CarbanEl—A sustainable solution to triclocarban in water

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I. INTRODUCTION

Pharmaceutical and personal care products (PPCPs) like cosmetics, soaps, disinfectants, and detergents comprise many chemical classes, including antimicrobial compounds. PPCPs containing these compounds are consumed globally at a rate of 0.1 million tonnes per year and often find their way into wastewater treatment plants (WWTPs) through washing, direct disposal, or human excrement [1].Due to its high hydrophobicity and chemical stability, it becomes challenging to degrade TCC thoroughly once flushed off into the WWTPs, and 76-79% of TCC is released with sewage sludge.

At local WWTPs in Udupi, India, and Mangalore, India, the average concentration of TCC recorded in the sludge was 21,000 ng/g dw and 13,000 ng/g dw, respectively [3]. The concentration of TCC detected in Indian aquatic environments (including surface waters or even drinking water) was 1119 ng/L [2]. On a global scale, the median values of detected TCC in surface water worldwide ranged around 10-100 ng/L, 51-347 ng/L, and 7.5-102 ng/L TCC were monitored in tap and treated drinking water, respectively [4].

TCC is found to accumulate within our flora and fauna, causing hormone disruption. Several studies indicate that humans absorb a significant portion of TCC in soaps during and after showering. TCC exposure occurs through water or food consumption [5]. The maternal and umbilical cord sera have reported around 2.75 and 0.82 µg/L of TCC concentration. TCC could potentially inhibit the human soluble epoxide hydrolase (sEH), which is involved in the biological regulation of pain, inflammation, and blood pressure [6]. It has also been demonstrated to induce oxidative stress and cause physical dysfunctions in animals and humans [7,8].

Among majorly affected species, exposure to 5-10 mg/L of TCC for 21 days reduced mobility, feeding, aggression, fecundity, or even caused death in fathead minnows [2]. In zebra fishes, exposure to 6.6 µg/L of TCC altered the expression of proteins related to nervous system development and immune response, binding and metabolism, skeletal muscle development, and function[2]. In food crops cultured in hydroponic media, after four weeks of exposure, roots accumulated 86–1,350 mg kg^{-1} of antimicrobials, and shoots had accumulated 0.33-5.35 mg kg^{-1} of antimicrobials, including triclocarban [9]. The uptake and accumulation of TCC due to using contaminated biosolids has been detected in several food crops —carrots, green peppers, tomatoes, and cucumbers—and has been proven to hinder proper crop metabolism [2].

Standard pollutant removal practices are also implemented for triclocarban but have proven inadequate. Although activated carbon adsorption has been considered, its disadvantages include high costs, lack of selectivity, and a limited regenerative capacity [10]. Ultraviolet light (UV), chlorine oxidation, ozone, and electron-Fenton are usually considered promising methods. However, inorganic ions, like sulfate and nitrate, often found in wastewater, were found to hinder the photolysis of TCC by UV radiation [11]. Chlorine oxidation often results in the incomplete degradation of the compounds and the production of hazardous byproducts. Chemical approaches are generally more expensive, while ozone-based techniques have been proven to be less effective. It was found that most methods currently utilized by WWTPs in India aren't adequate for removing TCC [2].

Further research elucidated the toxic nature of the chloroaniline byproducts formed using these methods. According to studies conducted by the World Health Organisation and Centers for Disease Control and Prevention, 4-chloroaniline and 3,4-dichloroaniline act as carcinogens and cause methemoglobinemia—which is a condition characterized by an abnormal form of haemoglobin in the blood leading to cyanosis and hypoxia.

Thus, we are working with the TccA gene from *Ochrobactrum sp.* which codes for an amidase enzyme that breaks down TCC into toxic byproducts, 4-chloroaniline and 3,4-dichloroaniline[4]. To combat this, we would introduce the gene into an organism that inherently possessed the pathways to naturally degrade the chloroaniline byproducts. Finally, this will form non-toxic daughter compounds that enter the TCA metabolic cycle. Our designed bacteria would degrade this compound into harmless byproducts in a modeled packed bed bioreactor at a wastewater treatment plant, avoiding the negative impact of TCC in the real world. We plan to integrate biocontainment strategies and kill switches into our project design to address concerns regarding safe and responsible use of

genetically modified organisms.

II. MATERIALS AND METHODS:

Chemicals, bacterial strains and culture conditions: TCC (99% purity) and high performance liquid chromatography (HPLC) grade acetonitrile were purchased from Sigma-Aldrich (St. Louis, MO, USA). 3,4-Dichloroaniline (98% purity), 4-chloroaniline(98% purity) was purchased from the Tokyo Chemical Industry. All other chemicals used were of analytical grade. Required enzymes for DNA manipulation were all purchased from New England Biolabs. Stock solutions of TCC (1%, 150 w/vol), 3,4-DCA (1%, w/vol) and 4-CA (1%, w/vol) were prepared in acetonitrile (1:1; vol/vol). Competent *Escherichia coli* DH5α and *E. coli* BL21 were cultured in Luria-Bertani (LB) medium at 37°C at 180rpm. Plasmid vectors pET 22b+ were used for DNA cloning and gene expression. For growth curves and study of the chassis, *Pseudomonas putida* (MTCC, India), *Pseudomonas fluorescens* (MTCC, India) and *Acinetobacter baylyi* cultured in (NCMR, India) cultured in Luria-Bertani (LB) medium at 30°C at 180rpm.

Mineral salt medium (MSM) consisted of NaCl (1.157 g/L), NH4Cl (1 g/L), K2HPO4 (1.5 g/L), KH2PO4 (0.5 g/L), MgSO4·7H2O (0.2 g/L), pH 7.0. The media were sterilized by autoclaving at 121°C for 30 min before use. Ampicillin (Amp; 100 mg/L) was supplemented to the media as necessary.

In Silico Analysis: All DNA constructs were designed using the SnapGene software. The same platform was used to simulate cloning and other modifications in DNA. Stem-loops, homodimers, and heterodimers were analyzed using the IDT OligoAnalyzer tool. The dimers that are likely to form were highly unstable.

Plasmid Construction and transformation: Plasmid pET22b+TccA was constructed by cloning the 1.5-kb fragment of TccA gene into the BamHI-XhoI site of plasmid pET 22b+ via Gibson assembly cloning kit from New England Biolabs.

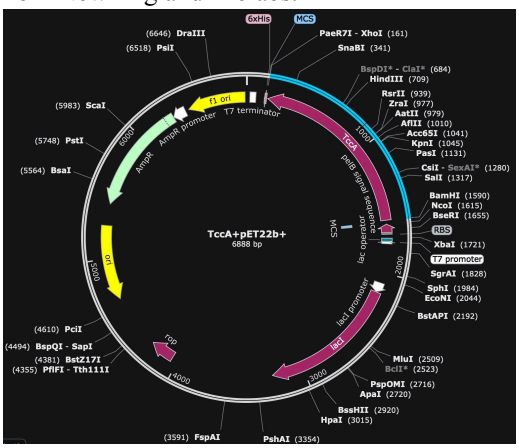

Fig. 1. Plasmid design of pET22b+TccA

The TccA gene was codon optimized for *E.coli* and synthesized by Integrated DNA Technologies. The plasmids were then transformed into *E.coli* by heat shock method and plated onto Luria-Bertini agar supplemented with Ampicillin (100mg/L).

Expression of Plasmid pET22b+ in E. coli: *E. coli* BL21 cells harbouring plasmid pET 22b+TccA were grown at 37°C in LB medium supplemented with ampicillin (100 µg/ml) to an OD600 of 0.6 and then induced with isopropyl β-d-thiogalactoside (1 mM). E. coli strain BL21 harbouring plasmid pET 22b+ was used as a control. Aliquots of 1 ml were withdrawn at several time intervals after induction, and the cells were harvested by centrifugation (8,000 × g for 1 min). The cell pellet was resuspended in 100 µl of extraction buffer (60 mM Tris·HCl/1% 2-mercaptoethanol/1% SDS/10% glycerol/0.01% bromophenol blue, pH 6.8) and heated in boiling water for 5 min. Samples (20 µl) were subjected to SDS/PAGE (10% acrylamide) , and the separated proteins were stained with Coomassie brilliant blue.

Growth curves: For the growth curve measurements conducted in duplicates, 250 mL flasks containing Luria Bertani broth at the required pH or with addition of TCC were first sterilized by autoclaving at 121°C for 20 mins. The broth was inoculated with 1% of overnight culture.

To measure the growth of bacteria, 1000 µL of the bacterial cultures were transferred to a 10mm plastic cuvette. The cuvette was placed in a UV/Vis spectrophotometer (Eppendorf) to monitor the OD at 600 nm of the bacteria. The plates were maintained at 30°C and rotated at 180 rpm. The OD of the flasks were read every hour for 24 hours.

Making of a Raschig Ring: The biochar is initially obtained as a powder or in granular from a local rice mill. The shaping process involves forming the material into the characteristic cylindrical ring shape. We hypothesize that this can be achieved through the method of pressing of biochar with additional binding agents. The shaped rings are fired in a furnace at high temperatures. However, biochar produced at high pyrolysis temperatures (greater than 500°C) leads to the unsuccessful formation of pellets and rings and results in the degradation of biochar's organic matter. This makes the biochar brittle, and therefore, further optimization is required for firing and heating temperatures for biochar to make raschig rings [12-14]. This optimization includes testing the stability and strength of the ring under a bioreactor sparger when different binding materials and ratios are used. The ideal ring should be able to withstand its shape under the reactor conditions.

Proposed Immobilization of Bacteria on Raschig Rings for future implementation: Bacteria was cultured at 30°C at 150 rpm for 12 hours in a Minimal

Salt Medium containing 0.66 g/L NaH2PO4.2H2O, 0.58 g/L Na2HPO4, 0.5 g/L NaCl, 2 g/L NH4Cl, 3 g/L KH2PO4, and 0.25 g/L MgSO4.7H2O at pH 7.0. The bacteria-inoculated Minimal Salt Medium (MSM) was then centrifuged at 6000 rpm for 10 minutes, and the supernatant was discarded. The particle was resuspended and rinsed with 0.85% NaCl. To increase the concentration of the bacteria, 10 mL of MSM is added. Biochar was mixed with MSM with TCC and concentrated bacteria to immobilize cells. The mixture was cultured at 150 rpm for 48 hours at room temperature. The cells are immobilized on the surface of the biochar. Two methods can be employed to immobilize the microorganisms on the Raschig rings:
1. Adsorption- In this process, we gently shake or swirl the raschig rings with the bacterial culture to allow the bacteria to attach to the ring surface.
2. Dipping- In this method, we dip the raschig ring into a bacterial culture for a fixed time to allow the bacteria to adhere to the surface.

III. RESULTS

A. To find the most suitable chassis

In order to discern the best possible model organism for the degradation of TCC, growth curve experiments were performed with our three possible model organisms, *Acinetobacter baylyi, Pseudomonas putida* and *Pseudomonas fluorescens* to study which had best survivability in wastewater treatment plant conditions. To this end, the bacteria were inoculated in media that mimics the conditions of wastewater treatment plant's sludge tank. These conditions include the varying pH range usually exhibited in a sludge tank, the temperature range and concentration of TCC observed in the sludge. Growth curves were plotted for the organisms in media containing acetonitrile as well as it is used to dissolve TCC. The growth was measured by checking the arbitrary units of optical density at 600nm. The observations from the experiments are given below:

1. pH range:

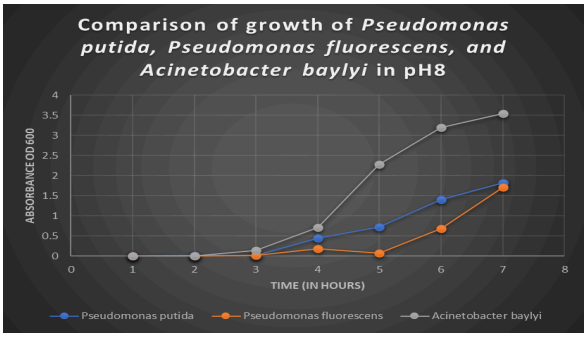

Fig. 2. Comparison of the growth of *Pseudomonas putida, Pseudomonas fluorescens and Acinetobacter baylyi* at pH 8.

2. TCC (sludge concentration levels)

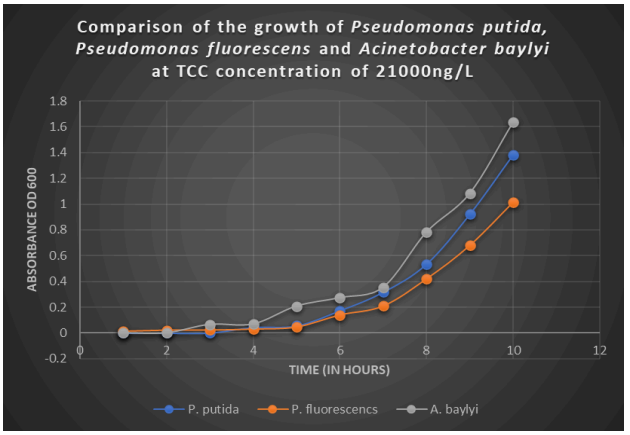

Fig. 3. Comparison of the growth of *Pseudomonas putida, Pseudomonas fluorescens and Acinetobacter baylyi* at TCC concentration of 21,000ng/L.

After analyzing these growth curves, we were able to come to the following conclusions:

a. Pseudomonas putida :
- We found that it showed optimal growth at 30ºC and 7.5pH
- Its growth is stunted under the presence of TCC.
- It shows reduced growth at 37ºC.

b. Pseudomonas fluorescens :
- shows good growth at 30ºC, 7.5 pH.
- growth is stunted under the presence of TCC.

c. Acinetobacter baylyi :
- The lag phase for the secondary inoculation was about 2-3 hours.
- We found that 30ºC, 7.5 pH were the ideal conditions for growth and surprisingly, the presence of such high concentrations of TCC did not impede its exponential growth. Hence, *A. baylyi* can proliferate and grow uninhibited in the presence of TCC.

From these observations, we came to the conclusion that *Acinetobacter baylyi* is the best possible choice for the ideal model organism due to its ability to proliferate in environments containing near-toxic levels of TCC as well as with all the other parameters that it would be subjected to within the sludge tank of a wastewater treatment plant. The other two bacteria were promptly rejected due to their growth being stunted in the temperature, pH range, and range of TCC concentration observed in a sludge tank.

B. To study the characteristics of the amidase enzyme produced by TccA gene

To study the TccA amidase characteristics, the pET22b+ plasmid was isolated and Gibson Assembly was used to insert the TccA amidase gene. The competent *E.coli* DH5alpha cells were prepared by

CaCl2 method and the cells were transformed.
After a confirmatory colony PCR to check the presence of our insert, we transformed the Gibson-assembled plasmid into *E.coli* BL21, and then we moved towards expression studies.
Double digestion and colony PCR was then performed on the isolated plasmid of the transformed cells to confirm the presence of the fragment, which was successful; distinct bands were observed. We then proceeded to do expression studies.

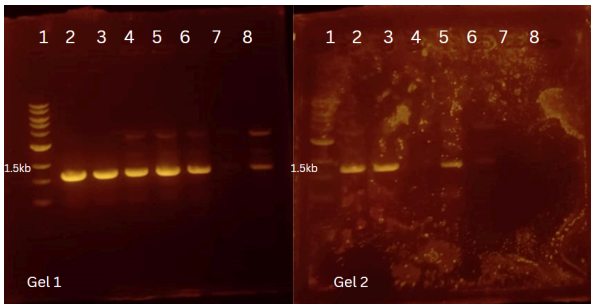

Fig. 4. Lane 1 of gel 1 and 2- 1kb DNA ladder,Lane 2-7 of gel 1- Colony PCR of colonies 1-7,Lane 2-4 of gel 2- Colony PCR of colonies 8-10 , Lane 5 of gel 2- Positive control

SDS-PAGE was carried out, but distinct bands were not observed. This could have been due to the extended time for destaining. Further experiments and assays need to be carried out to check the expression and efficiency of the TccA amidase.

IV. INFERENCES

Despite previous indications that all three organisms had the capability to degrade TCC in different environments, the results of our experiments contradicted these expectations.

Pseudomonas putida KT2440 and *Pseudomonas fluorescens* strain MC46 were known for their ability to facilitate the removal of TCC. However it underwent self-substrate inhibition at a concentration of 30 mg/L [12]. Contrary to expectations, when the growth curves were analyzed with TCC concentrations ranging from 13 microg/L to 21 microg/L, both *Pseudomonas fluorescens* and *Pseudomonas putida* strains either failed to efficiently break down TCC into simpler compounds or exhibited self-substrate inhibition, resulting in stunted growth. In contrast, *Acinetobacter baylyi* growth is not hindered by the concentrations typically found in wastewater treatment plants. By inserting the TccA gene into *A.baylyi*, a sustainable solution to degrade TCC to non-toxic byproducts is possible.

The promising performance of *Acinetobacter baylyi* in utilizing TCC without growth inhibition implies its potential for application in wastewater treatment processes.

V. MODELING

The bioreactor modeling consists of three main segments -

- Growth kinetics of the untransformed chassis
- Adsorption kinetics of the TCC onto the biochar raschig ring
- Degradation of the TCC into non-toxic cis-muconic acid, which will enter the Krebs cycle.

All these kinetics are discussed below.

I. Growth kinetics

As *Acinetobacter baylyi* follows Monod kinetics, we use the Monod equation to calculate growth of organisms.

The Ks value was obtained from the growth curve data via experimentation and a few assumptions were made to develop a design equation for the proposed bioreactor model which can be accessed in the supplementary material.

To check the growth of our chassis utilizing LB broth in the presence of TCC.

Growth Rate: $\frac{1}{X}\frac{dX}{dt} = \mu = \frac{d(\ln X)}{dt}$ where, (1)

X is the concentration of cell/biomass concentration

μ is the specific growth rate

For the Batch Reactor:

Accumulation = Input - Output + Generation + Consumption

$$\frac{d(XV)}{dx} = 0 - 0 + Vr_x + 0$$

Here the input and output is 0 because it is a closed system. The consumption is 0 because the biomass is not consumed.

V is the constant volume

r_x *is the growth rate of cell biomass due to division*

Vr_x is the generation of biomass due to the multiplication of cells.

$$\frac{dX}{dt} = r_x = \mu X \quad (2)$$

$$\frac{dX}{dt} = \frac{\mu max.S}{S+K_s} X \quad (3)$$

- S is the final amount of LB broth (limiting substrate)
- K_s is the Monod half saturation constant

- S_o is the initial amount of LB broth

$Y_{X/S}$ = Growth yield coefficient = $\frac{X-X_o}{S_o-S}$ (4)

$Y_{X/S}$ (S_o – S) + X_o = X (final concentration at time t) — (1)

$\frac{dX}{dt} = \frac{\mu_{max}(S)}{S+K_s}$ X— (2) (5)

Therefore, from the above two equations,

$$t(\mu_{max}) = A\left(ln\left(\frac{X}{X_o}\right)\right) + B\left(ln\left(\frac{S_o}{S}\right)\right) \quad (6)$$

This equation calculates the time required for the exponential phase of growth of the chassis in the presence of TCC at ppm levels in our ideal batch reactor.
In the equation:

- A= $\frac{K_S(Y_{X/S})}{X_o+S_o(Y_{X/S})} + 1$ (7)
- B = $\frac{K_S(Y_{X/S})}{X_o+S_o(Y_{X/S})}$ (8)

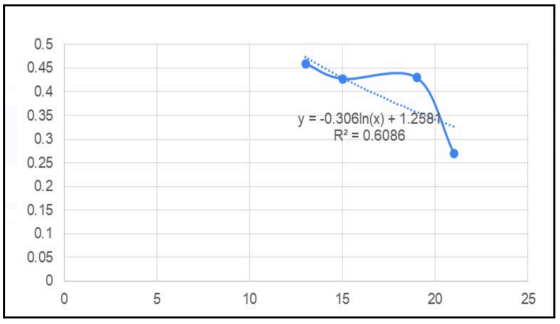

Fig. 5. Monod curve of *Acinetobacter baylyi* in TCC

From the Monod curve obtained from growth curve experiments on *Acinetobacter baylyi,* the value of μ_{max} were obtained

μ_{max} = 0.45 h^{-1}

$\frac{\mu_{max}}{2}$ = 0.225 h^{-1}

Assumption: Initial concentration of substrate (LB) is 100% while as the biomass increases, 90% of the initial amount of LB decreases, which means only 10% of the initial amount of LB is left as the final substrate concentration.

Hence, $S = 0.1S_0$ (9)

$X_0e^{\mu t} = X$
(10)

In batch, growth change in the biomass concentration with respect to time in the exponential phase is given by equation (10). (Assumptions mentioned in supplementary material.)

$\mu = \frac{\mu_{max}S}{k_s+S}$ (11)

assume k_s << Hence $\mu = \mu_{max}$

To calculate the yield coefficient of our bacteria

$Y_{x/s} = \frac{X-X_0}{S_0-S}$ (from equation 4)

X = 0.04155 $\frac{g}{mL\ of\ broth}$

$X_0 = 5.65\ x\ 10^{-4}\ g/mL$ of broth

$S_0 - S = S_0 - 0.1S_0$

$= 0.9S_0$

We know $X = X_0e^{\mu t}$ as $\mu = \mu_{max}$

$X = X_0e^{\mu_{max}t}$

$\ln(\frac{X}{X_0}) = \mu_{max}t$

Here, t is the batch time

$$t = \frac{1}{0.45} ln\left(\frac{0.04155\frac{g}{mL\ of\ broth}}{5.565\ x\ 10^{-4}\frac{g}{mL\ of\ broth}}\right) \quad (12)$$

t = 9.55 hrs. Which is the amount of time required by the chassis to grow and degrate TCC efficiently.

II. Adsorption Kinetics

To treat 1.2 liters of sludge, a few assumptions are taken as mentioned in supplementary material.

Material balance: TCC lost from sludge = TCC gained by biochar, displayed in equation number (13)

$$L(y_o - y_1) = S(q_1 - q_o) \quad (13)$$

Assumption: 90% of TCC gets adsorbed

$y_o - \frac{90}{100}y_o = y_1$ (14)

Since 1.2 liter is the volume of our lab-scale batch-packed bed bioreactor so L = 1.2 L

$1.2\left(y_o - y_1\right) = S(q_1 - q_o) \rightarrow (1)$ (15)

Case 1: Taking y_o as 21 ppm

$for\ n = 1$(Cycle 1)

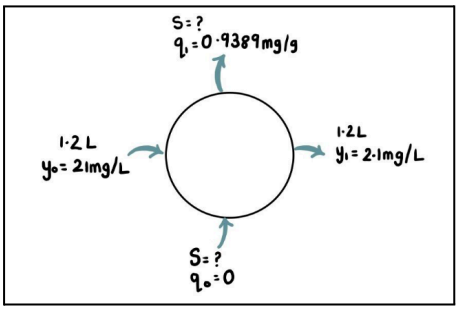

Fig. 6. Material balance of cycle 1 of adsorption kinetics.

This is the material balance of cycle 1 of adsorption kinetics. Using the equation $\frac{t\,k_s q_e^{\,2}}{1+tk_s q_e} = q_1$ for t= 48 hours (since we have kept the batch time as 2 days),

$q_1 = \frac{(1^2 \times 0.32 \times 48)}{1+(0.32\times 48)} = 0.9389 \frac{mg}{g}$

Hence: $1.2(21 - 2.1) = S_1(0.9389 - 0)$

$S_1 = 24.1559\ g$

S_1 gives us the amount of biochar needed to adsorb 90% of TCC from sludge in cycle 1.

Assumption: 90% efficiency for degradation.

$(21 - 2.1 = 18.9 mg/L\ is\ getting\ adsorbed)$

Therefore, 90% of 18.9 $\frac{mg}{l}$ is getting degraded by our bacteria(again taking 90% degradation efficiency).

$\frac{90}{100} \times 18.9 \frac{mg}{L} = 17.01 \frac{mg}{L}$ (17)

$17.01 \frac{mg}{L}$ is the total amount of TCC getting degraded

$18.9 - 17.01 = 1.89 \frac{mg}{L}$ of TCC is left behind on the biochar. Converting the units of $1.89 \frac{mg\ TCC}{L\ of\ soln}$ to $\frac{mg\ TCC}{g\ of\ biochar}$

$q_1 = 1.89 \frac{mg}{L} \times \frac{1.2\ L}{24.1559\ g}$

$q_1 = 0.0939\ \frac{mg}{g}$ (18)

q_1 is the mg of TCC left behind per g of biochar after cycle 1.

$n = 2$ (Cycle 2)

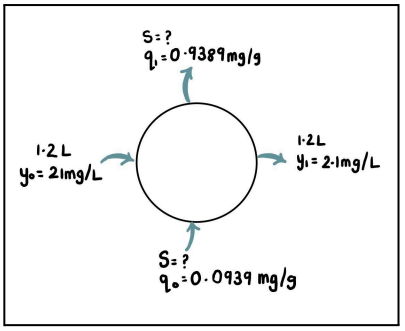

Fig. 7. Material balance for cycle 2 adsorption kinetics

Here, 18.9 $\frac{mg}{L}$ is getting adsorbed again by biochar every cycle in 48 hours(each cycle is for 48 hours).

$1.2(21 - 2.1) = 22.68$

$22.68 = S_2(0.9389 - 0.0939)$

$S_2 = 26.84\ g$

Assumption: 90% efficiency for degradation.

$(21 - 2.1 = 18.9 mg/l)$

Therefore, 90% of 18.9 $\frac{mg}{l}$ is getting degraded.

$\frac{90}{100} \times 18.9 \frac{mg}{L} = 17.01 \frac{mg}{L}$

$18.9 - 17.01 = 1.89 \frac{mg}{L}$ of TCC is left behind on the biochar.

$1.89 \frac{mg\ TCC}{L\ of\ soln}$ converting the units to $\frac{mg\ TCC}{g\ of\ biochar}$

$q_2 = 1.89 \frac{mg}{L} \times \frac{1.2\ L}{26.1559\ g\ biochar}$

$q_2 = 0.0867\ \frac{mg}{g}$

$\left(S_1 + S_2 + S_3 ..... + S_n\right) = 1211\ g$

We get a value of n = 46, where n is the number of cycles that can treat 21 ppm of TCC with 1211 g of biochar. For 46 cycles, our packed bed can treat about 46x1.2L of sludge = 55.2 L of sludge. Each cycle is run for 2 days, so 46x2 = 92 days of run time. Hence, 55.2L of sludge can be treated in 92 days, after which our packing material needs to be replaced.

Degradation kinetics

To optimize the method, we decided to do the calculations based on the highest (21 ppm), lowest (10 ppm), and average concentration (15 ppm) of TCC in sludge. We have gone with 90% degradation efficiency in each case.
To simplify our calculations, we have assumed that no

interference with the degradation of TCC.
For varying concentrations (ppm):

21 ppm	15 ppm	10 ppm
$t = -\int_{CA0}^{CA} \frac{dC_A}{(-r_A)}$ $-r_A = 0.112C_A$ $t = -\int_{21}^{2.1} \frac{dC_A}{0.112C_A}$ $= -\frac{1}{0.112} ln \frac{2.1}{21}$ $=$ 20.56 hrs.	$t = -\int_{15}^{1.5} \frac{1}{0.112} \frac{dC_A}{C_A}$ $= -\int_{15}^{1.5} \frac{dC_A}{0.112C_A}$ = 20.558 hrs ~ 20.56 hrs.	$t = -\int_{10}^{1} \frac{dC_A}{-r_A}$ $= -\int_{10}^{1} \frac{dC_A}{0.112C_A}$ $= -\frac{1}{0.112} ln \frac{1}{10}$ = 20.558 hrs ~ 20.56 hrs.

The time to degrade 90% of the initial TCC concentration is the same in all three cases(20.56 hrs), as it only considers the degradation kinetics and not the biomass needed in each case. To degrade 21 ppm of TCC, the amount of biomass required is much more than 10 ppm of TCC, which is not considered in the pure degradation kinetics but is considered in the growth kinetics. Unlike other models available, the time required for growth has also been considered along with the degradation kinetics [15].
The time calculated here for 21 ppm will be used to model the bioreactor.

FUTURE IMPLEMENTATION

The concentration of Triclocarban (TCC) in the sludge (21000 ng/g dry weight) of a wastewater treatment plant is much higher than in the effluent (13000 ng/g dry weight) [3], and this is difficult to degrade due to the compounds' chemical stability and high hydrophobicity. To combat this, a packed bed batch process bioreactor can be set up in the sludge treatment plant and efficiently degrade TCC into non-toxic compounds using our chassis - a modified *Acinetobacter baylyi* GFJ2. This bioreactor will treat the primary sludge which is still 95-97% water. The bioreactor will allow a closed, secluded environment for the chassis to perform complete degradation of the harmful TCC and give the chassis the required amount of time for the complete degradation to occur while following biosafety.

An additional advantage of the chassis is its ability to degrade a broad variety of substrates, apart from TCC[15] which will be aided by the packing material of the bioreactor. It will be made of biochar, which is a carbon-rich substance produced by the thermal combustion of organic waste such as rice or corn stalks in an oxygen-limited environment. It is a highly nutritious environment for the growth of bacteria. Biochar has a high surface area, due to which it exhibits micropollutant and cell adsorption properties. It has high cation exchange capacity and stability, making it useful for packing material for the packed bed bioreactor. Biochar is a simple, affordable, and effective choice for the adsorption of our target contaminant, triclocarban (TCC)[16]. The biochar has the perfect pore size of 0.6 to 0.8 nanometers required to adsorb TCC and can be modified by adding KOH to increase the adsorption efficiency. Biochar can adsorb other hydrophobic compounds quite efficiently as well. With the chassis immobilized on the biochar, it can then degrade not only TCC but other toxic compounds such as diflubenzuron, 3,4-DCA, 4-CA, 4-chlorophenyl-urea, carbanilide, etc.[15]

The packing material will be made in the shape called a Raschig ring which is a hollow cylinder of equal outer diameter to height ratio each being 2 centimeters as shown in fig.8[17]. This is the most ideal shape as its hollow aspect can prevent any chance of the bioreactor choking on the sludge and increases surface area that will be in contact with the sludge which will allow higher adsorption and hence higher degradation efficiency[18,19].

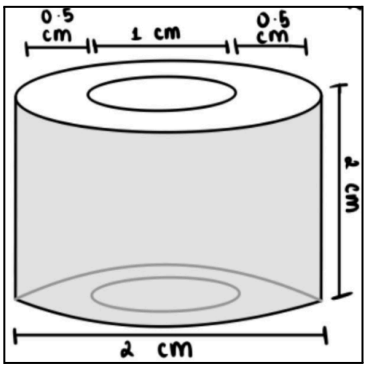

Fig. 8. Raschig Ring

The design of the packed bed reactor (Fig. 10) [18] would consist of the above mentioned packing material with immobilized bacteria between two perforated plates. The primary sludge would enter from the top of the reactor and flow to the bottom with the help of gravity. This eliminates the need for a pump to fill the sludge in the reactor. A sparger at the bottom center would aerate the sludge and promote mixing to help increase the mass transfer efficiency of the TCC onto the biochar.

The sparger (Fig. 9) will have a valve to prevent the backflow of sludge into it, and the sparging will begin before the inflow of sludge into the reactor begins. The outlet pipe will also have a valve to prevent sludge from leaving the tank before the specified time. The valve in the inlet pipe is to control the flow rate of the sludge entering the tank. The reactor would be fitted with a pressure gauge to measure and maintain the pressure drop across the reactor. The reactor will be operated in batch mode in unsterile conditions.

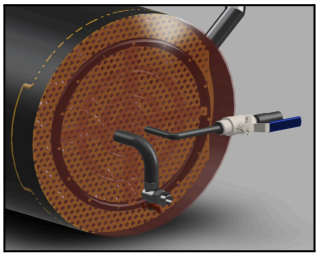
Fig. 9. CAD design of the sparger and perforated plate

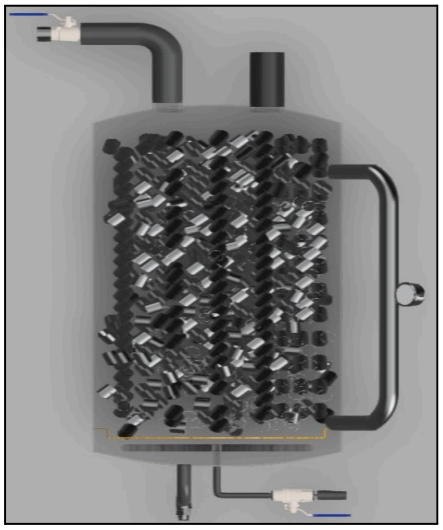

Fig. 10. CAD design of the bioreactor with randomized packing

Mechanism

To treat the sludge that is continuously generated from the wastewater treatment plant, a toggling mechanism can be used, where the system will consist of two bioreactors, but only one will work at a time while the other will get regenerated. The toggling time is kept in such a way that our bacteria will have time to grow and degrade TCC with up to 90% efficiency. After studying the growth kinetics of the chassis and taking into consideration the time taken for TCC to get adsorbed on the biochar and get degraded by the bacteria with 90% efficiency, the time for which the sludge remains in one of the bioreactors before it is switched to the other bioreactor, is 2 days at the maximum.

VII. HARDWARE (SENSOR)

An additional aspect of the bioreactor will be a sensor that can detect the concentration of TCC in the sludge entering the bioreactor and exiting the bioreactor.

The sensor consists of a laser, photodiode, and infrared filter placed inside a sample collector. The initial and final feed samples would be taken out manually from the inlet and outlet pipe of the bioreactor via valves. The sample would then be placed into the sample collector, where monochromatic light from the laser would interact with the compound.

If the presence of photons within a specific wavelength range is identified, the photodiode coupled with an infra-red (IR) filter would capture these photons and confirm the presence of triclocarban in the given sample. Typically, multiple wavelength ranges are isolated for a particular compound using Raman spectroscopy, and these wavelength ranges can be integrated to design a photodiode particular to the compound.

By attaching a microcontroller to the photodiode, data can be wirelessly transmitted to software, where reduction of background noise and quantification of triclocarban would be done.

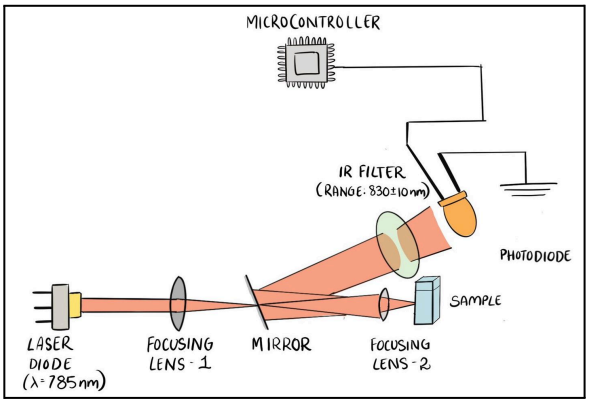

Fig. 11. Block diagram of the proposed sensor to detect TCC

The proposed design of the sensor is proven via Raman spectroscopy to identify specific bond vibrations (peaks) in the structure of triclocarban[20]. Raman spectroscopy, unlike other elemental analysis, is a non-destructive method and does not change the samples composition or structure and offers higher resolution compared to other spectral methods.

Using an excitation laser of wavelength 785 nm, Surface Enhanced Raman Spectral analysis of triclocarban was analyzed. Using this data, we can come to a conclusion that the Raman Shift should be around 750-760 cm-1 (wavenumber).

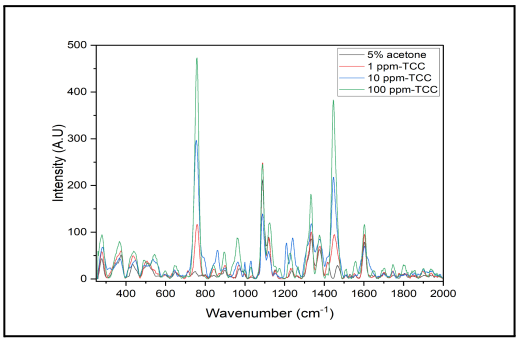

Fig. 12. Raman Spectroscopy results

The wavelength was calculated as 834.9185 nm. The specific wavelength can be acquired using an IR filter of wavelength range of 820nm to 840 nm. Additionally, a silicon PIN photodiode within the range of 350nm to 1100 nm was selected. A microcontroller attached to this photodiode would wirelessly transmit the data to software which would

be programmed to quantify triclocarban using the standard calibration curve.

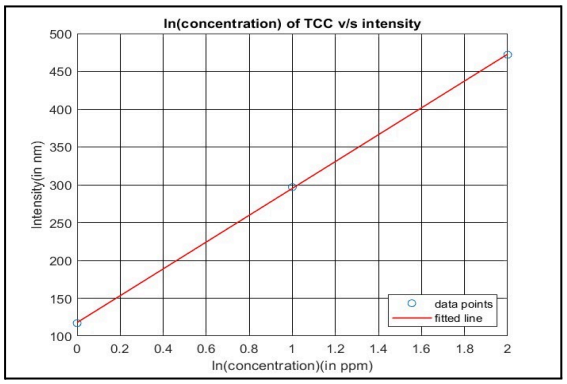

Fig. 13. Calibration curve for TCC

VIII. KILL SWITCH FOR BIOCONTAINMENT

The project outlines the development of a CRISPR-based kill switch for the biocontainment of a genetically modified organism, *Acinetobacter baylyi*. The primary goal is to prevent the escape of the modified bacteria into the environment. The proposed "Light-Induced Kill Switch" utilizes a specific wavelength of blue light as a trigger for bacterial containment. It builds upon a kill switch designed by iGEM USTC, NEU-China, and Unesp_Brazil, incorporating genetically engineered photoreceptors to induce cell death. The guide RNA targets essential genes in Acinetobacter baylyi's natural metabolism, ensuring specific cell death. The ACIAD1150 gene is identified as a crucial and relatively specific target.

To enhance the kill switch stability, the plasmid carrying it will be engineered to be antibiotic-independent. Extensive laboratory and environmental testing are planned to validate the kill switch's efficacy under blue light conditions, simulating real-world scenarios. As an alternative, if the light-induced switch proves ineffective, a chlorine-responsive CRISPR-based kill switch is proposed[21,22]. This two-input system aims to detect elevated chlorine levels and activate a containment mechanism for the genetically modified bacteria.

IX. HUMAN-CENTERED DESIGN

We aimed to keep the crux of our project as human-centered design. The “IMPACT” framework which stands for Ideation, Mobilization, Purpose, Adaptation, Collaboration and Transparency, has been followed to ensure that we keep in mind the requirements and needs of the people around us. We interacted with the local people to gauge an understanding of their opinions, aligning our ideas with community needs and taking inspiration from them.

The project's evolution relies significantly on the involvement of stakeholders. Our inclusive strategy pinpointed a diverse group, encompassing not only the ultimate end users—the public—but also engaging with legal professionals, policy makers, and environmental experts. This outreach aimed to gain comprehensive insights into the real-world rules and regulations influencing our project's implementation.

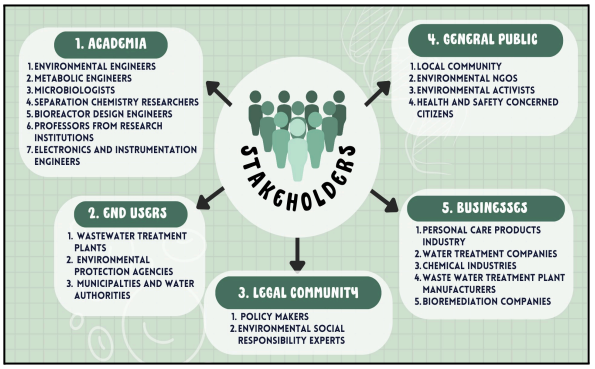

Fig 14. Stakeholder Map for Human Centered Design.

Consequently, our focus shifted to biocontainment after interactions with our stakeholders and we designed a separate bioreactor and a CRISPR-Cas9 kill switch activated by light. Visits to local wastewater treatment plants and industries enriched our understanding, inspiring new ideas and refining our approach as well.

One of the main objectives during the project was to spread an awareness and understanding of the persistent issue of chemicals in daily use products, along with basic concepts of synthetic biology and genetic engineering. We crafted educational content tailored for a broad audience, including specially-abled individuals. Additionally, we sought advice from various experts in the field, whose insights contributed significantly to the development of our project. Thus, this human centered approach proved to be extremely beneficial and important, without which our project would not have reached its current level of progress.

X. CONCLUSION

The integration of synthetic biology in the field of bioremediation has led to significant breakthroughs. Our project has also discerned a comprehensive solution to the pressing global issue of securing access to potable water. Through our research, we aim to contribute to environmental solutions, thereby substantiating the rationale behind incorporating bioreactors into the wastewater treatment plant. Beyond the scientific aspect, our project has expanded the outreach initiatives and educational elements, exhibiting a commitment to raising awareness about

bioremediation and water security. The long-term goal of our project is to inspire future work by employing synthetic biology for bioremediation.

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