A Novel Atrazine Degrading Bacterium - Proposal

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Abstract

Atrazine, despite being banned in the EU due to its toxicity and damage to the environment, is still used as a cheap and effective herbicide in many countries. Due to its widespread availability, it is unlikely that individual local legislatures will have any lasting effect on the atrazine build up in waters worldwide. Due to this, bioremediation through enzymatic biodegradation of the herbicide is a promising option. This paper proposes use of an optimized version of the pADP-1 plasmid found in *Pseudomonas sp*. This simplified plasmid containing the genes for the enzymatic breakdown of atrazine into a nitrogen source using enzymes atzA,B,C,D,E,G,F, can then be inserted into a fast replicating bacterium, the *E.coli.* Using this genetically modified organism, we plan to apply these locally to reduce atrazine concentrations to ensure a healthier surrounding population and environment, in an effective way requiring one time human intervention.

1. Introduction

1.1. Background

Atrazine is a toxic pesticide (Srijit et al., 2023) widely used in areas with less strict agricultural regulation such as China, where atrazine usage exceeds 10 '000 tons annually (Sun et al., 2019). Atrazine, once dissolved in water, enters plants through their roots and inhibits Photosystem-II, causing oxidative stress. (Zhu et al., 2009). Consequently, the concentration of atrazine in soil and its accumulation can lead to extensive toxicity in plants (Sánchez et al., 2017). Atrazine has been used worldwide for decades, and even in countries such as the United States, it is still the second most widely used pesticide, making it into the drinking and food supply of up to 40 million Americans.

As shown in a study by Hayes, atrazine is a disruptor of the endocrine system and affects development of gonads across several species of vertebrates (Hayes et al., 2011). A review paper published by NRDC (Wu, Mae, et al, 2010), states that the Midwestern United States is “pervasively contaminated by atrazine”, and drinking water is mostly contaminated.

Recently biodegradation of compounds like atrazine, which are toxic in the environment, has been more intensively studied. The bacteria - while facing many challenges - are important alternatives to conventional degradation. This is an enzymatic reaction which delivers the bacteria an intermediary which can be used as carbon, nitrogen, or phosphorus source. (Li et al., 2021)

One of the challenges facing current bioremediation attempts (the main bacterium used for these is *Pseudomonas sp.*) lies in the unstable gene expression of atzA, atzB and atzC, meaning that the first three enzymes in the metabolic pathway of atrazine breakdown are often not expressed (Thompson et al. 2010),(De Souza et al. 1998).

1.2 Current Degradation Methods

Currently, the atrazine breakdown process encompasses artificial chemical and physical methods, as well as natural microbial degradation. Species such as *Pseudomonas sp.* possess atrazine-degrading metabolic enzyme pathways

encoded by genes, atzA,B,C etc, that are located on the self-transmissable pADP-1 plasmid. The expression of the atz genes is regulated by atzR, a transcription factor that is triggered by environmental factors, which predominantly refers to the concentration of cyanuric acid, an intermediary product of atrazine breakdown. This breakdown pathway is crucial for mitigating atrazine action. However, the highly substrate-dependent expression and atrazine-degrading phenotype fluctuates due to large disrupted DNA sequence, which is caused by insertion of transposon(de Souza et.al).

1.3. Research Objectives

In this paper, we propose inserting custom designed plasmids containing enzymes from *Pseudomonas sp.*'s metabolic pathway of atrazine breakdown into *E.coli* BL21 DE3. Our research objective is to develop a stable and efficient host with a high rate of expression for atzA,B,C,D,E and F proteins for atrazine degradation.

1.4. Rationale for Choice of *Escherichia coli* BL21 (DE3)

Escherichia coli BL21 (DE3) is used as the host bacterium for the plasmid. It is engineered to express T7 RNA polymerase, which in turn allows for a stable expression of genes under the T7 promoters, leading to high yield of the recombinant protein products — the atrazine degrading enzymes. What is more, the B strain suggests that this *E. coli* lacks protease, which breaks down the expressed proteins, ensuring sufficient yield and quantities of the recombinant protein products. Currently, *E. coli* is used extensively in scientific research due to its relatively small and well-studied genome, the simplicity of which gives rise to the compatibility and predictability of this species, making it an ideal host of the recombinant plasmid. Additionally, the established gene editing protocol for *E. coli* warrants the developed equipment and techniques necessary to perform this experiment. Last but not least, *E. coli* readily adapts to a wide range of environmental conditions, where it undergoes rapid replication and protein production process to efficiently synthesize sufficient desired recombinant protein products, for degrading the atrazine present in the environment.

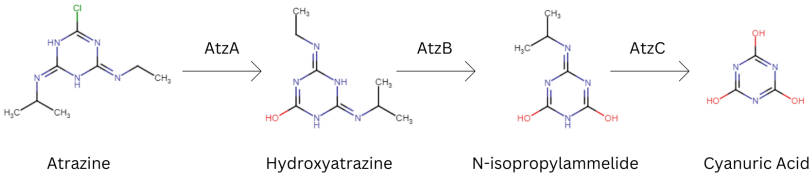
1.5. Significance

This paper suggests the creation of a novel bacterium which would overcome current challenges (mainly the unstable gene expression of most other bacteria, such as *Pseudomonas sp.*) of atrazine biodegradation.

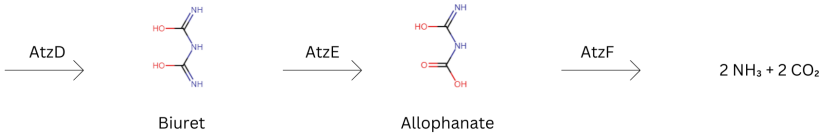
2. Mechanism of action

2.1. Atrazine Breakdown

Atrazine breakdown is used as a nitrogen source for *Pseudomonas sp*., using 6 different enzymes entitled atzA,B,C,D,E,F. These enzymes are encoded on the pADP-1 plasmid of *Pseudomonas sp*.



The first step of the biodegradation process, atrazine chlorohydrolase (atzA) catalyses the breakdown of atrazine to hydroxyatrazine by dechlorinating the substrate.



Hydroxyatrazine ethylaminohydrolase (atzB) acts on the carbon-nitrogen bonds in hydroxyatrazine in the presence of water, converting it to N-isopropylamide.
N-isopropylammelide isopropylaminohydrolase (atzC) catalyzes the final step of atrazine breakdown into its main intermediary, cyanuric acid.
Cyanuric acid hydrolase (atzD), an amidase which opens the ring of cyanuric acid, results in formation of biuret.
Archetype of a recently discovered fold entitled the "Toblerone" fold (Esquirol et al., 2018).
atzG, a subunit of the atzE protein, is recently discovered and required for soluble and active atzE.
AtzE is an amidohydrolase which catalyzes the reaction of biuret into 1,3-dicarboxyurea, which results in allophanate. Recently discovered to contain another small protein by the name of atzG, which is necessary for solubility and function of atzE.
The final step of cyanuric acid degradation, atzF catalyzes the reaction of allophanate to carbon dioxide and ammonia.

2.2. Mechanism of Plasmid Function

The custom plasmid has been digitally designed using the atrazine degradation segments from pADP-1 plasmid from bacterium *Pseudomonas sp*, constructed *in vitro*, and inserted into the host *E. coli* BL21 (DE3)

T7 promoter:It is well-known for efficient transcription, because the T7 RNA polymerase specifically binds to the promoter, allowing for high levels of transcription of the downstream genes.

rpoN-1: A sigma factor that recognises and binds to specific promoters (T7) to initiate transcription. It is involved in regulating metabolic pathways related to nitrogen fixation of the utility of alternative nitrogen sources.

NtrC: transcriptional regulator that functions in response to nitrogen availability. It regulates a variety of genes, including those responsible for nitrogen compound uptakes and utilisation.

GlnK1: Signal transduction protein that interacts with nitrogen regulatory proteins such as NtrC to affect their activity. It acts as a sensor for the availability of nitrogen. Under conditions when nitrogen availability is limited, GlnK1 influences the activity of proteins involved in nitrogen assimilation, helping the plant to adapt to nitrogen availability.
atzA,B,C: Located on the self-transmissible pADP-1 plasmid in *Pseudomonas sp*. These three genes code for the function of atrazine catabolism to cyanuric acid.

atzR: A regulator responsible for activation of atzDEF genes, which carry out the remaining steps of the atrazine breakdown pathway from cyanuric acid to the end, where ammonia and carbon dioxide are released. atzR is activated in response to limited nitrogen or the presence of cyanuric acid.

atzD,E,F: Functions to catabolise cyanuric acid to ammonia and carbon dioxide.

3. Materials and methods

3.1. Materials

Item Type	Item	Quanitity
Organism	E. coli BL21	
Plasmids	pET 28 Plasmid	x1
Restriction enzymes	NEB NcoI (100 µg/ml)	x1
	NEB MreI (100 µg/ml)	x1
	NEB HindIII (100 µg/ml)	x1
	NEB XhoI (100 µg/ml)	x1
	NEB NgoMIV (100 µg/ml)	x1
	NEB AflIII (100 µg/ml)	x1
	NEB AvrII (100 µg/ml)	x1
	NEB BmtI (100 µg/ml)	x1
Reagants/consumables	LB Solution	
	Kanamycin solution (30 µg/mL)	

3.2. Method

3.2.1. Insertion and immediate confirmation

3.2.1.1. Choice of Plasmid and Organism of Expression

E. coli BL21 (DE3) was selected as the host for atrazine degradation pathway expression due to its facultative anaerobic metabolism, broad temperature tolerance (7.5–49°C), and T7 expression system, allowing controlled high-level protein production. Its deficiency in Lon and OmpT proteases enhances protein stability, while its genetic tractability ensures efficient modifications.

The pET28GST-LIC (Plasmid #26101, ADDGENE) vector was chosen for its low-copy number, minimizing replication-transcription conflicts and promoting proper protein folding. Its T7 promoter system, in conjunction with BL21 (DE3), enables high-yield, tightly regulated expression of atrazine degradation enzymes. The inclusion of an N-terminal GST-tag followed by a 6X His-tag facilitates protein solubility and purification. The kanamycin resistance marker ensures robust selection with minimal metabolic stress. Its design supports large operons, accommodating multiple genes essential for atrazine degradation and nitrogen sensing.

Together, *E. coli* BL21 (DE3) and pET28GST-LIC form a highly efficient, stable, and adaptable expression platform suitable for controlled laboratory applications and field-based bioremediation efforts, addressing key challenges in atrazine degradation with optimized enzyme production and regulatory control.

The atzA, atzB, and atzC genes were inserted into the plasmid for constitutive expression, ensuring continuous atrazine conversion to cyanuric acid. In contrast, atzD, atzG, atzE, and atzF were placed under an operon-regulated array to optimize resource allocation, activating only when needed. Regulation involves constitutive expression of ntrC, rpoN, and glnK-1 upstream of atzABC, and activation/autorepression at the inserted PatzR-PatzDGEF promoter region.

AtzDGEF expression is governed by AtzR, a LysR-type transcriptional regulator (LTTR) activated by cyanuric acid and nitrogen limitation. NtrC enhances atzR transcription through a σ54 (rpoN product) -dependent promoter, while GlnK modulates NtrC activity based on nitrogen levels. AtzR further activates atzDGEF by binding to its promoter while repressing its own expression, ensuring a balanced regulatory response. This integration of nitrogen sensing and atrazine degradation enables efficient cell resource utilization, activating atzDGEF only when both signals are present.

3.2.1.2. Primer Design

As the coding sequences are from a plasmid of different host origin (Pseudomonas sp.), they are prepared for insertion in a plasmid suitable for use within the E.Coli BL21. Prior to the creation of the primers, the coding sequences were codon optimised to allow for optimal expression of the genes in the operon. Primers were created for the coding sequences of Atz A, B, C, D, E, F, and G, and the glnK-1, ntrC and rpoN, individually, in which separate fragments were made for the glnK, rpoN, ntrC, AtzA, AtzB, and AtzC coding sequences, with the cut sites:

- NcoI
- MreI
- HindIII
- XhoI
- NgoMIV
- AflIII
- AvrII
- BmtI

in order, proposed for use in the ligation of the fragments. The cut site at the forward primer of rpoN (NcoI) being used to ligate the fragment to the backbone. An additional longer primer sequence is made for AtzDEFG, ligated to the first fragment (reverse primer of AtzC) at the cut site AvrII, and the backbone through BmtI

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3.2.1.3. Molecular Cloning:

Biobrick assembly is proposed as the means of ligating the fragments to the plasmid, with Gibson assembly also standing as a potential means of assembly as well from the creation of complementary overhangs.

Cognizant of the limitations of the Biobrick assembly method, such as the potential misalignment at multiple cut sites, the sections of the plasmid will be fragmented, amplified via PCR, and subjected to Sanger sequencing. Moreover, it shall be subjected to a run-through of the BLAST algorithm to ensure that there are no significant mutations to which the sequences may have experienced.

3.2.1.3. Plasmid Transformation

The recombinant plasmid shall be inserted into the E.coli BL21 through heat shock transformation. Following the heat shock transformation at 43 °C, the transformed cells are incubated in 1.5 mL LB solution at 37 °C overnight without kanamycin present within the solution to allow for recovery, in which expression of the Kanamycin resistance gene begins. Following this the BL21 cells are swabbed onto plates with 30 µg/mL kanamycin and left to culture overnight at 37 °C as a means of selective isolation of the bacteria which were successfully transformed. Successful cultures are then cultured further in LB solution containing 30 µg/mL Kanamycin at 37 °C, shaken at 250 rpm for 6 hours, allowing for its further growth of the bacteria and the amplification of the inserted plasmids.

3.2.2. Long term viability testing

3.2.2.1. Influence of Time

The long term functionality and effectiveness of the operon shall be tested through controlled time intervals in vitro to determine the long term reliability of the system, with the aim of having the system run at the scale of weeks, if not months.

3.2.2.2. Specificity determination

To determine the specificity of the operon to the degradation of atrazine, the bacterium will be exposed to other common contaminants that may be present within the environment, such as other pesticides, or other natural nitrogenous compounds to ensure that activity is not influenced by untargeted substrates.

3.2.2.3. Concentration tests

To determine the effectiveness of the operon based on the concentration of the contaminant, the bacterium will be subjected to different concentrations of atrazine solution in an array based on existing environmental conditions, from the detection limit at ~0.03 ng/m3 to average concentrations post application of the herbicide, at 0.20–0.32 µg/m3.

3.2.2.4. Influence of pH and Temperature

To determine the effects of the environmental conditions of pH and temperature of the environment of the stability of the enzymes. The aim of this experiment is to determine the sensitivity of the proteins in the operon to changes away from optimum conditions within its environment for breakdown of the atrazine to ammonia and carbon dioxide, ensuring that there is long term reliability of the system against variable conditions.

4. Implementation and applications

To overcome the issue of atrazine contamination in the environment, the present study is focused on the design, modification, and application of genetically modified *Escherichia coli* BL21 (DE3) as a bioremediation tool. The implementation steps are explained below in detail:

4.1. Construction of the tailor-made plasmid

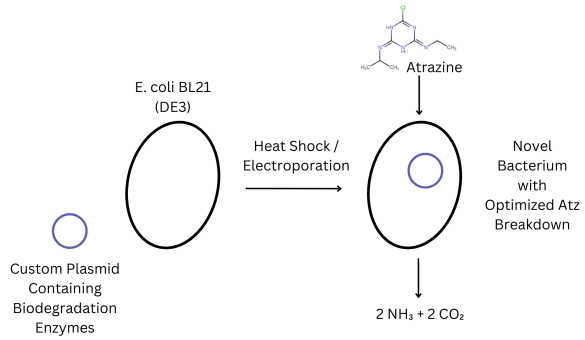
A tailor-made plasmid was in silicon designed using atrazine degradation pieces from the pADP-1 plasmid of *Pseudomonas sp*. The plasmid contains genes atzA, atzB, atzC, atzD, atzE, atzF, atzG along with regulatory elements for the maximal expression of genes.

- **T7 Promoter:** The promoter ensures an extremely efficient transcription of genes due to its precise interaction with T7 RNA polymerase present in E. coli BL21 (DE3) (Studier and Moffatt, 1986).
- **rpoN-1:** Enables transcription associated with nitrogen metabolism (Reitzer and Schneider, 2013).
- **NtrC:** Controls transcription based on the availability of nitrogen (Amon et al. 2010).
- **GlnK1:** Controls the nitrogen assimilation pathways for full activity during nitrogen limitation (Leigh and Dodsworth, 2007).
- **atzR:** Act as a regulatory protein to increase the transcriptional response to atrazine in the environment based on its concentration (de Souza, et al.1998).

4.2. Transformation into *E. coli* BL21(DE3)

The recombinant plasmid was transformed into *E. coli* BL21(DE3) using heat-shock or electroporation transformation protocols. The rationale behind the use of this strain includes:

- *E. coli* BL21(DE3) T7 RNA polymerase system supports stable and high-level expression of atz genes (Studier and Moffatt, 1986).
- The high rate of replication of the bacterium ensures rapid accumulation of the transformed populations (Neidhardt, et al.1994).
- The stability and simplicity of the *E. coli* BL21(DE3) genome facilitate the accurate manipulation of genes with minimal likelihood of unintended effects (Blattner et al. 2007).



4.3. Optimization of Recombinant Expression

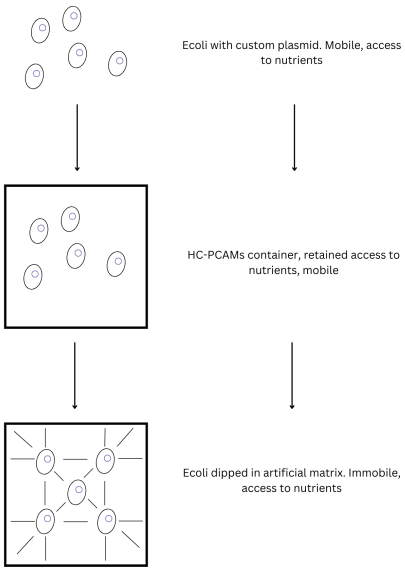
Upon transformation, the recombinant *E. coli* BL21(DE3) was cultured under optimized conditions for maximum yield of protein. IPTG (isopropyl β-D-1-thiogalactopyranoside) was used in inducing the activity of T7 RNA polymerase for high-level expression of atz enzymes, The growth conditions were optimized for maximum bacterial replications, plasmid stability, and enzyme production.

4.4. Killswitch Integration

For biosafety purposes and to prevent unforeseen ecological effects, an additional plasmid with a killswitch function can be incorporated. The function, triggered by the detection of specific environmental signals such as the lack of atrazine or detection of specific temperatures/pH ranges, ensures the final death of the recombinant bacterium once atrazine levels have been reduced to tolerable levels. (Wright, et al. 2013).

4.5 Biocontainment:

With the E.coli containing Atz enzyme metabolic system, there is the need for implementation of the E.coli in the environment. Atrazine is widely contaminating water bodies as well as agriculture-used soil. The E.coli package can be buried in soil and can also be put in certain checkpoints along the river. The purpose is to let the metabolic system perform its job, and this needs the normal function of *E .coli*. To make sure *E. coli* cells survive and function sustainably, there needs a bio-container to conserve the *E. coli*.



4.5.1 Container material

E.coli will be conserved in hydrogel shells when it is being put out in the natural habitat. Artificial polymer hydrogel shell was tested for its efficiency of holding cell activity. HC-PCAMs, a kind of reaction holder that is permeable, allows E coli to have access to water, oxygen, and nutrients. (Girolamo, Salvatore Di, et al.)

4.5.2 Immobilization of *E. coli*.

Movement of E.coli will affect its remediation efficiency. Edited *E. coli* will be dipped into artificial matrix to be put in the container. Possible matrices can be synthetic polymers. Polyvinyl alcohol has been considered as an efficient immobilizer for biodegradation (Zhong, Zhi-Hao, and Yu-Qing Zhang).

4.5.3 Selection for *E. coli*

If the biopackage is used for atrazine degradation in soil, there are other species of bacteria that may be attached to the matrix. The artificially edited E.coli with Atz enzyme should be immune to Kanamycin due to Khan-resistence DNA segment on the plasmid. Kanamycin should be added to the matrix so that other bacteria won't be clutter on the matrix to impede *E. coli*'s degrading efficiency.

4.5.4 Waste Absorption and Management

The metabolic byproduct at the end of the metabolic pathway is ammonia and CO2. Moreover, the package will also be influenced by the metabolic activity of other bacteria and cells when it is applied to natural environments. Zeolite will be added to the hydroshell for absorbance of excess ammonia. Zeolite can also be reused over time. ("Removing Ammonia and Ammonium with Natural Zeolite.") Also, zeolite is not soluble in water, so it can function normally even if the package is applied in water bodies.

4.6 Uncontrollable variables and monitor system.

E.coli's optimum growth temperature is 37 ℃ and can also survive under 4℃. (Doyle, M. P., and J. L. Schoeni.) pH of E.coli survival ranges from 4.4-9 (Suehr, Quincy J., et al.)However, these variables cannot be controlled in the natural environment. Experiments would need to be conducted to investigate how temperature and pH will impact E.coli's survival and function in the biopackage. However, even with every possible preparation, the fluctuation of the variables will influence the progress. Therefore, there needs to be a sensory system to monitor the performance of E.coli. During the primary testing stage of E.coli, when they will be tested in a mini-ecosystem. Monitors for ammonia like Ammonia Ion-Selective Electrodes can be used to test the product after a certain amount of reaction time. More investigation on practical carry out needs to be investigated when experiment moves on to more realistic natural environments.

5. Biosafety

The system we're applying possesses little biosafety risk, based on the following adoptions.

5.1. Microcapsules

Although the recombinant *E. coli* BL21(DE3) populations are contained in the packages, HC-PCAM, the microcapsules that were used to encapsulate recombinant *E. coli* BL21(DE3), is an artificial permeable

microcapsules which can make the modified organisms stay within the compartment while the synthesized enzymes and their required elements can pass through. (Di Girolamo, S., et al. 2020) This material nature significantly lowers the risks of modified organisms being released into the environment, however, there are still risks of release when the microcapsules are broken. Multiple factors that could lead to the breakage of microcapsules are listed:

- **Mechanical Stress:** Any physical forces that are over tolerance might deform or break the HC-PCAM, for instance, agitation, shear stress, and compression. (Di Girolamo, S., et al. 2020)
- **Environment pH:** HC-PCAM would be weakened or broken as the pH level in the water environment would affect the charge interaction inter-layers. (Bukreeva, Tatiana V., et al.2022)
- **Osmotic Pressure:** The tolerance to osmotic pressure of HC-PCAM membrane depends on the elasticity of the multilayer polyelectrolyte we made. If its elasticity is not flexible enough, the structure of HC-PCAM might be caved or broken. (Gao, C. Y., et al. 2001)
- **Enzymatic Degradation:** Alginate lyase, a kind of enzyme naturally produced by many soil bacteria to degrade organic matters as food (Sangi, Sibghatullah, et al.). This kind of enzyme could break the alginate of HC-PCAM down into oligosaccharides, which would destroy HC-PCAM and the formed compartments. (Li, Jinmeng, et al. 2023)

5.2. Kill switch

Given the potential risks of microcapsules' breakage, kill switch related genes are adopted in the plasmid. Kill switch is a conditional suicide system, the condition could be set during processing. This switch makes the organisms destroy their own genetic materials when a particular situation happens. (Balan, Andrea & Ana Clara G Schenberg. 2005) The recombinant *E. coli* BL21(DE3) would be ordered by the set trigger situations to conduct suicide. Hence, the system can lower the further risks about affecting the original environment by exposed recombinant *E. coli* BL21(DE3) population when microcapsules are broken or after the atrazine degrading work is done. Specifically, to prevent release, the first trigger situation should contain all of the factors that can lead to microcapsules' breakage, making the breakage considered a readable order for recombinant *E. coli* BL21(DE3) to conduct suicide. The second trigger situation should be under a particular atrazine concentration as an ending up to stop the useless work afterward. More importantly, it can lower the chance of recombinant E. coli BL21(DE3) being released after the work is done, by the absence of it.

5.3. Chosen organisms

None of the species or strains we use are pathogenic to human, common crops and livestock under regular conditions according to "CDC", "Gogec Biosafety and Biosecurity Blacklist: https://www.gogecconference.org/biosecurity-whitelist", and "Biosecurity Law of the People Republic of China". These organisms possess little chance to harm human's livelihood, however, more research on potential pathogenicity to other species that are also vital to the ecosystem should be conducted before the system is practiced.

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