Bio Ink

Project: lab journal
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THURSDAY, 7/25/2019

Abstract:

Instead of printing a QR code stamp then use it to print bacteria on agar with various complications for blending the various strains with various growth conditions while maintaining the randomness in the strain pattern on the QR code, what if we could print a bio QR code directly using a 3D printer?

Desining a bio ink serves the following reasons

a. We can put strains in ink and the diffusion pattern creates randomness of strains on the code.
b. Adding Sodium alginate and CaCL2 to the bacterial solution and the media to be printed on, when combined gives an invisible QR on the surface which can fully be decrypted if the recipient of the QR has knowledge of all growing conditions.
c. with changes in concentration to CaCl2 we can also print 3D biological objects with safe GMO s expanding the horizons of this project.

Concentration balance

	Sodium alginate	CaCl2	Method	Results
1	1%	1%	paper dipped in Sod.Alg. Cacl2 used as ink	diffusion on paper no visible pattern
2	1%	2%	paper dipped in Sod.Alg. Cacl2 used as ink	diffusion on paper no visible pattern
3	1%	1%	paper dipped in CaCl2. Sod Alg used as ink	no diffusion slow solidification visible pattern
4	1%	2%	paper dipped in CaCl2. Sod Alg used as ink	better solidification, distinct visible drawing pattern

Conclusion : Sodium alginate solution can be used as printing material with the bacterial solution while printing on a paper doused with CaCl2 solution.

Assuming the better solidification rates in higher concentrations of CaCl2 we proceed to check the survivability of Vibrio Natrigens in the Sodium alginate solution.
Inoculating 1% and 2% sodium alginate solution with bacteral solution containing different concentration of media.

Survivability of Vibrio in different concentrations of Sodium Alginate

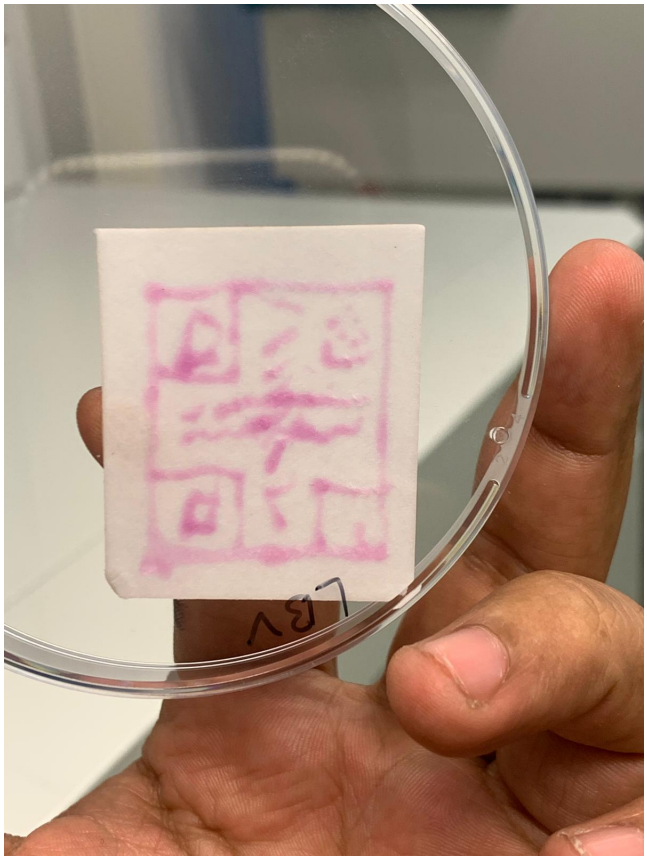
	Na.Alg with vibrio Lbv	OD	Results	D
1	overnight culture 1% NaAlg	1.840	Faster growth	
2	overnight culture 2%NaAlg	1.145	Slow yet sustained growth	
3				
4				

After checking the survivability of Vibrio in Ca.Alginate we proceed to print the "bio ink" on a piece of paper and check the solidifying and absorption parameters. to check this we make the Vibrio cultures with Na.Alginate and print it on paper lightly brushed with CaCl2

Drawing with bio ink

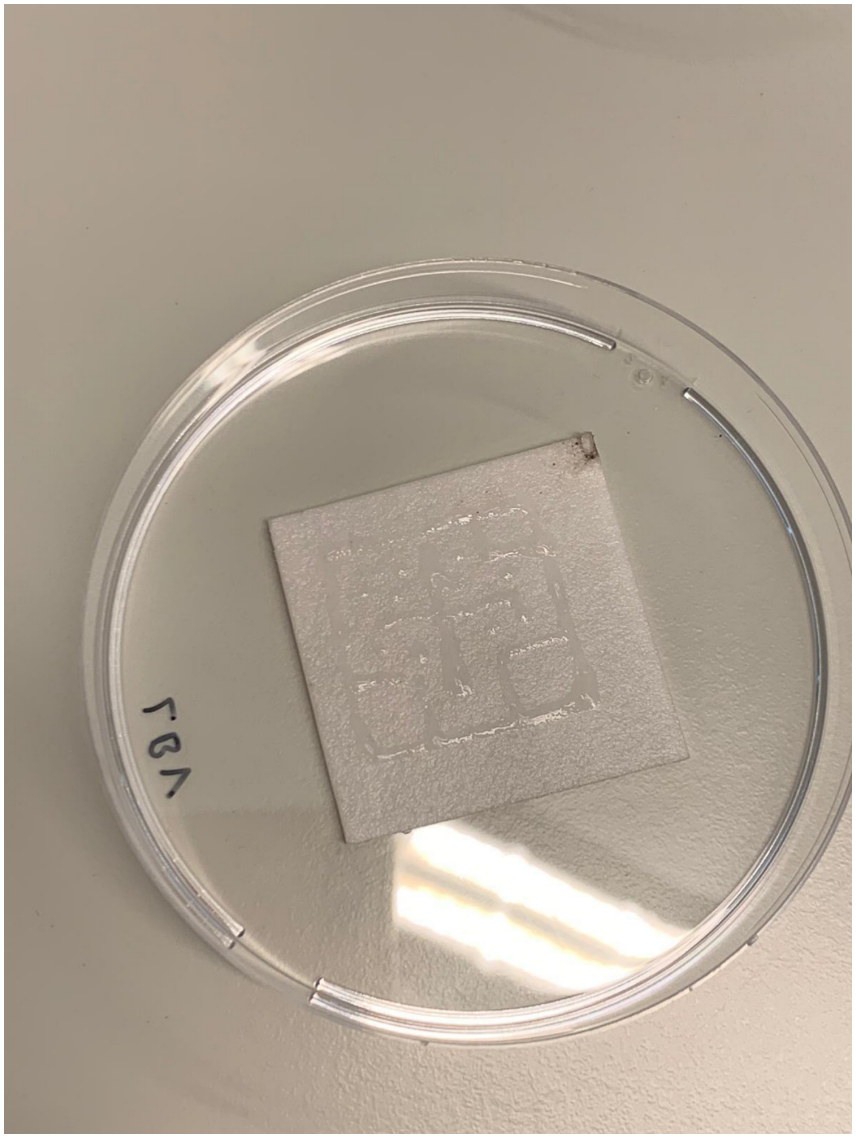
	Bio Ink	Solidification	Absotption	Results
1	1%	takes a few mins to solidify	spread and absorption on paper	distortion while drawing
2	2%	solidifies within a minute	low spread quick setting time	very low distortion promising results

Hand Drawing.jpeg



The first image is with addition of loading dye to the NA.Alg solution, but as we observed later on there was no growth in the drawings with the dye. Hence we skipped the dye in later experiments.

Fine drawing.jpeg

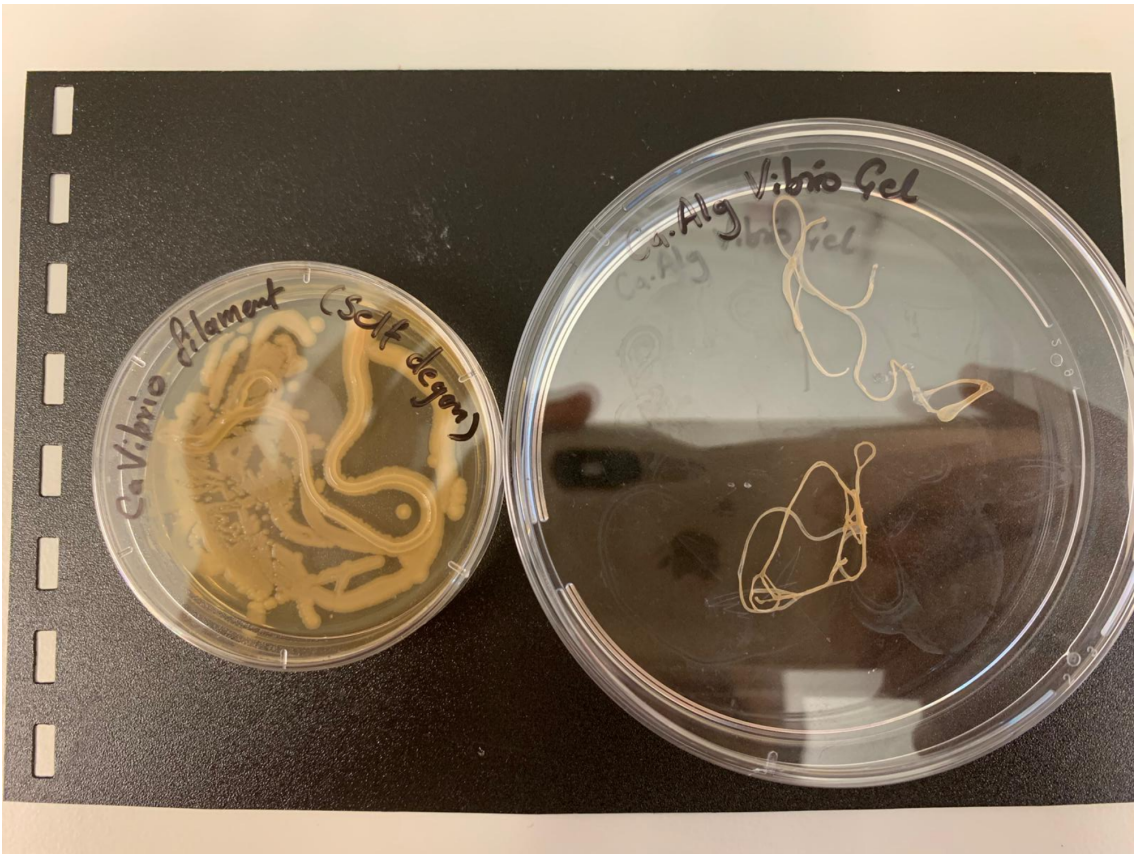


As we figure out the proper conc to encapsulate Vibrio in CaAlginate, we need to first check the survivability of the vibrio in the encapsulation. for that we make Beads and strands of vibrio encapsulated in CaAlginate and test the survivability of vibrio in alginate.

Survivability

	Encapsulation	Self degeneration	EDTA Degeneration	Results
1	Beads	normal growth	restricted growth	Vibrio survives
2	Strands	expansive growth	restricted growth	Vibrio survives
3	leftovers	expansive growth all over the plate	broken strands show restricted growth	Vibrio survives

Strands.jpeg



Survivability.jpeg

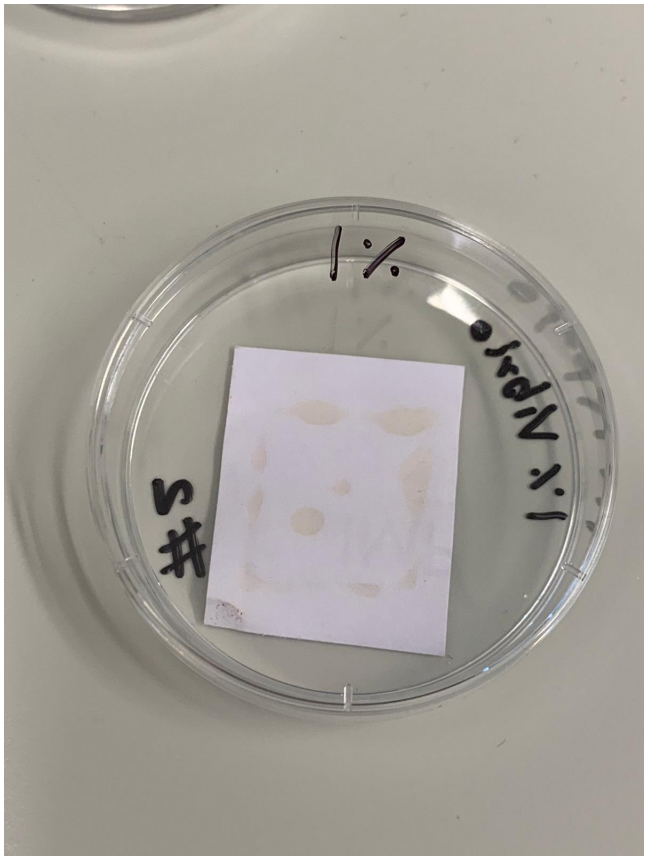
Now as we know that Vibrio encapsulated in CaAlginate survives we proceed to recover growth after letting the vibrio being encapsulated overnight in NTP conditions. The possible compounds able to re-solubilize CaAlginate would be EDTA and NaCitrate dihydrate. so to check which one works the best we try re-solubilizing and retrieving the growth of encapsulated vibrio. we do this by two methods:

1. spraying the drawn paper with EDTA/NaCitrate and then placing it on LBV agar plates
2. placing the drawn paper face down on LBV agar plates and spraying EDTA/NaCitrate over the top

EDTA recovery

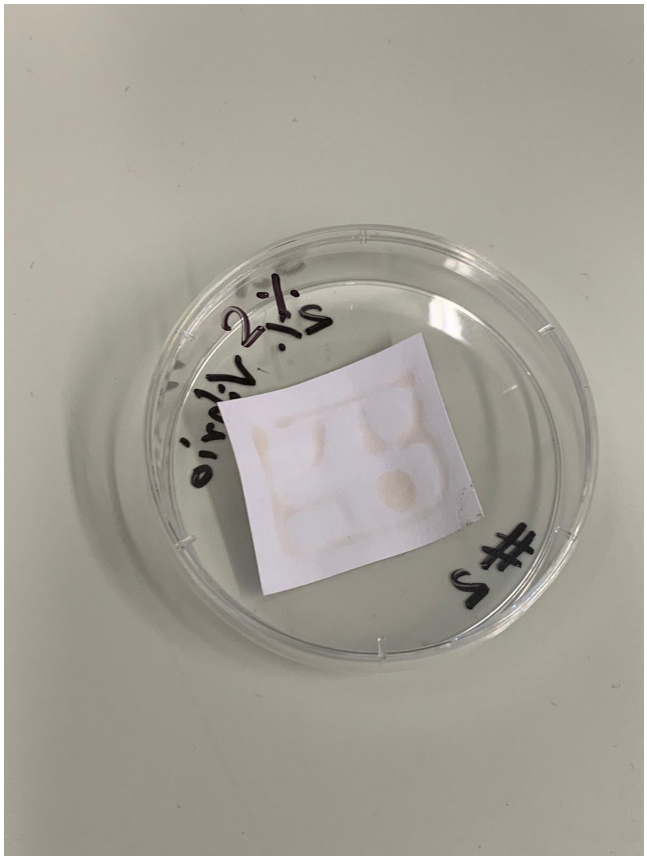
	Ink used	Approach	Observations on plate	Observations on printed paper	Results
1	1% NaAlg + vibrio	spray and stamp	no growth	no growth	method cannot be used
2	1% NaAlg + vibrio	stamp and spray	growth with reasonable resolution	no growth	Encapsulation methon is viable.
3	2% NaAlg + vibrio	spray and stamp	no growth	no growth	method cannot be used
4	2% NaAlg + vibrio	stamp and spray	growth with poor resolution	no growth	Encapsulation methon is viable.

1% paper.jpeg



1% Rev stamp.jpeg

2% paper.jpeg



2% Rev stamp.jpeg

x

THURSDAY, 8/8/2019

REsolubilization in Citrate experiment
Different concentration of citrate solutions
From bioink paper:
Bacterial Survival. To determine bacterial survival, 5 mL of an overnight culture of E. coli K12 MG1655 (OD600 of approximately 2.5) was spun down at 4000 rpm for 3 min and the supernatant discarded. The bacterial pellet was resuspended in 5 mL of fresh LB medium containing 2% w/v sodium alginate by vortexing until all ingredients were entirely dissolved. A portion of the bioink was used to print a 6-layered rectangular quadrangle. The gel was allowed to solidify for 30 min before the first sample was taken. The remainder of the bioink was incubated in unsolidified liquid form at 37 °C as a positive control showing planktonic growth. After 0, 24, or 48 h of incubation at 37 °C, 0.3 g of the gel was removed and dissolved in 1.75 mL of 1 M sodium citrate solution. For planktonic samples, 200 μL samples were removed, containing approximately the same volume of bioink as that sampled from the printed gel. Colony forming units (CFU) were determined following the protocol of Karas et al.31 In short, each sample was serially diluted three times (in 10-fold increments from 10−1 to 10−8), and 5 μL of each dilution was pipetted in triplicate onto a LB-agar plate. The plates were incubated at 37 °C for 24 h, and visible colonies were counted.

METHOD:
a 1% and 2% solution of sodium alginate in BHI was used. The 10 mL solution was inoculated with a loopfull of an overnight culture of *V. Natriegens.* grown on LBV medium. (NO v2 SALTS WERE ADDED, 4 hours later, v2 salts were added.

At OD 2.1, the alginate solution was dropped in 2% CaCl2 solution, now you should see pretty beads, (leave them overnight)

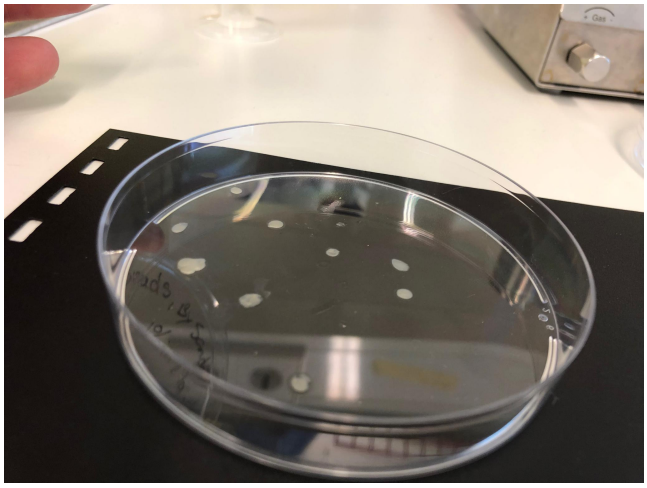
RESULTS

Time to dissolve

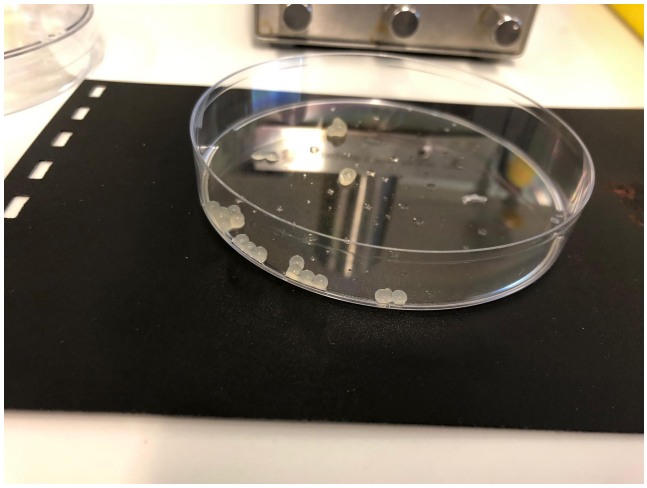
	A	B	C	D	E
1	M citrate ->	1.0 M	0.5 M	0.1 M	
2	2% alginate	3 min	4 min	12 min	
3	1% alginate	5 min	5 min	7 min	6
4	Growth				
5	2%	x	Yes	Yes	
6	1%	x	x	Yes	

The 1% alginate gel dehydrated and did not look like beads anymore after 1 day of leaving them in RT on my bench.

1% alginate beads after 1 day



2% alginate beads after 1 day



As follows, a 2% solution of alginate seems appropriate. Especially because higher concentrations are extremely non-viscous

TUESDAY, 8/13/2019

Print on paper soaked in CaCl2 (2a l%)

Methods

A V. Natriegens culture was started by inoculating 10 mL of LBV2 media with a colony from a spread out plate that was grown overnight. 5 mL of the overnight culture (OD600 = 2.6) was spun down at 7000 RPM for 4 minutes. The supernatant was discarded and 5 mL of fresh LBV2 medium supplemented with 2% sodium alginate was added. The bacteria were resuspended by vortexing. The now freshly prepared bio-ink was used in the following way

2% CaCl2 solution was used to soak a sheet of paper. (wood was also used to create some sort of stamp)
A figgure was pipetted on the paper and was allowed to solidify for 30 minutes. (a 100 uL pipette was used to create the patterns)
Now, the figures/paper were placed on an agar plate, where 150 uL of a 0.5 M solution of EDTA or Na Citrate were spread out on.
(in the case of the papers, 150 uL was spread out over the paper and the paper was placed on top of the agar.)

ALSO, a 1 M solution of CaCl2 was made and spread out over an agar plate. Now, the previousy prepared bioink was used to draw a figure on the agar.

Results

Beads/Snakes survivability

A V. Natriegens culture was started by inoculating 10 mL of LBV2 media with a colony from a spread out plate that was grown overnight.. The now freshly grown bio-ink was used in the following way.
Beads and snakes(stripes) were made by adding a drop of bio-ink (1 or 2 %) to a 2% solution of CaCl2.

EDTA1 0.069 g (per 4 beads)
EDTA2 0.066 g (per 4 beads)
CIT1 0.075 g (per 4 beads)

CIT2 0.074 g (per 4 beads)

EDTAsnake 0.270 g
EDTAsnake/self (0.097 g)
Citrate 0.154 g Citrate
Citrate/self 0.170 g

Ad 0.5 uL of 0.5M EDTA or citrate solutionwere added per bead
the snakes got 10 uL.

THe plates were incubated overnight.

What happened??

WEDNESDAY, 8/14/2019

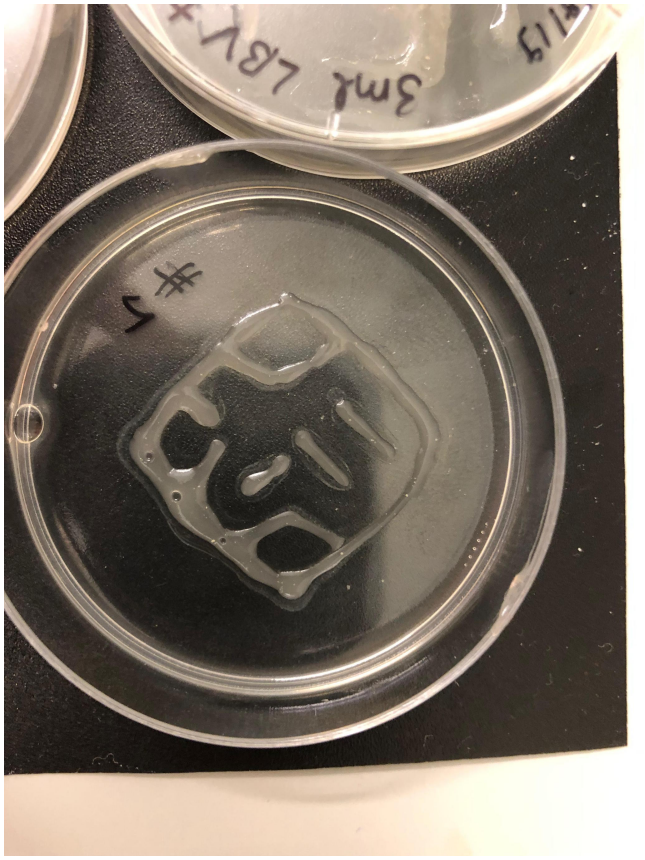
M&M of the PRINTED stuff

Printer Bioink. To obtain 10 mL of bioink containing E. coli, 10 mL of overnight bacterial culture (OD600 of approximately 2.5) were spun down at 4000 rpm for 3 min and the supernatant discarded. The cells were resuspended in 5 mL of sterile LB medium (Sigma-Aldrich). A 5 mL mixture of sodium alginate (5% w/v, Sigma-Aldrich) was added to the solution, followed by vortexing.

Printing Substrate. A Petri dish (150 mm × 15 mm) was filled with 20 mL of agar (1.5% w/v) dissolved in LB medium. The printing surface was prepared by the equally distributed application of 500 µL 1 M $CaCl_2$ onto the agar surface.

Checking the reproducibility of the 1% and 2% NaAlg LBV vibrio using the rev stamp method. This time measuring the lowest amounts of EDTA necessesary for resolubalization. Also since EDTA shows restrictive growth we are going to do the same experiments with NaCitrate using the previous

printed on CaCl2.jpg



THURSDAY, 8/15/2019

Resolubilization viability of V natriegens in printed cultures.

M&M

Ink was prepared as stated before. A 10 mL liquid culture was inoculated with a loopfull of stationary phase bacteria. The culture was grown overnight and the bacteria were spun down, Now, new LBV2 media was added with 1 or 2% Sodium Alginate respectively.

Drops were dropped in a tube containing CaCl2, due to spheriphication, ink bubbles were created.

These bubbles were left outside on the bench overnight and were then placed in 10 mL media containing Citrate (0.1M, 0.5M, 1.0 M). Time to dissolve was measured and the culture vials were cultured for =/- 4 days.

Growth and time to disolve were measred and were as folows:

Results

	A	B	C	D	E	F	G
1	Concentration citrate	1.0M	Growth	0.5M	Growth	0.1M	Growth
2	Time to dissolve of 2% alg	3 min	X	4 min	Yes	12 min	Yes
3	Time to dissolve of 1% alg	5 min	X	5 min	X	7 min	Yes
4							

MONDAY, 9/9/2019

Inducibility of bio-ink.

Faulty print plates: LB broth with 5, 2 and 1M of $CaCl_2$ were supplemented with 2, 3.5 and 7% of agar, however after drying for 30 minutes the agar still did not solidify. Solid plates are a must in microbiology, so we moved to lower concentrations. (potentially dry them in a laminar flow cabinet??) because these are really high calcium concentrations and as it is known that high concentrations of calcium chloride exert osmotic stress on bacteria, we decided to move to 0.05M $CaCl_2$ for future plates.

Making print plates: 200 ml LB agar was prepared with 3.1 g LB-broth and 3 g agar (1,5 %w/v). 2 mL of a 5M $CaCl_2$ stock solution was added for a final concentration of 0.05M $CaCl_2$. 3 mL of this agar was pipetted in a tiny petri dish. The plates were allowed to dry for 30 minutes in a laminar flow cabinet.

Bio-ink. *E.coli* and *E.coli* bearing a plasmid conferring ampicillin resistance and tetracycline inducible RFP were used to inoculate 25 mL of LB agar supplemented without and with 100 ug/mL ampicilin respectively. The cultures were grown overnight at 37*C and were shaken at 275 rpm. The next day the OD_{600} was over 2.5. Now, the bacteria were spun down for 3 minutes at 6500 rpm. The supernatant was discarded and was replaced with 25 mL liquid LB medium containing 2% sodium alginate (2% w/v) supplemented with 100 ug/ml ampicillin for the plasmid containing *E. Coli*.

Printing: 100 uL of the aforementioned bio-ink was dispersed randomly or as droplets over the print-plates using a 100 uL pipette. The ink solidified within a minute when comming into contact with the print plates. After 15 minutes, 100 uL of different concentrations of tetracycline or anhydrotetracycline, an inactive tetracycline mimic, shown in the table were applied to the plate using a 100 uL pipette, aiming to deposit liquid on all the areas where bio-ink was present. (for future, use spray??) After 30 minutes, the plates were incubated for 12 hours in a 37*C incubator and then kept at RT. The plates were observed after 12 and 36 hours.

Results:

After 12 hours

	A	B	C	D	E	F	G	H	I
1		no	no	Tetracycline	Anhydrotetracyclin				
2	Concentration	-	-	100 ng/mL	125 ng/mL	250 ng/mL	500 ng/mL	250 ug/mL	2.5 mg/mL
3	Empty	x	x	x	x	x	x	x	x
4	RFP	x	x	x	x	x	x	x	x

36 hours

	A	B	C	D	E	F	G	H	I
1		no	no	Tetracycline	Anhydrotetracyclin				
2	Concentration	-	-	100 ng/mL	125 ng/mL	250 ng/mL	500 ng/mL	250 ug/mL	2.5 mg/mL
3	Empty	x	x	x	x	x	x	x	x
4	RFP	x	x	x	x	x	x	Yes	Yes

Results: Indeed, induction is shown at 250 ug/mL and 2.5 mg/mL, generally people induce with lower concentrations, however, this is not seen. Potentially this is caused by the anhydrotetracycline having to diffuse through the calcium alginate hydrogell before being taken up by bacteria. Furthermore, inducibility is only seen after 36 hours. Potentially this is also a result of the diffusion process. Or, the bacteria residing in the alginate have a slower metabolism. Further investigations of the inducibility of the bacteria in the ink need to be conducted, using different techniques and different concentrations of the inducer (spray??) It is known that RFP usually develops after 16 hours, so other reporters should also be employed in future experiments.
Add pictures of these plates!

FRIDAY, 9/20/2019

Are they inducible inside the ink? YES!

Making print plates: 200 ml LB agar was prepared with 3.1 g LB-broth and 3 g agar (1,5 %w/v). 2 mL of a 5M $CaCl_2$ stock solution was added for a final concentration of 0.05M $CaCl_2$. 3 mL of this agar was pipetted in a tiny petri dish. The plates were allowed to dry for 30 minutes in a laminar flow cabinet.

Bio-ink. *E.coli* and *E.coli* bearing a plasmid conferring ampicillin resistance and tetracycline inducible RFP were used to inoculate 10 mL of LB agar supplemented without and with 100 ug/mL ampicilin respectively. The cultures were grown overnight at 37*C and were shaken at 275 rpm. The next day the OD_{600} was over 2.5. Now, the bacteria were spun down for 3 minutes at 4000 rpm. The supernatant was discarded and was replaced with 10 mL liquid LB medium containing 2% sodium alginate (2% w/v) supplemented with 100 ug/ml ampicillin for the ink containing *E. Coli* with a plasmid.

Printing: 100 uL of the aforementioned bio-ink was deposited on the print-plates as a droplet using a 100 uL pipette. The ink solidified within 3 minutes when comming into contact with the print plates. After 30 minutes, 100 uL of different concentrations of tetracycline or anhydrotetracycline, an inactive tetracycline mimic, shown in the table were applied to the plate using a 100 uL pipette, aiming to deposit liquid on all the areas where bio-ink was present. (for future, use spray??) After 30 minutes, the plates were incubated overnight in a 37*C incubator,

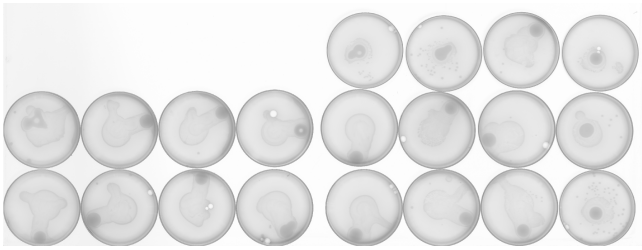
Results: After 24 hours, the plates were imaged in a Typhoon FLA 9500 imager using Alexa Fluor 488 nm excitation wavelength and the intensities were measured using ImageJ FiJI software imaging processing software. Plates that have a discontinuous ink droplet were excluded from the measurement. The ink droplet was selected in the software and the average intensities were measured. The relative average intensities were calculated by substracting the average intensities of all the negative controls from the average of the respective plates and are shown in table1.

Relative average intensities, corrected for background noise:

Table1

	A	B
1	100 ng/ml TC	704
2	250 ng/ml aTC	964
3	125 ng/ml aTC	692.5
4	62.5 ng/ml aTC	410
5	0 ng/ml aTC	359

GFP24hrsindutiontest-[Alexa Fluor 488].png



Map of plates

	A	B	C	D	E	F	G	H
1	x	x	x	x	B empty 0	A empty 0	B empty 62.5	A empty 62.5
2	B GFP 100 TC	B GFP 250 aTC	B GFP 125 aTC	B GFP 62.5 aTC	B GFP 0	B empty 100 TC	B empty 250	B empty 125 aTc
3	A GFP 100 TC	A GFP 250 aTC	A GFP 125 aTC	A GFP 62.5 aTC	A GFP 0	A empty 100 TC	B empty 250	B empty 125 aTc

RAW data:

inducibility 24h bioink.xlsx

SATURDAY, 9/21/2019

Inducibility after 24,72h, 1 week in the gel (DID NOT WORK!)

Making print plates: 200 ml LB agar was prepared with 3.1 g LB-broth and 3 g agar (1,5 %w/v). 2 mL of a 5M $CaCl_2$ stock solution was added for a final concentration of 0.05M $CaCl_2$. 3 mL of this agar was pipetted in a tiny petri dish. The plates were allowed to dry for 30 minutes in a laminar flow cabinet.

Bio-ink. *E.coli* and *E.coli* bearing a plasmid conferring ampicillin resistance and tetracycline inducible RFP were used to inoculate 10 mL of LB agar supplemented without and with 100 ug/mL ampicilin respectively. The cultures were grown overnight at 37*C and were shaken at 275 rpm. The next day the OD_{600} was over 2.5. Now, the bacteria were spun down for 3 minutes at 4000 rpm. The supernatant was discarded and was replaced with 10 mL liquid LB medium containing 2% sodium alginate (2% w/v) supplemented with 100 ug/ml ampicillin for the ink containing *E. Coli*.

Printing: 100 uL of the aforementioned bio-ink was deposited on the print-plates as a droplet using a 100 uL pipette. The ink solidified within 3 minutes when comming into contact with the print plates. After **24 hour, 72 hour and 1 week,** 100 uL of 250 ng/ aTC (anhydrotetracycline), an inactive tetracycline mimic, were applied to the plate using a 100 uL pipette, aiming to deposit liquid on all the areas where bio-ink was present.

Results: After 24 hours, the plates were imaged in a Typhoon FLA 9500 imager using Alexa Fluor 488 nm excitation wavelength and the intensities were measured using ImageJ FiJI software imaging processing software. Plates that have a discontinuous ink droplet were excluded from the measurement. The ink droplet was selected in the software and the average intensities were measured. The relative average intensities were calculated by substracting the average intensities of all the negative controls from the average of the respective plates and are shown in table2.d

pretty inconclusive thanks to leakyness, or harmfullness of 250 ng aTc. might try 100 TC or 125 aTC for next measurement.

Typhoon fluorescence induced after 24 h-[Alexa Fluor 488].png

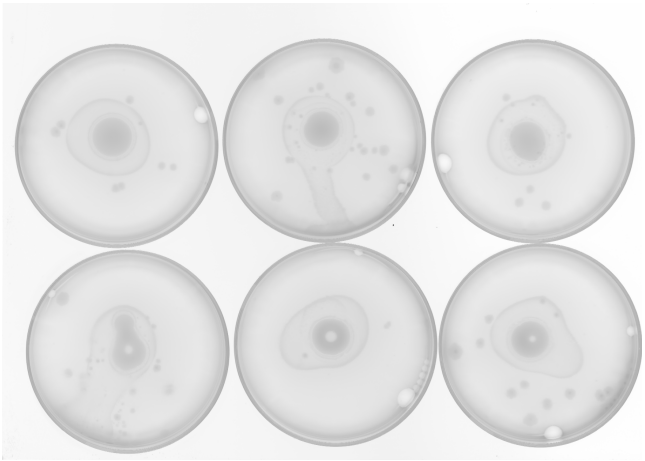


table 2 (results)

	A	B
1		corrected for neg. control
2	GFP + aTc	235
3	GFP - aTc	374

Raw data:

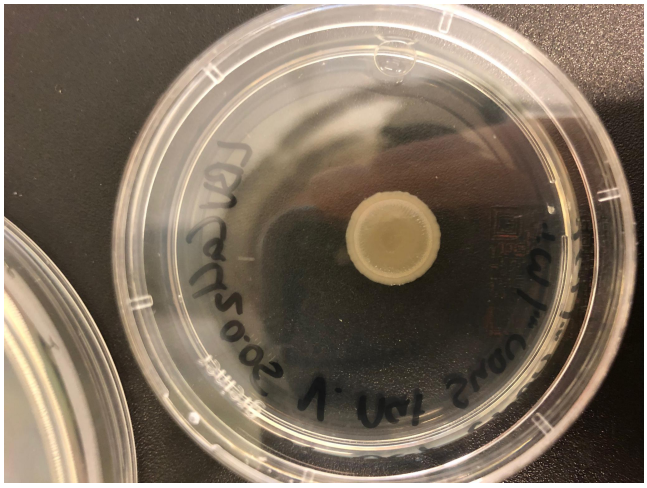
after 24h.xlsx

MONDAY, 9/23/2019

Survivability in the unprinted ink and printed Gel on a plate

Observation: The bacteria grow outside the gel on the plate, make biofilm?

IMG-9862.JPG



Making print plates: 200 ml LB agar was prepared with 3.1 g LB-broth and 3 g agar (1,5 %w/v). 2 mL of a 5M $CaCl_2$ stock solution was added for a final concentration of 0.05M $CaCl_2$. 3 mL of this agar was pipetted in a tiny petri dish. The plates were allowed to dry for 30 minutes in a laminar flow cabinet.

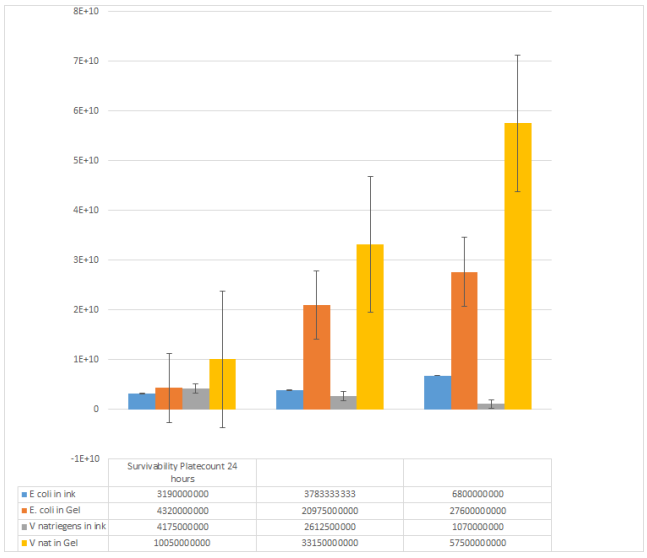
Bio-ink. 2 overnight cultures of *E.coli* and *V. Natriegens* grown overnight at 37*C, 275 rpm, OD_{600} was over 2.5 were spun down for 3 1/2 minute at 4000 rpm. The supernatant was discarded and was replaced with 10 mL liquid LB or LBV medium containing 2% sodium alginate (2% w/v).

Print : 100 ul was used to make a blob on the plates. The residual ink was stored at room temperature, the plates were kept 6h, 24h, 72h and 1w on the bench.

Sampling: after said induction times, samples were taken. The 100 ul blob was removed from the plate using tweezers in placed in 0.9 mL LB/LBV supplemented with 0.1 M sodium Citrate solution to chelate the calcium ions. after complete chelation and solution, the mixture was serially diluted twice and 10 uL was used to inoculated big plates. 100 uL was sampled form the unsolidified ink and also placed in citrate, before being serially diluted twice. Plates were incubagted at 37*C o
vernight and visible colonies were counted.

IMG-9860.JPG

image.png



	Survivability Platecount 24 hours		
E coli in ink	3190000000	3783333333	6800000000
E. coli in Gel	4320000000	20975000000	27600000000
V natriegens in ink	4175000000	2612500000	1070000000
V nat in Gel	10050000000	33150000000	57500000000

error bars don't seem to work in these kind of graphs.

RAW DATA:

Plate count 0 24 and 72 hours.xlsx

Same experiment, now on plates without LB, so just CaCL2 and agar.

Making print plates: 200 ml agar was prepared with 3 g agar (1,5 %w/v). 2 mL of a 5M $CaCl_2$ stock solution was added for a final concentration of 0.05M $CaCl_2$. 3 mL of this agar was pipetted in a tiny petri dish. The plates were allowed to dry for 30 minutes with the lid half open near a flame.

Bio-ink. 2 overnight cultures of *E.coli* and *V. Natriegens* grown overnight at 37*C, 275 rpm, OD_{600} was over 2.5 were spun down for 3 1/2 minute at 4000 rpm. The supernatant was discarded and was replaced with 10 mL liquid LB or LBV medium containing 2% sodium alginate (2% w/v).

Print : 100 ul was used to make a blob on the plates. The residual ink was stored at room temperature, the plates were kept 6h, 24h, 72h and 1w on the bench.

Sampling: after said induction times, samples were taken. The 100 ul blob was removed from the plate using tweezers and placed in 0.9 mL LB/LBV supplemented with 0.1 M sodium Citrate solution to chelate the calcium ions. after complete chelation and solution (15 mins), the mixture was serially diluted twice (10-1, 10-8) and 10 uL was used to inoculated big plates, the fluid was spread using a drigalski spatula. Plates were incubagted at 37*C overnight and visible colonies were counted.

RAW DATA:

platecount after 0h.xlsx

TUESDAY, 10/1/2019

Printing on empty vs full plates, simulated induction with H20

Bio-ink. 2 overnight cultures of *E.coli* and *V. Natriegens* grown overnight at 37*C, 275 rpm, OD_{600} was over 2.5, were spun down for 3 1/2 minute at 4000 rpm. The supernatant was discarded and was replaced with 10 mL liquid LB or LBV medium containing 2% sodium alginate (2% w/v).

Printin plates were prepared with LBV+0.05 M CaCl2, LB + 0.05M CaCl2, and just 0.05M CaCl2 (1.5% agar w/v)

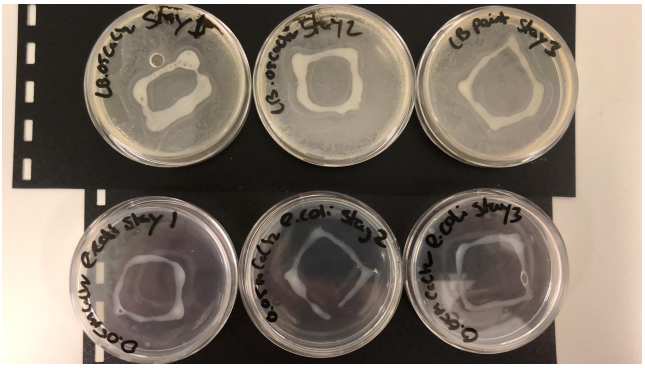
A square was deposited (100 ul of ink was used) in triplicate, after 30 min, 200 uL milliQ was added and the plate was gently swirled to spread the liquid over the complete surface. The moisture was allowed to evaporate for 30 min with the lid half open before being incubated at 37 degrees o/n.

Results:
The plates were observed the next day: t
The plates that had no supplemented medai showed no sign of bacterial growth outside of the ink!!!!!!!!!!! WE CAN USE THIS to stamp/print with high precision and increase robustness.

PICS: (few examples)

pics.zip

60AB9B6E-7EAD-4F85-B4F3-D4C2AAD30485.JPG



WEDNESDAY, 10/2/2019

Biofilm induction

Plates were made with LB and with rhamnose (0.5% w/v) and without LB and with ramnose (0.5% w/v).

Overnight culture of bacteria harbouring pAM413 (GFP + CsgA+)
and pAM 419 (GFP, CsgA-) were made into ink, (see procedure before (allthough usually i keep 0.5ml of media in there, resuspend, and then add the alginate media then vortex some more.

100 uL of ink was then deposited in a square on the respective dishes (in duplo)

After 1d, 2d, 3d, 4d, the plates were observed. then, 5 ml of 0.1M sodium Citrate solution was added to the petri dishes. The dishes were placed in a water bath (without water) and gently shaken at 70 RPM for 2 hours.
After 2 hours, the liquid was observed and the plates were observed again.

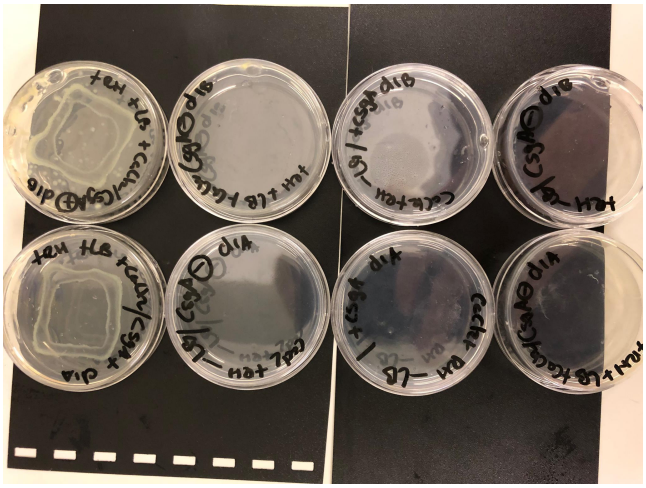
MAP:

Table2

	A	B	C	D
1				
2	CcsgA?			
3	LB	+	+	
4		-	-	
5	empty	+	+	
6		-	-	

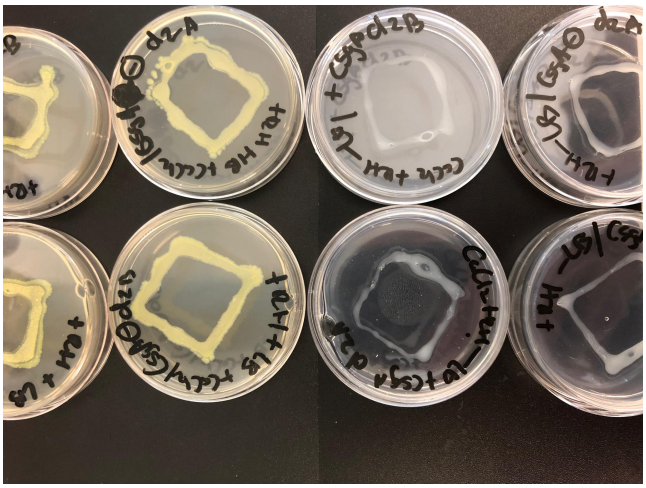
day 1:

induction after d1.JPG



Day 2:

IMG-9933.JPG



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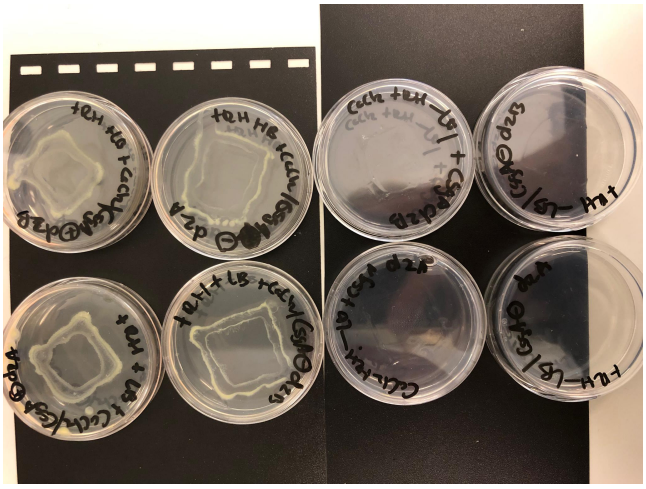

day 2: succes

Typhoon:

Biofilm after 2 and 3 days.2-[Alexa Fluor 488]PNG.png