Isolation, Characterization and Identification of Antibiotic Producing Bacteria

BACKGROUND

Antibiotics are one of the most important discoveries of humankind in the modern world. Antibiotics are chemicals that kill or inhibit the growth of bacteria, and most are synthesized by bacteria or fungi, but some are synthesized in the lab by chemists (synthetic and semisynthetic antibiotics). What makes antibiotics so special are that they target bacterial cells, but not host cells. Thus, they are used to treat bacterial infections and have saved millions of lives both humans and animals. They can be administered in various ways such as by ingestion, intramuscular injection, intravenous injection or topically (applied to the surface). They differ from antiseptics and disinfectants in that antibiotics can be used internally. Both antiseptics and disinfectants are too harsh for internal use, but antiseptics may be used topically (for example certain peroxides). Disinfectants (such as bleach) can only be used on inanimate surfaces.

Certain antibiotics may kill the bacterial cells (bactericidal) and others may just inhibit their growth (bacteriostatic). Examples of targets affected by antibiotics are shown in the Figure below. For example, peptidoglycan is found in the cell wall of Gram-positive bacteria. Peptidoglycan synthesis is affected by beta-lactam antibiotics that target the enzymes that form the cross linkages between the glycan strands. As the bacterial cell grows it must make more cell wall, but in the presence of the antibiotic less cross linkages are formed and the newly synthesized structure is weakened. Water can enter the cell by osmosis and due to the weakened cell wall, the cell bursts and dies. Another common target of antibiotics is the bacterial ribosome which is composed of a 30S and a 50S ribosomal subunit. Each subunit is composed of various ribosomal proteins and rRNA species. Certain antibiotics target a specific bacterial subunit that affects the proper functioning of the ribosome and inhibits protein synthesis. Eukaryotic ribosomes are composed of 40S and 60S subunits (each containing a different set of proteins and rRNA) and are therefore not targeted by these antibiotics. Other possible targets of antibiotics include the bacterial RNA polymerase, DNA gyrase, cell membrane components and certain bacterial metabolic processes.
The initial discovery of antibiotics occurred in the late 1920s and 1930s and the first useful antibiotic was penicillin (a beta-lactam antibiotic). Penicillin was "accidentally" discovered by Alexander Fleming. He noticed that a fungus (*Penicillium*) was producing a substance that prevented the growth of bacteria on a discarded agar medium plate. Howard Florey and Ernst Chain took this discovery to the next step which led to the successful application of penicillin in the treatment of infections. All three were awarded the Nobel Prize in 1945 for their work with penicillin. Selman Waxman adapted Fleming's observations into a screening method to find other microorganisms that produced similar antibacterial substances. His screening technique involved growing potential antibiotic producing microorganisms on the same medium as a pathogen, and looking for inhibition of growth of the pathogen by the other microorganisms. This technique led to the discovery of streptomycin from *Streptomyces griseus* and a flood of other antibiotics in the 1940s to the 1960s. The Figure below shows the year of discovery of various antibiotics and the year of their application to clinical medicine.
The above Figure also shows that the discovery of new types of antibiotics have slowed in the 50 years since the golden age of antibiotic discovery. This is an unfortunate event in that during this same period of time, antibiotic resistance among bacterial strains has increased, thus making it more difficult to treat bacterial infections. Resistance to antibiotics can arise by various means. For example, the bacterial target of an antibiotic can be modified by a genetic mutation or by a bacterial enzyme, so the target no longer interacts with the antibiotic. An antibiotic can also be modified or completely destroyed by a bacterial enzyme so it no longer can function (for example, some bacteria synthesize beta-lactamases that cleave beta-lactam antibiotics to neutralize their activities). An antibiotic can also be prevented from entering the bacterial cell or if the antibiotic gets into the cell, it is pumped back out. Examples of specific pathogens that have become multiply resistant to several antibiotics include methicillin resistant *Staphylococcus aureus* (MRSA) and extensively drug resistant (XDR) *Mycobacterium tuberculosis*. Needless to say, new methods of antibiotic discovery must be pursued in order to keep up with the evolving rise in bacterial resistance.

Several approaches to discover new antibiotics have been implemented with some success. High-throughput miniaturized screening methods can be automated to assay millions of different bacteria for the production of antimicrobial substances. This is basically a brute force approach to find that
rare one-in-a-million antibiotic-producing bacterium that has not been discovered previously. Another approach is to search for microorganisms that live in types of niches that have not been explored before. Only a small fraction of the earth's total microbiota has been sampled for antibiotics, and there is potentially a vast amount of microbes yet to be tested. Combinatorial biosynthesis involves genetically introducing new metabolic pathways into a microorganism. It is possible that the interaction of these new pathways with the host's metabolic pathways could result in the production of novel compounds with antimicrobial activities. The relative ease and low cost of DNA sequencing of whole bacterial genomes makes genome mining a promising technique. Basically it involves searching the entire genome sequence for genes or certain DNA motifs related to the synthesis of anti-bacterial products. This was recently accomplished by screening genomes from 10,000 Actinomycetes for the production of new phosphonic acid products with antibiotic activities (Ju et al., 2015).

The genus *Streptomyces* is still one of the most prolific antibiotic producing microorganisms known. Some have estimated that the total number of possible antimicrobial agents produced by the genus *Streptomyces* is about 100,000 (Arch Microbiol (2001) 176:386-390). *Streptomyces* belong to the general group of bacteria called Actinomycetes. Actinomycetes are a very diverse group of aerobes, also known as high G+C Gram-positive bacteria found in the soil. These organisms are typically characterized by their filamentous growth phase and complex developmental lifecycle involving cellular differentiation and the formation of desiccation and heat resistant spores. However, these spores (which can be killed by boiling) are not as heat resistant as those studied in the "Endospore-Forming Bacteria" Exercise you performed earlier in the course. A medium to grow Actinomycetes was described by El-Nakeeb and Lechevalier (1962) and can be modified for use in an enrichment procedure based on the bacterium's resistance to dessication and their ability to utilize the amino acid arginine as a source of nitrogen. Note: this method is not entirely selective for Actinomycetes, but instead is a true enrichment in that most, but not all of the organisms that grow on this medium will be of the desired class.

Following isolation and growth of a bacterium from an environmental sample, the identity of a bacterial isolate can be determined by classical approaches involving morphological and biochemical studies (see the "Identification of Bacteria" Lab Exercise). In addition, more modern techniques have been developed involving antibodies and DNA studies. In 1977, Carl Woese at the University of Illinois, was studying certain microorganisms that survived extreme environments and found that they had characteristics similar to both bacteria and eukaryotes. This was discovered when analyzing the sequences of certain rRNA genes from different microorganisms. This led to the important discovery of the three domains of life: bacteria, eukarya and archaea. Woese was later awarded the Crafoord Prize in Bioscience (comparable to the Nobel Prize) in 2003 for this discovery. Today, rRNA is routinely used to characterize and identify microorganisms. The rRNA gene is essential for all cellular life forms and is
transcribed into rRNA that makes up ribosomes. Certain portions of the rRNA gene sequence are conserved among microorganisms (for example the ends of the genes), whereas other internal portions are variable. DNA primers that anneal to the conserved regions can be used to amplify the whole rRNA gene by polymerase chain reaction (PCR). The amplified gene can then be sequenced and the variable regions analyzed to form phylogenetic trees depicting genetic relatedness between isolates to determine the identity of the unknown microorganism. Additional information about an unknown microorganism can also be elucidated by sequencing its entire genome and analyzing it for specific gene sequences (as previously mentioned with regards to genome mining). Not only would its identity become clearer, but also its metabolic capabilities could be uncovered.

In this Laboratory Exercise you will enrich for Actinomycetes from a soil sample and generate pure cultures of those microorganisms. Those microorganisms will be screened for the production of anti-bacterial substances and the identity of those microorganisms will be determined. Molecular genetic studies will assist in both the identification of the bacterial isolate (16S rRNA gene sequencing) as well as the assessment of their potential to produce antibiotics (genome mining).

Additional articles to be read related to this Exercise are provided in the MCB301 website and include the following:

El-Nakeeb, M.A. and H.A. Lechevalier (1963) Selective Isolation of Aerobic Actinomycetes. Appl. Microbiol. 11:75-77. This article describes an AGS medium to grow Actinomycetes and was modified for use in an enrichment protocol to be performed in the MCB301 Lab.


Ju, K.-S. et al. (2015) Discovery of phosphonic acid natural products by mining the genomes of 10,000 actinomycetes. PNAS Early Edition. 1-6. This is a recent journal article from the research group at Carl R. Woese Institute of Genomic Biology, University of Illinois, Urbana, Illinois, led by William Metcalf and Wilfred van der Donk. Although the details of this article are beyond the scope of this course, briefly look it over and just focus on understanding how genome mining was utilized in their research.
ENRICHMENT PROTOCOL

1. Label the side of a clean aluminum cup with your initials. Carefully transfer soil to the cup. Dry soil for 48 hours in the drying oven.

2. Transfer dried soil to a sifter and sift soil into a clean petri plate.

3. Transfer a spatula full of sifted soil to a clean 50 ml centrifuge tube. Add approximately 35 ml of sterile water to the tube and mix by vortexing vigorously until soil is fully resuspended.

4. Transfer 1 ml of the liquid portion of the suspension to a clean microcentrifuge tube. Prepare a 1:10 dilution of the suspension (100 µl of undiluted liquid suspension + 900 µl sterile saline) and a 1:100 dilution of the suspension (100 µl of the 1:10 dilution + 900 µl sterile saline).

5. Spread plate 100 µl of undiluted suspension and 100 µl of each dilution onto separate AGS + cyclohexamide plates and incubate at 30°C for 3-10 days. Cyclohexamide inhibits the growth of eukaryotic fungi, whose spores can also survive desiccation.

6. Pick two different colonies to purify by plate streaking. The colonies are often brightly pigmented (yellow, red, purple, green, black) due to the production of secondary metabolites (natural products), which often have useful medicinal properties including antibiotics, antifungals, immunosuppressive compounds and more. Streak separate AGS plate with each of your selected colonies. Incubate at 30°C for 3-10 days.

7. From your initial isolation plate prepare a Gram stain slide and a wet mount slide. Record your observations. Streak your characterized colonies onto fresh LB plates and incubate them at 30°C for 48 hours.

8. From your second streak plates, prepare a Gram stain slide and a wet mount slide. Record your observations. Pick a well-isolated colony from each of your plates into separate bottles of sterile LB media, and incubate them at 30°C for 48 hours.

DETERMINATION OF ANTIMICROBIAL SPECTRUM OF SOIL ISOLATES

1. Obtain three trypticase soy agar plates and label each of them as follows: a) the soil isolate identification number, b) positive control sample, and c) negative control sample.
2. Using sterile technique, make a single streak inoculation of each of the above bacteria on the surface of the appropriate agar medium plate, so as to divide the plate in half.

3. Incubate the plates in an inverted position for 3-5 days at (30°C).

4. Following incubation, on the bottom of the plate draw 4 lines perpendicular to the bacterial growth of the soil isolate.

5. Using sterile technique, make a single streak inoculation of each of the 4 test cultures (overnight trypticase soy broth cultures of *E. coli* (Gram-negative), *Staphylococcus aureus* (Gram-positive), *Mycobacterium smegmatis* (acid-fast), and *Pseudomonas aeruginosa* (Gram-negative). Start the streak close to but not touching the growth of the soil isolate and ending near the side of the plate.

6. Incubate the plates inverted overnight at 37°C.

7. Assess the inhibition of growth of the test cultures by the soil samples versus by the controls.

**DNA ISOLATION PROTOCOL**

You will be preparing a total of 4 extractions of DNA:

1) Isolate #1
2) Isolate #2
3) *Streptomyces* sp. (Positive control)
4) Extraction Buffer (Negative control; skip steps 1 and 2 below)

Given how easy it is to contaminate DNA preparations, it is important that you wear gloves throughout the procedure and strictly follow aseptic technique.

1. Briefly vortex your liquid culture.

2. Transfer 1 ml of culture to a sterile 1.5 ml microcentrifuge tube. Centrifuge 30 sec at 10,000 x g. Discard the supernatant. Repeat this step until you have a cell pellet approximately 35 µl in size.

3. Resuspend each pellet in 180 µl of lysis buffer (20 mg/ml lysozyme, 20 mM Tris, 2mM EDTA, 1.2% Triton X-100, pH 8) and mix by vortexing briefly. The lysozyme acts to degrade the thick peptidoglycan cell wall found in Gram-positive bacteria. Triton X-100 is a detergent which disrupts the integrity of membranes. The Tris buffers the pH, and the EDTA chelates divalent metal ions. Nucleases require the metal ions to function so EDTA limits spontaneous cleavage of DNA.
4. Incubate the tubes for 30 min at 37°C.

5. Add 200 µl of proteinase K (100 µg/ml). Proteinase K will degrade proteins released from the cells when you lyse them in the next step.

6. Add 200 µl of AL buffer and mix by briefly vortexing. This is an alkaline solution which will rapidly lyse the cells.

7. Incubate the tubes for 30 min at 56°C.

8. Add 200 µl of 100% ethanol to each tube and mix by vortexing briefly. This helps to precipitate nucleic acids in preparation for purifying by using the spin columns.

9. Transfer the contents of each tube to a clean QIAamp spin column and make sure the spin column is in a collection tube. The spin column contains a membrane that will bind the DNA.

10. Centrifuge the tubes for 60 sec at 6,000 x g and discard the flow through.

11. Add 500 µl of AW1 buffer to the spin columns, centrifuge for 60 sec at 6,000 x g, and discard the flow through. This step removes most of the proteins from your DNA preparation.

12. Add 500 µl of AW2 buffer to the spin columns, centrifuge for 60 sec at 14,000 x g and discard the flow through. This step removes most of the RNA from your DNA preparation.

13. Centrifuge the spin columns again for 60 sec at 14,000 x g. This step is important to remove as much residual buffer as possible to prevent interference in later procedures.

14. Transfer the spin columns to clean 1.5 ml microcentrifuge tubes and discard the used collection tubes.

15. Add 200 µl of AE buffer to each spin column and incubate for 5 min at room temperature. This buffer unbinds the DNA from the membrane found in the spin column.

16. Centrifuge for 3 min at 6,000 x g and discard the spin column. This elutes your purified genomic DNA.
PCR PROTOCOL

This PCR protocol is designed to amplify a region of the 16S rRNA gene ranging from position 27 to 1492. The primers used in this PCR are designed to anneal to highly conserved regions of the 16S rRNA gene so that they are able to amplify the gene from a very broad range of bacterial species. Given how easy it is to contaminate PCR preparations, it is important that you wear gloves throughout the procedure and strictly follow aseptic technique.

1. Add 100 µl of GoTaq Master Mix to a sterile 1.5 ml microcentrifuge tube.
2. Add 68 µl of sterile water to the tube.
3. Add 12 µl of the forward primer to the tube.
4. Add 12 µl of the reverse primer to the tube.
5. Mix by briefly vortexing.
6. Aliquot 47 µl of your prepared PCR mix to each of your 4 PCR tubes. If the last tube is a little short, that is fine. That tube is your negative control, and we are only interested in whether or not there is amplification, not yield.
7. Add 3 µl of DNA template to the appropriate PCR tubes:
   a. Tube #1 – Your Isolate DNA #1
   b. Tube #2 – Your Isolate DNA #2
   c. Tube #3 – Positive Control DNA
   d. Tube #4 – Negative Control Extraction
8. Give your PCR tubes to your TA who will place them in the thermocycler.

GEL ELECTROPHORESIS PROTOCOL

1. On a piece of clean parafilm, place 4 isolated 1.5 µl drops of gel loading buffer.
2. Remove 5 µl from each PCR tube, adding each to a different drop of loading dye. It would be in your best interest to keep track of which drop is which PCR tube.
3. Adjust the micropipette to 6 µl.
4. Gently and slowly pipette the first drop up and down 3-4 times to mix. Go slowly, you do not want to introduce a lot of bubbles.

5. Draw up the mixed drop and load it in the appropriate well on your gel.

6. Repeat steps 2-5 for each of your PCR tubes.

7. Load 5 µl of a prepared DNA ladder into the well immediately next to you negative control well.

8. Run the gel at 85 V for approximately 30-40 minutes (until the marker dye is between ½ to ¾ of the way down the gel).

9. Visualize on the gel imager. Your PCR product bands should be approximately 1.5 kbp in size, and you should have no band in your negative control lane.

**PCR CLEANUP**

1. Transfer the remaining PCR products from each of your PCR amplifications to separate clean 1.5 ml microcentrifuge tubes.

2. Add 250 µl PB buffer and mix by briefly vortexing.

3. Transfer the contents of each tube to a clean QIAquick column in a collection tube.
4. Centrifuge 60 sec at 14,000 x g and discard flow through.

5. Add 750 µl of PE Buffer to the spin columns.

6. Centrifuge 60 sec at 14,000 x g and discard flow through.

7. Centrifuge 60 sec at 14,000 x g and discard flow through.

8. Transfer spin columns to a clean 1.5 ml microcentrifuge.

9. Add 50 µl of EB buffer to the spin columns.

10. Incubate 5 min at room temperature.

11. Centrifuge for 2 min at 14,000 x g and discard the spin column.

12. Store purified PCR products at -20°C.
SEQUENCING OF THE PCR PRODUCT

Based on the results of your antimicrobial spectrum experiment, you and your partner will select one of your isolates for identification by DNA sequencing. Your TA will provide you with a label to affix to the tube so that your sequencing can be identified.
ENRICHMENT MEDIA

Modular AGS medium (per liter distilled water)

100X Freshwater Base: 10.0 ml
1 M MOPS, pH 7.2: 20.0 ml
Arginine monohydrochloride: 1.0 g
Glycerol: 12.50 ml
Trace elements solution: 1.0 ml
1 M Na$_2$SO$_4$: 0.2 ml
150 mM Potassium phosphate (pH 7.2): 1.0 ml
Agar: 15.0 g

Autoclave, then add:

1. 0.1 ml multi-vitamin mix solution
2. 0.1 ml Vitamin B$_{12}$ solution
3. 1.0 ml cyclohexamide from a sterile stock solution (50 mg/ml in 100% ethanol)

NOTE: Cyclohexamide is a biohazard and extreme caution needs to be taken when using it to avoid contact. It should be handled with appropriate Personal Protective Equipment (especially gloves and eyewear!) and within a chemical hood or biological safety cabinet.

100X Freshwater Base (per liter)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
<th>FW</th>
<th>100X Conc.</th>
<th>Final Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>100 g</td>
<td>58.44</td>
<td>1,711 mM</td>
<td>17.1 mM</td>
</tr>
<tr>
<td>MgC$_{12}$·6H$_2$O</td>
<td>40 g</td>
<td>203.30</td>
<td>197 mM</td>
<td>1.97 mM</td>
</tr>
<tr>
<td>CaC$_{12}$·2H$_2$O</td>
<td>40 g</td>
<td>147.02</td>
<td>14.7 mM</td>
<td>0.15 mM</td>
</tr>
<tr>
<td>KCl</td>
<td>50 g</td>
<td>74.56</td>
<td>671 mM</td>
<td>6.71 mM</td>
</tr>
</tbody>
</table>

Keep on shelf in clean bottle, not sterile (best to make fresh).

10,000X Na$_2$SO$_4$ (per 100 ml)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
<th>FW</th>
<th>1,000X Conc.</th>
<th>Final Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na$_2$SO$_4$</td>
<td>14.2 g</td>
<td>142.04</td>
<td>1,000 mM</td>
<td>1 mM</td>
</tr>
</tbody>
</table>
### 1,000X K$_2$HPO$_4$ (per 100 ml)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
<th>FW</th>
<th>1,000X Conc.</th>
<th>Final Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>K$_2$HPO$_4$</td>
<td>14.2 g</td>
<td>141.96</td>
<td>1,000 mM</td>
<td>1 mM</td>
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</table>

### 20X MOPS buffer (per 1000 ml)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
<th>FW</th>
<th>20X Conc.</th>
<th>Final Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOPS free acid</td>
<td>209.26 g</td>
<td>209.26</td>
<td>1,000 mM</td>
<td>50 mM</td>
</tr>
</tbody>
</table>

- Dissolve MOPS in approximately 750 ml H$_2$O, adjust pH with 5 M NaOH, adjust volume to 1,000 ml.
- Final pH can be any desired value between 6.8 and 7.6, (pKa of MOPS is 7.2 at 25°C).
- Note that some organisms can use MOPS as a carbon and/or sulfur source.
- Filter sterilize.
- Add to base medium after autoclaving.

### 1,000x Trace Elements Solution (HCl-Dissolved)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
<th>FW</th>
<th>1,000X Conc.</th>
<th>Final Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 mM HCl</td>
<td>1,000 ml</td>
<td>na</td>
<td>20 mM</td>
<td>20 µM</td>
</tr>
<tr>
<td>FeSO$_4$·7H$_2$O</td>
<td>2,100 mg</td>
<td>278.01</td>
<td>7.5 mM</td>
<td>7.5 µM</td>
</tr>
<tr>
<td>H$_3$BO$_3$</td>
<td>30 mg</td>
<td>61.83</td>
<td>0.48 mM</td>
<td>0.48 µM</td>
</tr>
<tr>
<td>MnC$_12$·4H$_2$O</td>
<td>100 mg</td>
<td>197.91</td>
<td>0.5 mM</td>
<td>0.5 µM</td>
</tr>
<tr>
<td>CoC$_12$·6H$_2$O</td>
<td>190 mg</td>
<td>237.93</td>
<td>6.8 mM</td>
<td>6.8 µM</td>
</tr>
<tr>
<td>NiC$_12$·6H$_2$O</td>
<td>24 mg</td>
<td>237.69</td>
<td>1.0 mM</td>
<td>1.0 µM</td>
</tr>
<tr>
<td>CuC$_12$·2H$_2$O</td>
<td>2 mg</td>
<td>170.48</td>
<td>12 µM</td>
<td>12 nM</td>
</tr>
<tr>
<td>ZnSO$_4$·7H$_2$O</td>
<td>144 mg</td>
<td>287.56</td>
<td>0.5 mM</td>
<td>0.5 µM</td>
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<tr>
<td>Na$_2$MoO$_4$·2H$_2$O</td>
<td>36 mg</td>
<td>241.95</td>
<td>0.15 mM</td>
<td>0.15 µM</td>
</tr>
<tr>
<td>NaVO$_3$</td>
<td>25 mg</td>
<td>121.93</td>
<td>2.0 mM</td>
<td>2.0 µM</td>
</tr>
<tr>
<td>Na$_2$WO$_4$·2H$_2$O</td>
<td>25 mg</td>
<td>329.85</td>
<td>75 µM</td>
<td>75 nM</td>
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<tr>
<td>Na$_2$SeO$_3$·5H$_2$O</td>
<td>6 mg</td>
<td>263.01</td>
<td>23 µM</td>
<td>23 nM</td>
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### 1000x Multi-Vitamin Mix Solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
<th>1,000X Conc.</th>
<th>Final Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM MOPS, pH 7.2</td>
<td>100 ml</td>
<td>10 mM</td>
<td>10 µM</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>100 mg</td>
<td>1.0 mg/ml</td>
<td>1.0 µg/ml</td>
</tr>
<tr>
<td>Biotin</td>
<td>30 mg</td>
<td>0.3 mg/ml</td>
<td>0.3 µg/ml</td>
</tr>
<tr>
<td>Thiamine HCl</td>
<td>100 mg</td>
<td>1.0 mg/ml</td>
<td>1.0 µg/ml</td>
</tr>
<tr>
<td>L-Ascorbic acid</td>
<td>100 mg</td>
<td>1.0 mg/ml</td>
<td>1.0 µg/ml</td>
</tr>
<tr>
<td>d-Ca-pantothenate</td>
<td>100 mg</td>
<td>1.0 mg/ml</td>
<td>1.0 µg/ml</td>
</tr>
<tr>
<td>Folic acid</td>
<td>100 mg</td>
<td>1.0 mg/ml</td>
<td>1.0 µg/ml</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>100 mg</td>
<td>1.0 mg/ml</td>
<td>1.0 µg/ml</td>
</tr>
<tr>
<td>4-aminobenzoic acid</td>
<td>100 mg</td>
<td>1.0 mg/ml</td>
<td>1.0 µg/ml</td>
</tr>
<tr>
<td>pyrodixine HCl</td>
<td>100 mg</td>
<td>1.0 mg/ml</td>
<td>1.0 µg/ml</td>
</tr>
<tr>
<td>Lipoic acid</td>
<td>100 mg</td>
<td>1.0 mg/ml</td>
<td>1.0 µg/ml</td>
</tr>
<tr>
<td>NAD</td>
<td>100 mg</td>
<td>1.0 mg/ml</td>
<td>1.0 µg/ml</td>
</tr>
<tr>
<td>Thiamine pyrophosphate</td>
<td>100 mg</td>
<td>1.0 mg/ml</td>
<td>1.0 µg/ml</td>
</tr>
</tbody>
</table>

- Titrate with 5 M NaOH (5-10 drops) until dissolved. Filter to sterilize and freeze in 10-ml aliquots.

- **Add to base medium after autoclaving.**
  
  Sterilize by filtration using a 0.22-micron filter.
  
  Store in the dark at 4°C.

### 1,000x Vitamin B₁₂ Solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>100 ml</td>
</tr>
<tr>
<td>Cyanocobalamin</td>
<td>100 mg</td>
</tr>
</tbody>
</table>

### Tryptic Soy Agar (1 liter, pH 7.3)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>15.0 g</td>
</tr>
<tr>
<td>Soytone</td>
<td>5.0 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0 g</td>
</tr>
</tbody>
</table>