Extraction of Violacein from *S. cerevisiae*

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The goal of these experiments is for students to isolate and extract violacein from *S. cerevisiae.* In the first three experiments, students can culture yeast genetically engineered to express violacein, autolyze the yeast cells, and extract the violacein. Optional experiments allow students and professional scientists alike to purify the compound and test its antimicrobial effectiveness. In the fourth experiment, violacein is purified by small-scale column chromatography, and in the fifth experiment, its antimicrobial effectiveness is tested through a Disk Diffusion Assay (DDA). Note that some of the chemicals used in the extractions are hazardous, and some steps must be performed quickly, so we suggest the extraction experiments for more advanced students.

OVERVIEW

In an era where more and more bacteria are building up antibiotic immunities, it is important that providers have access to many different pharmacological options. Researchers have already found that violacein, a hydrophobic compound naturally produced by the bacterium *C. violaceum*, has some form of antimicrobial activity. In a 2019 study, researchers found that the antimicrobial target of violacein is the cytoplasmic membrane of bacteria.[1] Violacein originates from a novel drug class known as cytotoxic drugs.[2] These biological compounds are known to have chemicals that destroy health cells and is the reason that violacein can be used as an anticancer agent.[3] Despite this research, it is unknown if violacein harvested from *S. cerevisiae* yeast has similar properties. This procedure is the beginning to answering that question.

Column chromatography is a common biochemical technique that is used to purify organic compounds. It helps to separate the violacein from other biological remnants that weren't taken out during chemical purification and autolysis. In this procedure, the dry method is used to prepare the column with aluminum oxide, known as the stationary phase. The sample is then passed through the stationary phase. As the sample moves down the column, the different components of it are separated and then collected at the bottom. This ensures that our final product is as close to pure violacein as possible.

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[1] Cauz, A., Carretero, G., Saraiva, G., Park, P., Mortara, L., Cuccovia, I. M., Brocchi, M., & Gueiros-Filho, F. J. (2019). Violacein Targets the Cytoplasmic Membrane of Bacteria. *ACS infectious diseases*, *5*(4), 539–549. https://doi.org/10.1021/acsinfecdis.8b00245

[2] *Violacein from Janthinobacterium lividum >98% (violacein (minimum 85% violacein) AND Deoxyviolacein, HPLC): Sigma-Aldrich*. >98% (violacein (minimum 85% violacein) and deoxyviolacein, HPLC) | 548-54-9. (n.d.). https://www.sigmaaldrich.com/US/en/product/sigma/v9389.

[3] *Safe handling of cytotoxic drugs in the workplace.* Safe handling of cytotoxic drugs in the workplace - Health and Social Care. (n.d.). https://www.hse.gov.uk/healthservices/safe-use-cytotoxic-drugs

MATERIALS AND EQUIPMENT

Below are the following materials that are necessary for performing **Experiments 1-3**:

Yeast strains
- *S. cerevisiae* strain expressing violacein from a plasmid

Equipment
- Incubator with temperature adjustable from 30-50°C
- Centrifuge
- P1000 micropipette and tips
- 2 - 10mL glass test tubes

Consumables
- YPD agar plate with G418 antibiotic
- YPD liquid media
- Sterile cotton swab and stick
- Parafilm
- Sterile scraping device
- Distilled water
- 1mL microcentrifuge tubes

Chemicals
- G418 antibiotic at a concentration of 200µg/1mL (if making your own YPD media)
- Saturated NaCl solution (6.14M)
- 100% isopropanol
- Dimethyl sulfoxide (DMSO) – **see WARNING**

Below are the addition materials and equipment necessary if performing **Experiment 4**:
- Pasteur pipette
- 25mL glass beaker
- 3mL sterile syringe
- 5ml Falcon® test tube
- Coffee filter
- Activated aluminum oxide (Alumina), basic. (Sigma CAS 1344-28-1)
- 10% bleach solution

Below are the additional materials and equipment necessary if performing **Experiment 5**:
- Liquid culture of *E. coli B* in LB media
- P10 or P20 micropipette and tips
- Sterile cell spreader
- Sterile forceps
- 2 sterile petri dishes (empty)
- 2 LB agar plates
- 2 - 6mm sterile pieces of construction paper

HAZARDS WARNING: DMSO is a combustible liquid, and hazardous. DMSO readily penetrates skin and may carry other dissolved chemicals into the body. If contacted, wash off immediately with plenty of water for at least 15 minutes.

EXPERIMENT 1. Culture violacein-expressing yeast.

Procedure:

1. Prepare a test tube of YPD with G418 antibiotic at a concentration of 200µg/1mL.
2. Inoculate with violacein-expressing yeast strain using a sterile stick or pipette tip.
3. Grow 1-3 days at 30°C.
4. Prepare one YPD agar plate with G418 antibiotic at a concentration of 200µg/1mL of media and distribute it in sterile 100mm x 10mm petri dishes.
5. Using a sterile cotton swab, inoculate the YPD-G418 plate from liquid culture yeast strain expressing violacein. Spread the culture over the entire plate.
6. Place culture into the incubator at 30°C for 94 hours.

Results: Check yeast plate daily and record any visible changes. Take pictures to compare the color over the growth period.

Note: If the culture is not dark purple or black after 94 hours, continue to let it grow at 30°C until pigmented. Try not to remove the sample from the incubator for prolonged periods. After the yeast has been grown, cultures plates can be wrapped in parafilm and stored in a refrigerator for up to six months.

EXPERIMENT 2. Autolyze the yeast cells.

1. Remove the plate from the incubator and record any changes you observe.
2. Turn the incubator up to 50°C.
3. Using a sterile scraping device, **gently** remove all of the yeast from the plate. Ensure that when collecting the yeast that none of the agar media is also scraped up with it.
4. Transfer the yeast on the scraper into a 10mL sterile glass test tube, washing the yeast into the tube by adding 8mL of distilled water.
5. Place the tube into a test tube rack and set it inside the incubator at 50°C.
6. Keep the sample at 50°C for 24 hours for autolysis to occur. The yeast cells will start to break down, so extracting the violacein pigment will be easier.

Results: Take notes and pictures of the sample before and after the autolysis is complete. Note any changes in color or appearance in the yeast pellet and the distilled water.

EXPERIMENT 3. Chemical extraction of the Violacein.

1. Centrifuge the yeast sample for one minute to ensure that the hydrophilic and hydrophobic contents are separated.
2. Remove the supernatant, taking care to not move or collect any of the pellet.
3. Add 1mL of a saturated NaCl solution to the pellet.
4. Centrifuge the suspension for 1 minute at 2000 rpm.
5. Remove and dispose of the supernatant.
6. **Repeat steps 3-5** three additional times, for a total of four NaCl washes.
7. Add 1mL of deionized water and resuspended the pellet.
8. Centrifuge for an additional minute at 2000 rpm.
9. Remove and dispose of the supernatant.
10. **Repeat steps 7-9** one additional time, for a total of two water washes.
11. Add 1mL of 100% isopropanol and resuspend the pellet. The mixture will become grainy. Try to distribute the liquid evenly, as much as possible.
12. Centrifuge for two minutes at 2000 rpm.
13. Remove and dispose of the supernatant.
14. To reconstitute the violacein, add 1mL of DMSO to the pellet. See Warning on page 2.
15. Centrifuge for one minute at 2000 rpm.
16. Move all of the liquid on top of the pellet into a new microcentrifuge tube.
17. **Repeat steps 14-16** until the pellet has no more black/purple pigment and is tan in color, or until the DMSO extract doesn't have pigment in it.

Results: Record the changes in color of the extract throughout the experiment. Take pictures and list your findings in your lab notebook.

Note: The violacein extract can be stored in a refrigerator for up to one month.

EXPERIMENT 4. Purification of the violacein by column chromatography.

1. Take a glass Pasteur pipette and place a small amount of a coffee filter as far down the tube as you can. A second Pasteur pipette can be used to help push down the filter as far as possible.
2. Fill the Pasteur pipette about ⅔ of the way with aluminum oxide.
3. Elevate the Pasteur pipette with a stand and place a 25mL glass beaker below it to collect fluid. This is the chromatography column.
4. Using a sterile syringe, wet the column with approximately 2mL of 100% isopropanol. Let it run completely through.
5. Once the isopropanol is running out of the bottom of the Pasteur pipette and there is no visible liquid on top of the column, immediately add the violacein yeast extract acquired from experiment 3. Ensure never to disturb your column. When adding any liquid to the Pasteur pipette, try to have it run down the side of the pipette instead of directly down the center.
6. Once there is no more purple liquid on top of the column, continuously add 100% isopropanol with the 3mL sterile syringe from step 4, refilling it as needed. Ensure that the column **never** runs dry and there is always some liquid on top of it.
7. Watch the collected fluid in the beaker until you see any sign of purple pigmentation. We recommend placing some white backdrop behind your column so the purple will be easier to identify as it runs down the narrow part of the pasture pipette.
8. As soon as you see purple pigment running out of the column, remove the beaker and replace it with a test tube to collect the purified violacein extract.
9. Continue to allow the test tube to fill until you see clear fluid come down the pasture pipette. As soon as this happens, remove the test tube and replace it with the beaker used earlier. The test tube now contains your purified violacein.
10. Let the column run dry and then add 10% bleach to it for 20 minutes before disposing of it. Dispose of liquid collected in the beaker with organic solvents, since it contains DMSO.

EXPERIMENT 5. Disk Diffusion Assay to test antimicrobial effectiveness of violacein.

1. Prepare a liquid culture of *E. coli B* in LB media and allow the bacteria to grow at 37°C.
2. Place a 6mm piece of sterile construction paper in each of the **empty** petri dishes. Label one plate violacein and one control.
3. Add 5 microliters of the purified violacein extract from Experiment 4 to the middle of the piece of paper in the violacein dish.
4. Add 5 microliters of 100% isopropanol to the middle of the piece of paper on the control dish.
5. Allow these disks to dry completely (about 30 minutes in a fume hood). You can proceed to the next step while the disks dry. **Do not** go further than step six until both disks are completely dry.
6. Add 500 microliters of the *E. coli B* culture to the middle of each of the two LB agar plates, one labeled violacein and one control. Using a sterile cell spreader, distribute the culture evenly on the plate and let sit lid up for at least 30 minutes.
7. After the disks are fully dried, and the cultures have set for 30 minutes, place the disks in the middle of their corresponding agar plate. Gently press down on the disk with sterile forceps to make sure it stuck to the agar.
8. Flip both plates upside down and incubate at 37°C for 48 hours.

Results: Note changes in the growth of the bacteria over the 48 hours. Measure the zone of inhibition (the radius of the circle in which bacteria do not grow, starting from the center of the disk) in millimeters. Conclude if your violacein is an antimicrobial against *E.coli* or not.