

Optimization of a Lithium Ion Battery Bioleaching Process Utilizing Organic Acids Produced by *Gluconobacter oxydans*

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I. ABSTRACT

Growing our global economic and environmental focus relies, in part, on our development of a sustainable system to recycle valuable metals. Idaho National Laboratory (INL) has been working towards this goal through an organic avenue by utilizing bacterium. To succeed in this low-cost and environmentally safe practice, *Gluconobacter oxydans* has been employed for its ability to produce a bio-lixiviant known for its ability to leach valuable metals from end-of-life consumer products or industrial wastes. *G. oxydans* is a rod-shaped, gram negative, acidophilic, and obligately aerobic organism. Because of its advantageous metabolic pathways, it was grown in a batch reactor setting to produce sufficient amounts of a low-pH bio-lixiviant so that leaching processes could be optimized. Following the bio-lixiviant production, various leaching experiments were conducted. These included adding different molar ratios of a redox agent (FeSO_4) to the bio-lixiviant, varying concentrations of lithium ion battery cathode powders (LiCoO_2), varying leaching time, and varying the temperature each leaching was performed at. The bio-lixiviant succeeded in retrieving, on average, 90% of the cobalt. If this strategy were to be scaled-up it would be a potential method to recycle critical materials from larger appliances such as electric vehicle batteries in an environmentally safe and economically profitable way.

II. INTRODUCTION

A number of critical elements are becoming more widely used in today's world for a multitude of devices that contribute to our growing movement towards a greener world. These appliances include wind turbines, permanent magnets, rechargeable batteries, and hybrid/electric cars along with many other applications. However, the predominate source of valuable materials and rare earth elements are not mined in the United States, and in the near future this could create an unsustainable situation resulting in insufficient amounts of critical materials for many appliances used in our society.^{[\[1\]](#)}

To mitigate this supply risk and to increase the available amount of these valuable materials, recycling is essential. These metals can be extracted from waste streams and/or from products that utilize them but have reached the end of their lives. Currently, in the United States, the metal

extraction methods being implemented are predominantly pyrometallurgical and hydrometallurgical. While affective, these methods can have a strong negative impact on the environment due to the hazardous wastes that they can create and are rarely profitable.^[10]

One such effective method utilizes inorganic acids, like hydrochloric (HCl) or sulfuric acid (H₂SO₄), to extract metals from various feedstocks. With efficiencies that can reach over 80%, these methods are popular but pose a large environmental threat.^[4] However, it has been shown that organic acids can also have comparably high efficiencies to inorganic acids but with a smaller negative environmental impact and economically be more profitable.^[8] In this case bioleaching can be utilized, which is a form of biohydrometallurgy. Biohydrometallurgy is a process that utilizes microorganisms and their ability to produce organic or inorganic products. In this case organic acids can be synthesized and used to leach critical materials from a desired source in an environmentally healthy way.^[2]

In this study, cobalt was leached from lithium ion battery cathode powders. Past studies have shown that gluconic acid can be utilized for this leaching process and *Gluconobacter oxydans* has also shown to predominantly produce gluconic acid as a byproduct because unlike many other aerobic microorganisms, it does not completely oxidize its carbon sources to water and carbon dioxide. Instead it incompletely oxidizes its substrates to aldehydes, ketones and organic acids.^[7,8] Because of this it was utilized for these experiments. To grow *G. oxydans* on pure glucose is, however, costly. A recent techno-economic analysis of bioleaching of rare earth elements from spent fluidized catalytic cracking catalyst using bio-lixiviant produced by *G. oxydans* from glucose estimated that 44% of the overall process expenses are due to the substrate requirements and of that, 98% of it was attributed to the cost of refined glucose. To mitigate and lower these costs other sources of glucose have been considered. Crain-Zamora et al. have shown that corn stover can be

successfully used to provide *G. oxydans* with a sufficient amount of glucose for growth and production of the desired bio-lixiviant. [\[3,10\]](#)

To continue optimizing the leaching process to decrease the recovery costs of cobalt from lithium ion battery powders, various other factors were evaluated to test their effect on the leaching capabilities of the bio-lixiviant. These factors included different added molar amounts of a redox agent, varying leaching time and temperature it was performed at, and varying the concentration of the lithium ion battery cathode powders. The resulting outcomes were then used to identify the most efficient combination of conditions.

It was hypothesized that *G. oxydans* would be successful in producing a bio-lixiviant that could reliably leach the cobalt from lithium ion battery cathode powders in an environmentally healthy and economically profitable way.

III. METHODS

a. Modified Pikovskaya phosphate medium preparation:

The *G. oxydans* was grown in modified Pikovskaya phosphate medium (Pkm), as described by [Reed et al. \(2016\)](#). To prepare a solution of Pkm that was five times its normal concentration (5x Pkm), all of the components in Table 1 were combined sequentially in 2 L of npH₂O using sterile technique (final volume ~2 L). Glucose, however, was prepared separately and added not to the Pkm directly, but to the bioreactor vessels. To prepare the 50% glucose solution, 250 g of glucose was added incrementally to 325 mL of warm npH₂O. Once all of the glucose was dissolved, npH₂O was added until the final volume of 500 mL was reached. After the solution had cooled it was then filtered twice (0.2 μm, PES). To dilute to 1x Pkm, 2400 mL of npH₂O was added to 600 mL of the

5x Pkm or 100 mL of 5x Pkm was added to the bioreactor vessels which had a final working volume of 500 mL.

Table 1: Pikovskaya's Modified Medium

<u>Order</u>	<u>Components</u>	<u>g/L</u>	<u>5X (2L)</u>
1	(NH ₄) ₂ SO ₄	0.5	5 g
2	MgSO ₄ x 7H ₂ O	0.1	1 g
3	MnSO ₄ x H ₂ O	1E-04	1 mL (1 mg/mL)
4	FeSO ₄ x 2H ₂ O	1E-04	1 mL (1 mg/mL)
5	KCl	0.2	2 g
6	CaCl ₂ x 2H ₂ O	0.046	0.46 g
7	KH ₂ PO ₄	0.37	3.7 g
8	Yeast Extract	0.5	5 g
9	Glucose	40	prepare separate

b. SixFors fermentation system setup:

Six separate batch reactors are controlled by the SixFors system. Temperature, air flow, stirring speed, and antifoam addition can all be manipulated and controlled. pH and dissolved oxygen (D.O.) content can also be controlled but were only monitored in this experiment. Before each bioreactor run, the pH and D.O. probe were autoclaved along with the rest of the vessel. Calibration of the pH probes were done with pH buffer solutions. D.O. probes were polarized overnight before they were calibrated by flowing air and nitrogen separately through the vessels to obtain low and high readings of oxygen. In this experiment the temperature was held at 30°C, the stirring speed was set to 600 rpm, the air flow was held at ~1.06 L/min, and no antifoaming solution was used.

c. Bio-lixiviant production:

Gluconobacter oxydans was grown in the modified Pikovskaya phosphate medium (Pkm).^[6] The modification step included the replacement of calcium phosphate with $\text{CaCl}_2 \cdot 7\text{H}_2\text{O}$ and KH_2PO_4 . This was done so that the calcium and phosphate would fully dissolve into the aqueous medium for use in the SixFors fermentation system.^[8] Before inoculating the reactors, *G. oxydans* was grown separately in a shake flask until it reached exponential growth (O.D._{600 nm} of 0.25-0.75, $1.9\text{E}+08$ - $7.5\text{E}+08$ cells/mL). It was then isolated and added to a Pkm solution that contained no glucose so that further growth was halted.^[5] Small aliquots (1 mL) were also set aside for preservation by freezing, following the addition of 70% glycerol (final glycerol concentration 20%).

The media that was used for growth in the reactors contained 100 mL of 5x Pkm (no glucose present), 40 mL of a 50% glucose solution (final concentration 40 g/L), and npH_2O was added until the final volume of 500 mL was reached (working volume of the SixFors reactors) in the reactors. The reactors were autoclaved prior to the glucose addition so that any contamination present in the solution would be eliminated and the glucose would not be caramelized. The 50% glucose stock was sterilized by filtering twice (0.20 μm PES). After inoculation with the previously frozen 1 mL aliquots and the addition of glucose, the *G. oxydans* was incubated for 36-40 hours at 30°C and 600 rpm. To collect the bio-lixiviant, the solution was centrifuged at 6000 times gravity for 30 minutes and filtered (0.20 μm PES). The final product was then kept refrigerated at 4°C until use in the bioleaching experiments.

d. Leaching process:

A redox reagent (FeSO_4) was added to the bio-lixiviant in an equal molar amount to cobalt, leaching times were varied from 1.5 to 24 hours, temperatures were varied between 20°C and 70°C, the concentration of the lithium ion battery cathode powders varied between 1.5% and 33%, and different acids were used instead of the bio-lixiviant.^[9]

e. Analytical technique:

The resulting leachate solutions were analyzed by a TXRF (Benchtop Total Reflection X-ray Fluorescence) spectrometer to determine REE concentrations. Samples were diluted (1:50) with 0.2% polyvinyl alcohol and a Selenium standard. These were then vortexed before 10 μL were placed onto disposable acrylic disks and dried. Before analyzing, it was imperative that each samples height was checked before being added to the TXRF spectrometer. If the samples were not flush with the plate and contained any bubbles, new ones were plated and dried again (damage to the TXRF spectrometer can result from samples that are tall and able to scratch the device). The standard was used as a basis for determining the concentration cobalt. Each sample was then analyzed for a minimum of 1000 seconds.

IV. RESULTS AND DISCUSSION

a. Bio-lixiviant production from various glucose concentrations:

Growth patterns for the cultures at each glucose concentration, based on absorbance readings done at 600 nm, are shown in FIG. 1. It is unknown why there are occasional sudden drops in cell density but the general decrease in cell density is believed to be due to the deceased bacterial cells being lysed. Determining a higher optimal concentration of glucose to grow *G. oxydans* on

would result in higher concentrations of organic acids in the bio-lixiviant and could result in larger bioleaching efficiencies.

Gluconobacter oxydans grew well with 80, 100, and 150 g/L glucose. When grown with 40 g/L glucose, acid concentrations ranged between 200 and 230 mM, however, with these increased concentrations of glucose, acid concentrations almost tripled. 635 mM of acid was produced with 150 g/L glucose. At glucose concentrations of 200, 250, and 300 g/L, little growth occurred though, and visible crystals formed in the medium. The pH still dropped to about 2.4, however, acid concentrations did not increase, but instead decreased (Table 2).

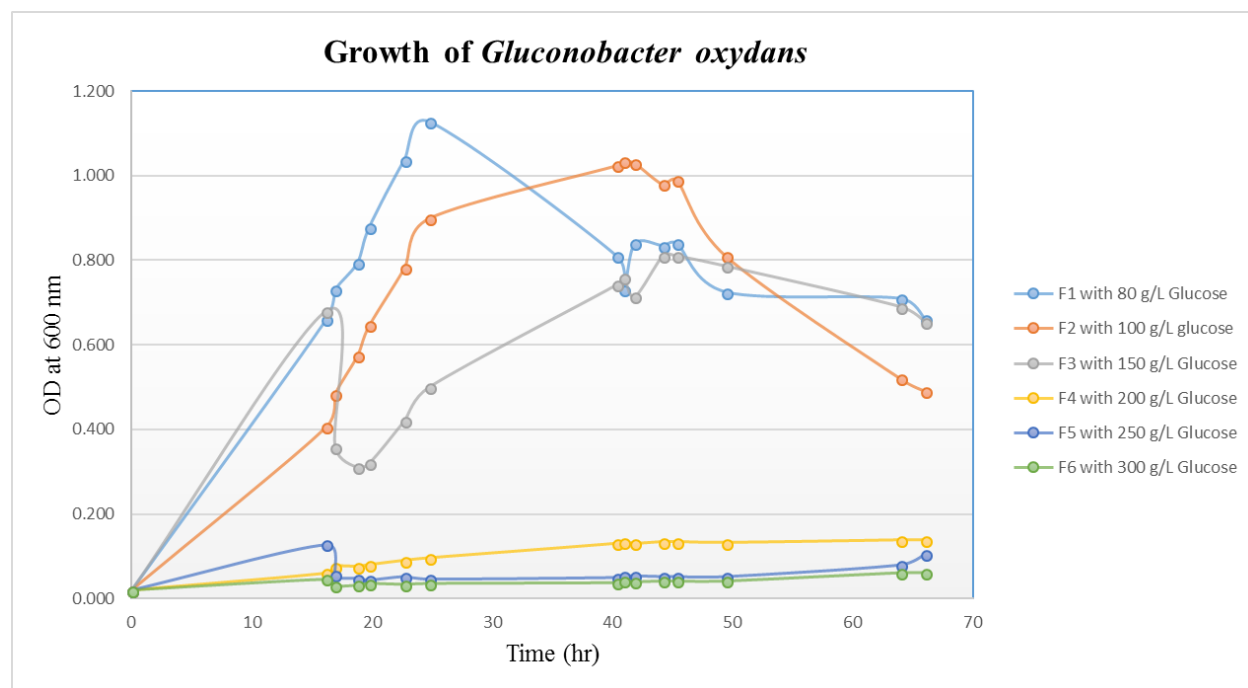


FIG. 1. Growth of *G. oxydans* on varying amounts of glucose

Table 2: Resulting acid concentrations produced by *G. oxydans* in varying amounts of glucose

Initial Concentration of Glucose (g/L)	Final Concentration (mM)	Final pH
80	583.20	1.86
100	589.97	1.87
150	635.19	1.81
200	237.80	1.96
250	128.25	2.21
300	84.08	2.35

b. Leaching of lithium ion battery cathode powders:

By comparing with previous data from Dr. David W. Reed, it has been deduced that the addition of a redox agent, in this case FeSO_4 , greatly increased the amount of cobalt extracted from lithium ion battery cathode powder. Shown in FIG. 2, extraction percentages were increased from 15% to 83% when FeSO_4 was present in the solution. During the leaching process the iron reduces Co^{3+} to Co^{2+} . However, this oxidizes the iron from Fe^{2+} to Fe^{3+} which can precipitate out of solution if the pH of the solution is greater than 4. The organic acid content of the bio-lixiviant, however, prevents this from happening. Along with the bio-lixiviant, sulfuric and gluconic acid were also tested to determine their efficiency in retrieving cobalt from LiCoO_2 . The retrieval of cobalt using the bio-lixiviant with the added redox agent increased the yield by 68%. Adding the redox agent in a one to one molar ratio with the cobalt present in the cathode powder, greatly increased efficiency, but higher iron concentrations only increased the efficiency by about 5% (FIG. 2).

For a basis, a standard leach was performed for 24 hours with the bio-lixiviant at a temperature of 30°C , agitated at 150 rpm, with 1.5% lithium ion cathode battery powder, and 260 mg of FeSO_4 .

In each experimental trial one factor was changed to determine its effect on leaching efficiencies. The amount of cobalt retrieved varied from 48% to 95% depending on how long the solution was incubated. The most efficient leach occurred when it was incubated for 18 hours. It resulted in an average 89% of cobalt being retrieved (FIG. 3).

To Determine the optimal temperature to perform the bioleaching, solutions were incubated at 20°C, 30°C, 50°C, and 70°C. The amount of cobalt extracted ranged from 78% to 99%, however the variation between each temperature was not large. It is likely that the bio-leaching process will be more profitable at lower temperatures (FIG. 4).

At higher concentrations of the lithium ion battery cathode powder, cobalt retrieval efficiencies decreased. This is thought to occur because of the lack of access that the bio-lixiviant had to the solid matrix. The solid could not distribute evenly enough to allow the bio-lixiviant to facilitate the required reactions (FIG. 5).

Along with the bio-lixiviant (~230 mM, pH ~2.10), sulfuric and gluconic acid were also tested to compare their leaching capabilities. Sulfuric acid was tested at a concentration of 220 mM (pH of 0.95) and at a pH of 2.15 to determine what the effects would be at similar concentrations and pH's to the bio-lixiviant. The gluconic acid was also tested since the bio-lixiviant is mainly composed of gluconic acid. It was at a concentration of 220 mM and had pH of 2.17. The bio-lixiviant performed the best with an average percent retrieval of 83%, while the gluconic acid

retrieved 77% of cobalt on average, the sulfuric acid at 220 mM retrieved 74%, and the sulfuric acid at a pH of 2.15 retrieved 27.6% of the cobalt (FIG. 6).

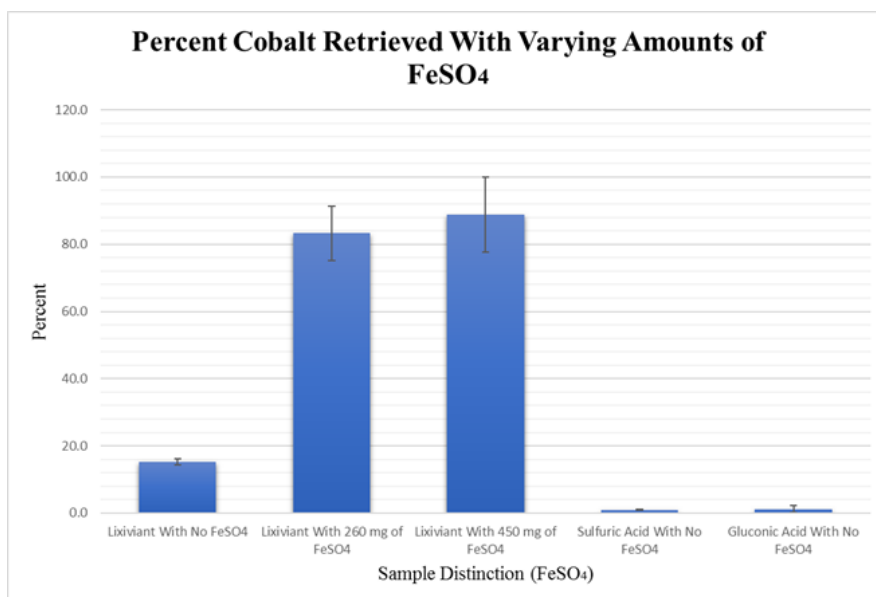


FIG. 2. Recovery of cobalt from solid LiCoO₂ powder with different amounts of ferrous sulfate

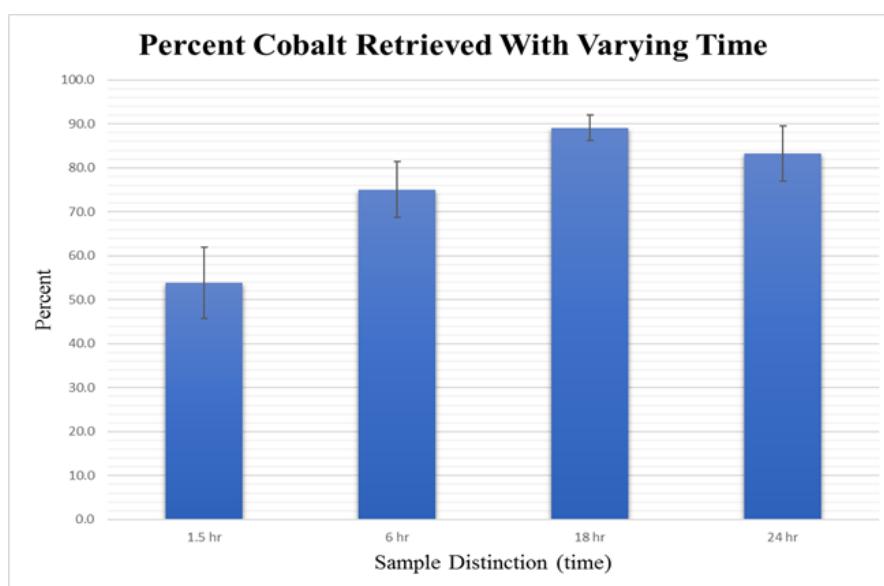


FIG. 3. Recovery of cobalt from solid LiCoO₂ powder with different leaching times

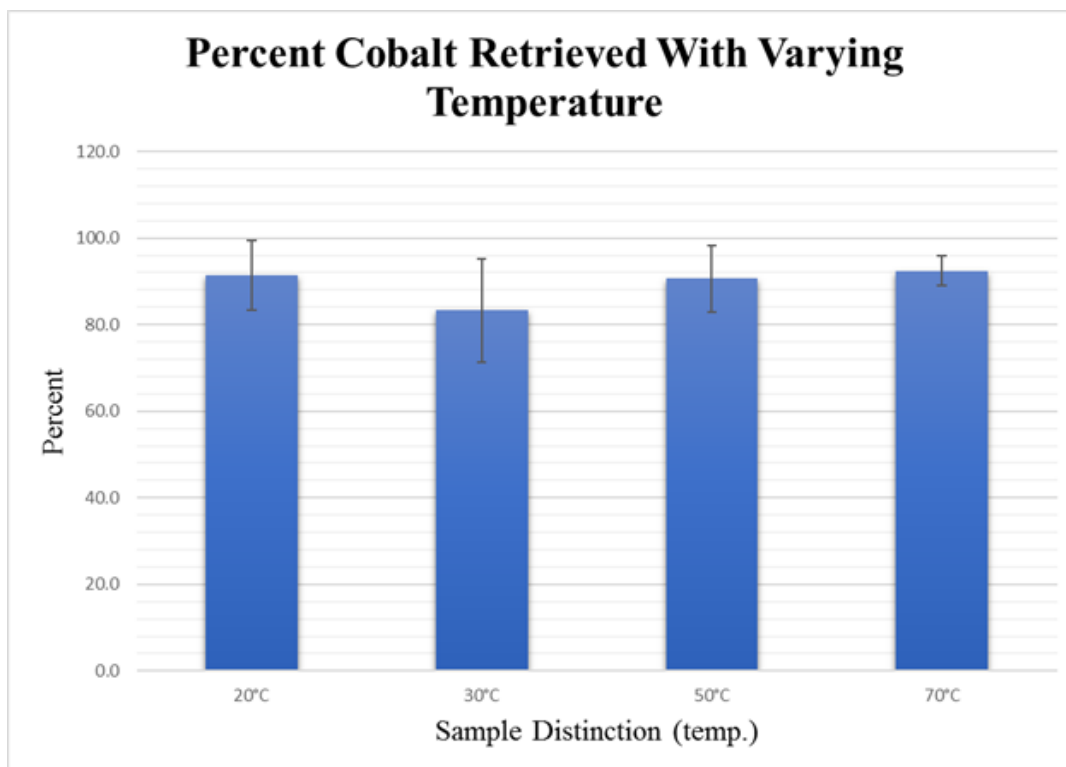


FIG. 4. Recovery of cobalt from solid LiCoO_2 powder with different leaching temperatures

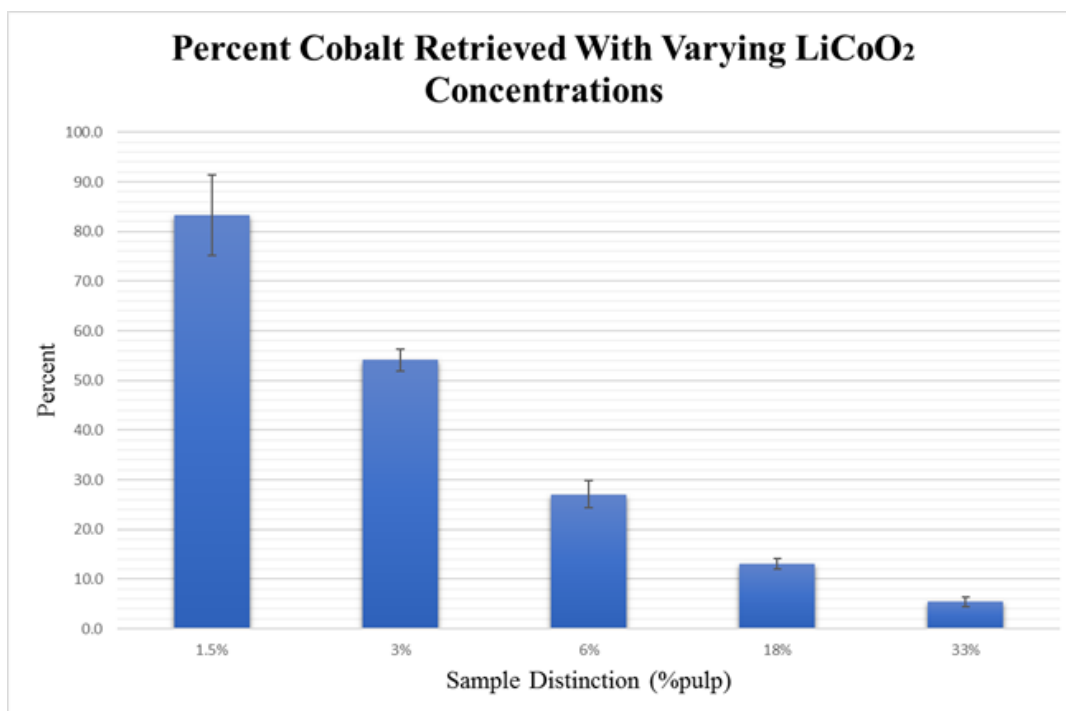


FIG. 5. Recovery of cobalt from solid LiCoO_2 powder with different concentrations of LiCoO_2

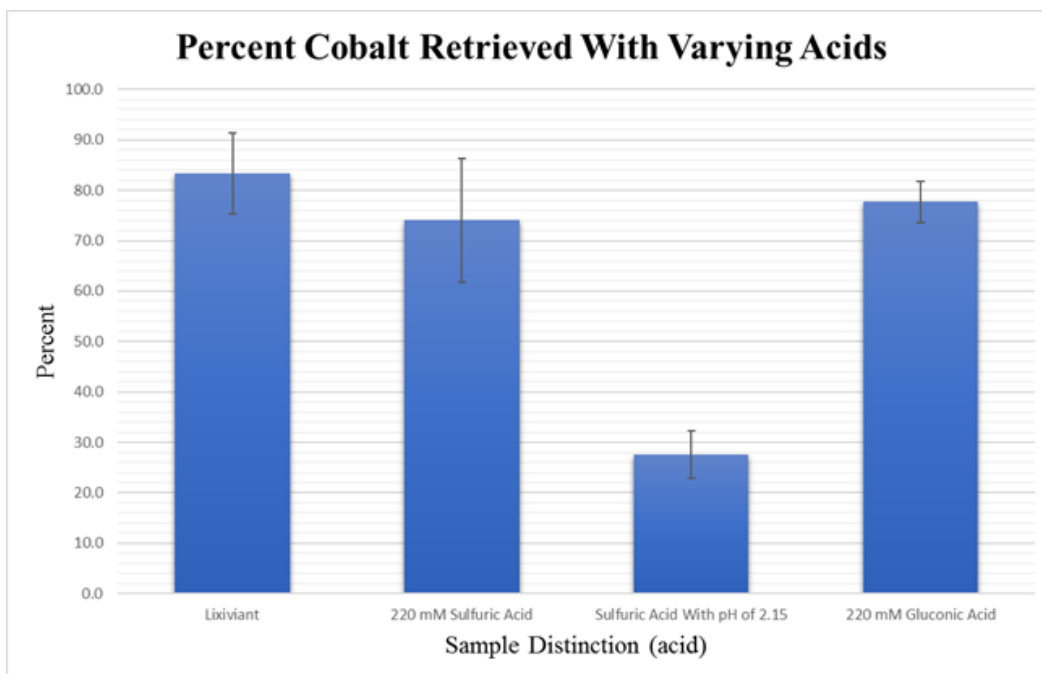


FIG. 6. Recovery of cobalt from solid LiCoO_2 powder with different acids

V. CONCLUSION

By combining the most efficient processes, a potential optimized procedure was developed. Utilizing the bio-lixiviant and performing the leaching processes at 20°C for 18 hours with 1.5% lithium ion battery cathode powder and 260 mg of ferrous sulfate, this procedure could be successful in retrieving the higher yield of cobalt from the battery cathode powder. However, this potential optimization still needs to be tested and is included in our future work. In addition to this, future work will also entail expanding this recycling process so that it can be scaled up to encompass other wastes such as electric vehicle batteries.

VI. ACKNOWLEDGMENTS

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VII. APPENDICES

APPENDIX A: Corn Stover hydrolysate preparation

1. To make a 10% solution of corn stover for one reactor run with only three fermenters being used, a final volume of 1400 mL is desired (140 grams of dry corn stover)
2. Dry the corn stover in an incubator at 60°C overnight
 - a. Separate the corn stover into multiple beakers so that it can dry completely
3. Making 0.1 M sodium acetate buffer
 - a. Prepare 0.1 M acetic acid by mixing 2.88 mL of glacial acetic acid and 497.12 mL of npH₂O
 - b. Prepare 0.1 M sodium acetate by mixing 6.8056 g of sodium acetate trihydrate and 500 mL of npH₂O
 - c. Now mix 413 mL of 0.1 M sodium acetate and 287 mL of 0.1 M acetic acid
 - i. pH needs to be 4.8 so adjust with acetic acid or sodium acetate as needed
4. Adding 0.1 M sodium acetate buffer to dry corn stover
 - a. In two 2800 mL Erlenmeyer flasks (**with screw tops**) combine 70 g of dry corn stover and 350 mL of 0.1 M sodium acetate buffer
5. To each flask add 1.358 mL of HTec2 and 13.44 mL of CTec2
 - a. These are Hemicellulase enzymes and will perform the hydrolysis of corn stover and release sugars
6. Adjust the volume to 700 mL with npH₂O
7. Incubate the solutions for five days at 50°C and 200 rpm
8. After incubation centrifuge the solutions
 - a. Centrifuge at 7000 times gravity for 30 minutes at 4°C
 - b. Do this four times
 - i. Transfer the supernatants to new 500 mL centrifuge bottles and rebalance them

- ii. Resuspend the pellets and dump them into the spent media tub
 - iii. Centrifuge bottles can be cleaned in the sink with soap
9. Sterilize the solution by filtration
 - a. Start with a pore size of 0.45 μm to remove larger particles so that the smaller filters won't be quickly clogged
 - b. Then filter the solution with 0.2 μm syringe filters using a 30 mL or 20 mL syringe
 - c. Finish with the 500 mL 0.2 μm filters
 - d. Store the hydrolyzed corn stover in the refrigerator
10. To find the sugar concentrations make up dilutions to send out
 - a. Neutralize a small amount of the hydrolyzed corn stover solution to a pH of about 5.5-6.0 with calcium carbonate solid
 - b. After neutralization, filter the solution with a 0.2 μm syringe filter
 - c. Obtain four 2 mL glass HPLC vials with 9 mm thread and pre-slit septa screw caps
 - i. Vial 1: undiluted
 - ii. Vial 2: 1 to 5 dilution
 - iii. Vial 3: 1 to 25 dilution
 - iv. Vial 4: 1 to 35 dilution

APPENDIX B: Detailed modified Pikovskaya phosphate medium procedure

1. In 2 L of npH₂O combine Pkm ingredients sequentially
 - a. Add 5 g of (NH₄)₂SO₄
 - b. Add 1 g of MgSO₄ x 7H₂O
 - c. Add 1 mL of MnSO₄ x H₂O and FeSO₄ x 2H₂O
 - d. Add 2 g of KCl
 - e. Add 0.46 g of CaCl₂ x 2H₂O
 - f. Add 3.7 g of KH₂PO₄
 - g. Add 5 g of yeas extract
2. Autoclave the solution for the required time on a liquid cycle
 - a. Let the solution cool after it's finished autoclaving
3. Adjust volume back to 2 L
 - a. Do this in a graduated cylinder with autoclaved npH₂O
4. Transport this to two 1 L bottles
 - a. Store the 5x Pkm solution on the bench top out of the way

APPENDIX C: Detailed *Gluconobacter oxydans* initial growth procedure

1. Obtain three 1 L flasks that are unbaffled
 - a. Add 350 mL of 1x Pkm with glucose (final concentration of glucose is 10 g/L)
 - i. For 350 mL of Pkm add about 7.5 mL of 50% glucose solution
2. Incubate for 29 hours at 30°C and 150 rpm

- a. Check that the flasks fit in the incubator
3. Check O.D. (absorption) at 600 nm
 - a. Should be between 0.25 and 0.75
 - b. Clean the cuvettes after use
4. Transfer to separate 500 mL centrifuge bottles
 - a. If temperature can be controlled place the range to 6°C - 8°C
 - b. Spin at 6000 times gravity for 20 minutes
 - c. Pour off excess once finished
5. Wash with 30 mL of Pkm that contains no glucose
 - a. Add Pkm to centrifuge bottles and vortex to resuspend
 - b. Spin at 6000 times gravity for 20 minutes
 - c. Pour off excess once finished
6. Make up a solution of 1x Pkm with 20% glycerol
 - a. Before adding glycerol filter with syringe (or prefilter a large amount that can be used many times)
 - b. Mix 20 mL of 1x pkm and 5 mL of 70% glycerol (25 mL is used assuming that the initial OD is about 0.25, this results in an OD of about 0.02 when 1 mL of the final solution is added to the reactors)
7. Add the Pkm glycerol mix to the pellets
 - a. Add some to the first pellet and resuspend, then add that to the next and so on
 - b. Add any remaining Pkm glycerol mix to the combined pellets
 - c. Make sure that solution is well mixed, and all cells are resuspended
8. Add 1 mL of the final solution to cryovials and freeze in blue isopropyl slow-freeze container for at least 2 hours
 - a. Cryovials can be found in lab A8

APPENDIX D: Detailed *Gluconobacter oxydans* growth in the SixFors

1. Check all O-rings, vacuum grease, D.O. probes, and condenser
 - a. O-rings are used in each mettle probe fitting, fermenter mettle tops, and tops of the stir shafts (check the white stoppers on the stir shafts for proper orientation)
 - b. Replace any that look worn or damaged
 - i. If replacing the large top O-ring, make sure that high temperature vacuum grease is added
 - c. Before adding the D.O. probes to the fermenters check them for any damage
 - d. Check the water level in the condenser machine
 - i. Water level should be midway filled or more
2. Add the media into each fermenter
 - a. Pour 100 mL of 5x Pkm to each one
 - b. Top off with 400 mL of npH₂O
 - c. Mark the water level in the fermenter after the top and probes are added
3. The mettle port cap should not be used for autoclaving!
 - a. Use a sponge stopper to cap the solid addition port
4. Adding the D.O. probe

- a. The mettle fitting that holds it should be left loose when inserting the probe
 - i. Wet the O-rings or probes so that the probes can slide in more easily
 - b. The probe is longer than the fermenters so make sure that the tip does not touch the bottom or magnets on the stir shaft
 - c. Finger tighten the mettle fittings once the probe is inserted to the correct level
 - d. Keep the end of the D.O. probe that connects to the SixFors capped throughout the autoclave process
5. Calibrate the pH probe
 - a. Connect the pH probe to the SixFors reactor
 - b. Wash the pH probes with distilled water so that any remaining Broadley James salt solution is removed
 - c. Go through the menus for calibrations
 - d. After calibration the probes can be added to the fermenters
 - e. Don't turn the SixFors reactor off!
 - f. Keep the end of the pH probe that connects to the SixFors capped throughout the autoclave process
6. Adding the pH probe
 - a. The mettle fitting that holds it should be left loose when inserting the probe
 - i. Wet the O-rings so that the probes can slide in more easily
 - b. Finger tighten the mettle fittings once the probe is inserted to the correct level
7. Check that the air pipe is close but isn't touching the stir shaft
 - a. The air pip is L-shaped
 - b. Use the small flat head screwdriver located in one of the draws under the SixFors reactor
 - c. Either wrap filters in tinfoil and autoclave them with the fermenters or use new sterile filters when starting up the SixFors
 - i. Filters are needed to sterilize the air being pumped into each fermenter since forced air has a higher risk of potential contamination
 - d. Mark the water level in the fermenters
8. Check how the condenser is oriented on the vessel
 - a. Orient the condenser so that the tubes are facing out over the volume markings
 - b. Also set the clamp in the same orientation
 - i. This allows the fermenters to sit in the SixFors easily
9. Cover all ends of any tubes
 - a. This includes any tips that are open to the air and any fittings that are joined together
10. Autoclave
 - a. Do a 60 min liquid run
11. After autoclaving let the fermenters cool down to room temperature
 - a. This can be done overnight or for about 3-4 hours
 - i. Cover the top of the fermenters with tinfoil
 1. This is done to prevent any contamination from falling onto the fermenters while they sit and cool
12. Place all of the fermenters into their designated blocks
 - a. Connect the pH and D.O. probes to their SixFors connections
13. Insert the temperature probes

14. Connect the condenser pipes up to the SixFors
 - a. Check that the water valves of each fermenter are in the open position
15. Let the D.O. probes polarize 4-6 hours or overnight
 - a. Cover the reactors with tin foil to prevent dust from settling on them
16. Add glucose
 - a. Using sterile techniques add 40 mL of 50% glucose to the fermenters for a final glucose concentration of 40 g/L
 - b. Fill to the water mark with npH₂O
 - c. Replace the sponge stopper with the mettles using the large flat head screwdriver
17. Check that everything is oriented properly in the reactor
 - a. The stir magnet shaft is sitting in the white stopper at the bottom of the vessel
 - b. The white stopper and small O-ring at the top of the stir magnet shaft is set properly
18. Start each fermenter
 - a. Check that only temperature and rpm are controlled for each fermenter as you turn them on
19. Set the temperature
 - a. Temperature should be set at 30°C
20. Turn the rpm on for each fermenter
 - a. rpm should be set to 200 for D.O. calibration
21. Turn the condenser machine on
 - a. Should be set at 17-18°C already but if not adjust the temperature
 - b. Calibrate the D.O. probe Adjust all air flows to 60 for each fermenter once the air tube and filter is connected and turned on
 - i. Let the nitrogen run for an hour or more
 - ii. Record this as your low reading
 - c. Turn the nitrogen off and turn the air on
 - i. Re-adjust the air flow to 60 (may have changed some with the switch)
 - ii. After about an hour record this as your high value
22. Adjust rpm to 600 for growth
23. Turn the computers on and start collecting data
 - a. Check the time on the computer and adjust if needed before collecting data
24. Inoculate the reactors
 - a. Take 6 frozen cryovials from the refrigerator and let them warm to room temperature
 - i. Suck up the culture in the cryovials with a 1 mL syringe and an 18-gage needle
 - ii. Take off the needle and replace its cap so that it can be safely disposed of
 - iii. Take off the tinfoil on the liquid addition tube connected to a fermenter and attach the syringe
 - iv. Discharge the culture fully
 1. After discharge, disconnect the syringe and take in air
 2. Reconnect the syringe and push air through the tube to expel all of the culture into the vessel
 3. Do this once more (twice total)

- v. Do this for each fermenter but there is no need to change the needle or syringe if they don't touch anything else
 - b. If added at 4:00 pm, 39 hours later will be 7:00 am two days later
 - i. Example: Monday 4:00 pm inoculation = Wednesday 7:00-8:00 am harvest
- 25. Clamp each liquid port tube to close them
 - a. They may leak if kept unclamped
- 26. Take samples from the fermenters to get OD₆₀₀ readings
 - a. Make sure that the spectrometer in lab A8 is turned on before taking samples
 - i. Power button is on the back
 - b. Use a 3 mL syringe to extract 1 mL of media
 - c. Place this into a small centrifuge vial
 - i. Found in one of the cabinets under the SixFors
 - ii. Grab a vial holder from lab A8 to hold all of the samples
 - d. Transport vials to lab A8 and take OD readings at 600 nm
 - e. Dispose of the vials in the autoclave waste and wash the cuvettes with DI water
- 27. Re-clamp each liquid port tube after a sample is taken
- 28. Check the air currents every once and a while
 - a. They may decrease over time (filters start to fill up with debris)
- 29. Turn the condenser machine and air off at the wall
- 30. Stop each fermenter by going through the SixFors menus
 - a. Transfer all of the data obtained by the computer to a flash drive
- 31. Turn off the SixFors
- 32. Disassemble the fermenters Part 1
 - b. Disconnect the condenser hoses from each fermenter and wipe up any water that may spill out of them
 - c. Shut the air off at each fermenter, then disconnect the air tubes
 - d. Take out the temperature probes
 - e. Disconnect pH probes
 - f. Starting with fermenter 6 disconnect the D.O. probes
 - i. Use the pliers to take off the caps that connect the D.O. probes to the SixFors
 - ii. As you disconnect a D.O. probe remove the fermenter and shut the water valves
- 33. Transport the fermenters to lab A8
 - a. Bring the rack that holds the Broadley James salt solution and cardboard sleeves
 - iii. Don't forget to grab the cups with the various probe caps
- 34. Pull the probes out of the fermenters and wash them
 - a. Wash the pH probe with wash water and rinse with DI water, dry with chem wipe, place in cardboard sleeve, insert into the Broadley James salt solution bottles, and wrap with parafilm
 - b. Wash D.O. probe with DI water, wipe down with 70% ethanol, quickly rinse with DI water, and replace the caps before storing
- 35. Disassemble the fermenters Part 2

- a. Unscrew all three port screws and wash with wash water and rinse with 70% ethanol and DI water
 - b. Remove the condenser from the top and run 70% ethanol through it, then rinse with DI water
 - c. Take off the top and remove all components
 - d. Clean everything with wash water and rinse with 70% ethanol
 - i. This includes the hoses
 - ii. Push 70% ethanol through the hose that comes out of the fermenter tops and goes right back in with no breaks by squeezing ethanol through the tips found on the bottom of the top
 - iii. With the other hose that comes out of the fermenter top and goes back in there is a connection that you can take apart
 1. Take this apart and push 70% ethanol through with the bottle
 - iv. Once ethanol has been pushed through all of the tubes, including the air tube, flush them with DI water
 - e. Rinse everything else with 70% ethanol and then with DI water
 - f. Set the fermenter vessels aside and cover with parafilm
 - i. Measure the amount of liquid in each fermenter visually
36. Harvesting the media
- a. Centrifuge at 6000 times gravity for 30 minutes
 - b. Clean the vessels with wash water and then rinse with 70% ethanol and DI water
37. Filter the supernatant with 0.2 μm , PES
- a. Resuspend the pellets and discard them in the biological waste tub in the hood by the sink
 - b. Store the lixiviant in the refrigerator
38. Assemble the clean fermenters and place them back by the SixFors
39. Autoclave the mettlet port caps and filters
40. To find the acid concentrations send out for analysis
- a. Place 2 mL of lixiviant from each fermenter into a HPLC vial
41. If you also want a sugar analysis done refer to the directions in the corn stover procedure but don't do the dilutions

APPENDIX E: Detailed bioleaching table of organization

Sample #	Vial #	Triplicate	Time	Temperature	RPMs	% Pulp	FeSO ₄ (g)	Added Acid (10 mL)
1	1	A	24	30	150	1.5	0.260	F2 Lixiviant
	2	B	24	30	150	1.5	0.260	F4 Lixiviant
	3	C	24	30	150	1.5	0.260	F5 Lixiviant
2	4	A	1.5	30	150	1.5	0.260	F2 Lixiviant
	5	B	1.5	30	150	1.5	0.260	F4 Lixiviant
	6	C	1.5	30	150	1.5	0.260	F5 Lixiviant
3	7	A	6	30	150	1.5	0.260	F2 Lixiviant
	8	B	6	30	150	1.5	0.260	F4 Lixiviant
	9	C	6	30	150	1.5	0.260	F5 Lixiviant
4	10	A	18	30	150	1.5	0.260	F2 Lixiviant
	11	B	18	30	150	1.5	0.260	F4 Lixiviant
	12	C	18	30	150	1.5	0.260	F5 Lixiviant
5	13	A	24	20	150	1.5	0.260	F2 Lixiviant
	14	B	24	20	150	1.5	0.260	F4 Lixiviant
	15	C	24	20	150	1.5	0.260	F5 Lixiviant
6	16	A	24	50	150	1.5	0.260	F2 Lixiviant
	17	B	24	50	150	1.5	0.260	F4 Lixiviant
	18	C	24	50	150	1.5	0.260	F5 Lixiviant
7	19	A	24	70	150	1.5	0.260	F2 Lixiviant
	20	B	24	70	150	1.5	0.260	F4 Lixiviant
	21	C	24	70	150	1.5	0.260	F5 Lixiviant
8	22	A	24	30	150	3	0.530	F2 Lixiviant
	23	B	24	30	150	3	0.530	F4 Lixiviant
	24	C	24	30	150	3	0.530	F5 Lixiviant
9	25	A	24	30	150	6	1.080	F2 Lixiviant
	26	B	24	30	150	6	1.080	F4 Lixiviant
	27	C	24	30	150	6	1.080	F5 Lixiviant
10	28	A	24	30	150	33	8.270	F2 Lixiviant
	29	B	24	30	150	33	8.270	F4 Lixiviant
	30	C	24	30	150	33	8.270	F5 Lixiviant
11	31	A	24	30	150	1.5	0.260	220 mM of Sulfuric
	32	B	24	30	150	1.5	0.260	220 mM of Sulfuric
	33	C	24	30	150	1.5	0.260	220 mM of Sulfuric
12	34	A	24	30	150	1.5	0.260	Sulfuric with pH of 2.15
	35	B	24	30	150	1.5	0.260	Sulfuric with pH of 2.15
	36	C	24	30	150	1.5	0.260	Sulfuric with pH of 2.15
13	37	A	24	30	150	1.5	0.260	220 mM of Gluconic
	38	B	24	30	150	1.5	0.260	220 mM of Gluconic
	39	C	24	30	150	1.5	0.260	220 mM of Gluconic
14	40	A	24	30	150	1.5	0.450	F2 Lixiviant
	41	B	24	30	150	1.5	0.450	F4 Lixiviant
	42	C	24	30	150	1.5	0.450	F5 Lixiviant
15	43	A	24	30	150	18	3.690	F2 Lixiviant
	44	B	24	30	150	18	3.690	F4 Lixiviant
	45	C	24	30	150	18	3.690	F5 Lixiviant

APPENDIX F: Detailed bioleaching procedure and analysis

1. Retrieve 50 mL conical vials to perform leaching experiments
 - a. Label each vial
 - b. Do each experiment in triplicate
 - c. Weigh the empty conical vial in lab B7 with the fancy scale
2. Add LiCoO_2 to the conical vial
 - a. Look to data tables for the amount required
3. Add FeSO_4 to vials
 - a. Look to the data tables for the amount required
4. Add 10 mL of the chosen bio-lixiviant or acid to each conical vial
5. Incubate the vials according to the data tables
6. After incubation weigh the vials to get the total mass
7. Centrifuge for 10 min at 10,000 times gravity (~9000 rpm)
 - a. Setting the centrifuge to 6°C seems to help the pellets set and not start to dissolve once the centrifuge stops
8. Filter supernatant through 0.2 μm syringe filters
 - a. Filter about 5 mL into 15 mL conical vials and add 50 μL of Nitric acid
 - i. This creates a 1% solution of Nitric acid for ICP analysis
 - b. Filter about 1 mL into small centrifuge tubes and transport these over to EIL to be tested in the TXRF spectrometer
 - c. Filter the remainder into a glass vial for pH readings
 - i. Calibrate the pH probe before you start to take readings
9. Dispose of the leftover spent solution
 - a. Label a 1 L glass orange topped bottle “Spent Lixiviant” and add the any leftover solution to it along with the amount used for the pH readings
10. Follow the TXRF directions provided to run the samples
 - a. In this case disposable disks were used so that the cleaning time could be eliminated
 - i. The disposable disks are acrylic, and more may need to be ordered depending on how many are left
 - ii. The acrylic disks have a thin film of plastic on each side that needs to be removed before use
 - iii. These disks are acrylic instead of quartz so when drying the sample only heat them to 50°C
 - b. The samples were diluted 1 to 50 before they were run
 - i. The final volume was 100 μL which contained 88 μL of 0.2% PVA, 10 μL of a Selenium standard, and 2 μL of your sample
 - c. When placing the disks into the TXRF orient them so that the sample is facing into the TXRF and the label that you placed on them is facing you
 - i. You should be able to read the label
 - d. The disks were run for 250 seconds when finding a good dilution but for the final analysis they were run for 1000 seconds

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