal DNA of the bacteria. Plasmid molecules are double stranded, circular entities, and generally exist as covalently closed circular molecule (supercoiled form), while chromosomal DNA molecule exists as linear double stranded molecule. So use is

made of the fact that linear double stranded DNA is denatured by exposing to high pH values of the lysing solution (in the range of pH 12–12.5). On the other hand, the covalently closed circular plasmid DNA in supercoiled form is resistant to these conditions. So when the pH is lowered in further steps, renaturation takes place and plasmid renatures faster (if denatured) than the chromosomal DNA. Because the renaturation is done at cool temperature and also pH lowering is sharp, as a result the whole chromosomal DNA forms an insoluble clump (aggregate) because of the mis-matched base-pairing. The aggregate DNA can easily be separated by centrifugation, as plasmid remains in the supernatant while aggregate chromosomal DNA forms pellet [5].

The need to adapt organic extraction methods to take account of the biochemical contents of different types of starting material has stimulated the search for DNA purifications methods that can be used with any species. This is one of the reasons why ion-exchange chromatography has become so popular. A similar method involves a compound called guanidinium thiocyanate, which has two properties that make it useful for DNA purification. First it denatures and dissolves all biochemicals other than nucleic acids and can therefore be used to release DNA virtually from any type of cell or tissue. Second, guanidinium thiocyanate allows DNA to bind tightly to silica particles. This provides an easy way of recovering the DNA from the denatured cell extracts [6]. One possibility is to add the silica directly to the cell extract but, as with the ion-exchange methods, it is more convenient. In addition to DNA, the cell extract contain significant quantities of protein and RNA. A variety of methods can be used to purify the DNA from this mixture. One approach is to treat mixture with reagents which degrade the contaminants, leaving a pure solution of DNA. The standard way to deproteinize a cell extract is to add phenol or a 1:1 mixture of phenol and chloroform. These organic solutions precipitate proteins but leave the nucleic acids (DNA and RNA) in an aqueous solution. The result is that if the cell extract is mixed gently with the solvent and the layers then separated by centrifugation, precipitated protein molecules are left as a white coagulated mass at the interface between the aqueous and organic layers. The aqueous solution of nucleic acids can then be removed with a pipette [7].

With some cell extract, the protein content is so great that a single phenol extraction is not sufficient to purify nucleic acids completely. This problem could be solved by carrying out several phenol extractions one after the other, but this is undesirable as each mixing and centrifugation step results in a certain amount of breakage of the DNA molecules. This can be solved by treating the cell extract with protease such as pronase or protease K before phenol extraction. These enzymes break polypeptides down into smaller units, which are more easily removed by phenol. Some

RNA molecules, especially mRNA, are removed by phenol treatment, but most remain with the DNA in the aqueous layer. The only effective way to remove the RNA is with the enzyme ribonuclease, which rapidly degrades these molecules into ribonucleotide subunits. A second useful method is drop dialysis, which can remove salt, SDS, and even some enzyme inhibitors. As such, it can be used with many methods involving DNA purification before or after enzymatic reactions. DNA fragments larger than a few 100 base pairs can be separated from smaller fragments by chromatography on a size exclusion column such as Sephacryl S-500. To simplify this procedure, the following mini-spin column method has been developed [6]. For fragments from 200 bp to 10 kb the agarose purification is ideal. For smaller fragments (20–400 bp), the acrylamide purification is preferred.

Ultra violet (UV) spectrophotometry is most commonly used for the determination of DNA concentration. The resonance structure of pyrimidine and purines are responsible for these absorptions. The DNA has a maximum and minimum absorbance at 260 nm. However, these are strongly affected by the degree of base ionization and hence pH of the measuring medium. If at A260/A280 the purity of DNA is out of the 1.8–2.0 range, then the DNA should be purified to remove contaminants. Absorbance measurements at wave lengths other than 260 nm are used for determination of DNA purity. The relevant spectrum for this purpose lies between 320 and 220 nm. Any absorbance at 320 nm indicates contamination of particular nature. Proteins absorb maximally 280 nm due to the presence of tyrosine, phenylalanine, and tryptophan and absorption at this wavelength is used for detection of proteins in DNA samples. This is usually done by determination of the A260/A280 ratio [8].

1.2 Materials

1.2.1. Bacterial (E. coli) *DNA Isolation*

1. Luria Bertani (LB) Broth
2. TE buffer—50 mM Tris, 50 mM EDTA (pH 8.0)
3. Tris (pH 8.0)—250 mM
4. Lysozyme—10 mg/ml
5. SDS—0.5 %
6. EDTA—0.4 M
7. Proteinase K—1 mg/ml
8. Phenol equilibrated with Tris (Phenol is a hazardous organic solvent. Always use suitable laboratory gloves when handling phenol containing solutions. Specific waste procedures may be required for the disposal of phenol containing solutions.)

9. Sodium acetate (pH 5.8)
10. Ethanol—95 %
11. RNase—200 μg/ml
12. Chloroform

1.2.2. Gram −ve Bacterial DNA Isolation by CTAB Method

1. Nutrient broth—25 ml
2. Tris EDTA (pH 8.0) (10 mM Tris-Cl, 1 mM EDTA)
3. 10 % SDS
4. 20 mg/ml proteinase K
5. 5 M NaCl
6. CTAB/NaCl Solution—Dissolve 4.1 g NaCl in 30 ml water and slowly add 10 g cetyltrimethylammonium bromide (CTAB) while stirring. If necessary, heat to 65 °C. Adjust to 100 ml
7. Chloroform
8. Isoamyl Alcohol
9. Buffered Phenol (8-Hydroxyquinoline, Liquefied phenol redistilled, 50 mM Tris-Cl, pH 8.0, TE buffer pH 8.0): Add 0.5 g of 8-hydroxyquinoline to a 2 l glass beaker. Gently add 500 ml liquefied phenol (crystals of redistilled phenol melted in a 65 °C bath). The phenol will turn yellow due to the 8-hydroxyquinoline, which is added as an antioxidant. Add 500 ml of 50 mM Tris base, cover with aluminum foil, and stir 10 min at low speed. Let phases separate at room temperature and gently decant the top (aqueous) phase into a suitable waste receptacle. Remove any residual aqueous phase with a glass pipette. Repeat twice with 500 ml each of 50 mM Tris-Cl, pH 8.0. Check pH of phenol with pH paper and repeat equilibration until pH = 8.0 and store at 4 °C in brown glass bottles or in clear glass bottles wrapped in aluminum foil
10. Isopropanol
11. 70 % (v/v Ethanol)

1.2.3. Plasmid DNA Isolation

1. Luria Bertani (LB) medium supplemented with proper antibiotic
2. Lysis buffer I
   (a) 25 mM Tris-Cl (pH 8.0)
   (b) 50 mM Glucose
   (c) 10 mM EDTA (pH 8.0)
   (d) 0.2 mg/ml RNase A

3. Lysis buffer II (always freshly prepared)
   (a) 0.2 NaOH
   (b) 1% (w/v) SDS
4. Lysis buffer III
   (a) 3 M potassium acetate (pH 5.5)
5. Chloroform: isoamyl alcohol (24:1)
6. Isopropanol
7. Ethanol (70 %)
8. TE buffer (pH 8.0)

1.2.4. Actinomycetes DNA Isolation

1. GYM broth
   (a) 4.0 g Glucose, 4.0 g Yeast extract, 10.0 g Malt extract, 1 L Distilled water, pH 7.4
2. SET buffer
   (a) 75 mM NaCl, 25 mM EDTA, 20 mM Tris, pH 7.5
3. SDS (10 %)
4. Lysozyme (10 mg/ml)
5. Proteinase K (20 mg/ml)
6. Rnase A (10 mg/ml)
7. 5 M NaCl
8. Phenol
9. Chloroform
10. Isoamyl alcohol
11. Isopropanol
12. Ethanol
13. Sodium acetate 3 M (pH 5.2)

1.2.5. Yeast DNA Isolation

1. Yeast extraction buffer A: 2 % Triton X-100, 1 % sodium dodecyl sulfate, 100 mM NaCl, 10 mM Tris–HCl, pH 8.0, 1 mM EDTA pH 8.0, Phenol: chloroform: isoamylalcohol: phenol is presaturated with 10 mM Tris–HCl, pH 7.5.
2. Prepare a mixture of 25:24:1 phenol:chloroform:isoamyl alcohol (v/v/v). This solution can be stored at room temperature for up to 6 months, shielded from light.
3. Glass beads, diameter range 0.04–0.07 mm. Suspended as 500 mg/ml slurry in distiller water.
4. Ammonium acetate (4 M).