Isoeugenol as an alternative selection marker for antibiotic substitution

García Alonso Claudia, García Kadala Sergio, Centeno Noriega Ana Sofía, Palacios Rivera Jennifer, Spínola López Amanda, Rivas Gutiérrez Regina, Jiménez López Brenda, Marín Jerónimo Karen, Soto Herrera Melchor, Andreivich Yaroslav, Muñoz Arteaga José Antonio, Arriola Gamboa Andrea, Huerta Torres Ana Laura, Antonio Pérez Aurora.

Introduction

Over time, humanity has widely used antibiotics principally for their high effectiveness and low cost. Beyond the treatment of infections, antibiotics are great selectable markers for molecular biology procedures. They can help identify cells that have been efficiently transformed [1]. Genetic transformation consists of the insertion of foreign DNA into a host cell, usually in an expression cassette. DNA delivery is artificially carried out in 2 ways: by direct DNA transfer either utilizing viral properties or microorganisms such as *Agrobacterium tumefaciens*. Regardless of the method used for the genetic transformation, a selectable marker must be used to verify the correct insertion of DNA [1].

There are different types of selectable markers and some are based on conferring microorganisms the ability to resist the presence of antibiotics [2]. With this mechanism, it is assured to only obtain growth of the microorganism conferred resistance to since all others will die; however, this has raised multiple concerns due to the risk it represents towards the environment and human health. Throughout the years, the number of microorganisms that have become resistant to commonly used antibiotics has increased, growing concern around the world because of the morbidity and mortality rates it is associated with [3]. In fact, a review commissioned by the United Kingdom government argued that by 2050, 10 million people per year will die due to antimicrobial resistance [4].

In 2016 Jasovský, Littmann, Zorzet & Cars determined that 214,000 neonatal deaths from sepsis per year were attributed to “drug-resistant” pathogens [5]. They also mentioned that “Drug-resistant pathogens could reverse the recent positive trend of falling global mortality rates from infectious diseases, which have decreased from 23% to 17% of total deaths over the last 15 years” being this alarming but also realistic conclusion [5].

In spite of this, antibiotics are necessary for synthetic biology experimental procedures. Therefore, the objective of this project was to create an alternative to antibiotic-based selection markers, the main focus being its functionality with the most commonly used microorganism *Escherichia coli*, and contribute to the reduction of antibiotic use in laboratories.

As replacement for antibiotics as an inhibitory compound, compounds in natural substances that were known for having antimicrobial activity were considered, such as honey, cinnamon, garlic, and others. It is crucial to find reported genes that could help the microorganism degrade the inhibitory compound. It is important to consider that the final product of the conversion of the chosen compound was not capable of causing cell death.

Cinnamon and clover essential oils were outstanding for their inhibitory action, related to the presence of the aromatic compound: eugenol, being present in over 70% in cinnamon essential oil [6]. Over all other inhibitory compounds, it is commercialized as a pure component, thus no extraction from cinnamon oil would be necessary, representing an advantage. However, the degradation pathway of this compound, many steps were involved in the transformation of eugenol, meaning that the bacteria express them all and code for those proteins in order to degrade it; and usually, the expression of too many genes is complicated [7]. With this in mind, isoeugenol, the isomer of eugenol was considered.

Isoeugenol belongs to the group of phenylpropenes and has shown comparable and even superior antimicrobial activity to its highly antimicrobial original form eugenol [8], probably owed to its free hydroxyl group and its position of double bonds in the α, and β positions of the side chain, and a methyl group in the γ position (**Figure 1**).

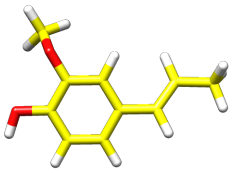

Figure 1. Isoeugenol structure

Isoeugenol's antibacterial activity covers a wide range of gram-positive and gram-negative bacteria, including

Escherichia coli, *Bacillus licheniformis*, *Micrococcus luteus*, *Pseudomonas aeruginosa*, *Salmonella* type B, and *Staphylococcus aureus*. [8]

As can be seen, Isoeugenol has a considerable antimicrobial effect, exhibiting the most pronounced inhibitory effect on the growth of different types of bacteria, in the same way various inhibitory effects have been carried out on fungi and yeasts, being *Saccharomyces cerevisiae*, *Pichia pastoris* and *Aspergillus niger*, inhibited by isoeugenol; this may mean that in the future it could function as a selection marker in other microorganisms. [8]

Nevertheless, despite the inhibitory effects that have been proved of isoeugenol, there are reports of other microorganisms that are able to metabolize it, such as *Pseudomonas bacteria* (known for being able to grow under harsh conditions) by the action of the enzyme Isoeugenol Monooxygenase, which allows a one-step conversion of it into one compound, vanillin. Isoeugenol monooxygenase (*IsoMo*), reported by Yamada, Okada, Yoshida & Nagasawa in 2008 besides from this, does not lead to the formation of undesired co-products that could have an inhibitory effect on *E. coli*, such as vanillic acid or acetaldehyde [9]. Vanillin, on its own, causes an inhibition of *E. coli* but at much higher concentrations than isoeugenol [10]. The reaction performed by the enzyme is represented in **figure 2**.

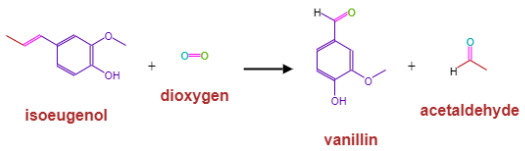

Figure 2. *IsoMo* reaction for the degradation of isoeugenol

Additionally, the methodology of the proposal requires an adequate incorporation of isoeugenol, an oleaginous compound into cell culture media in order for the compound to be available for the transformed cells to express the desired genetic cassette and render proof of the correct gene insertion. On the other hand, it is important to mention Isoeugenol's property of non-thermolability which facilitates some aspects of the sterilization process required for cell culture media. The proposal is valuable due to all the improvements and potential application both in molecular biology and media cell culture with oleaginous compounds.

Materials and Methods

1) *Accomplishment of a homogeneous distribution of isoeugenol culture mediums*

As a first stage of the project, experimental procedures were designed focused on the development of culture mediums with isoeugenol, in regard to the Minimum Inhibitory Concentration (MIC), to be able to move on to the next phase. For this purpose, different procedures were tested for both liquid and solid Luria Bertani (LB) culture mediums and different concentrations of isoeugenol.

1.1. Ordinary procedure

Figure 3. Methodology diagram for the ordinary preparation of culture mediums. 1) Treatment "A": Addition of isoeugenol before sterilization. 2) Treatment "B": Addition of isoeugenol after sterilization.

The methodology shown in Figure 3 was firstly tested with serial dilutions $x10^{-4}$ and $x10^{-5}$ of *E. coli* DH5α, to analyze the inhibitory effect of isoeugenol on the growth of *E. coli* using the concentrations reported in Table 1.

Table 1. Isoeugenol concentrations used for treatments A and B.

1	0 mM (Negative control)
2	1.8 mM
3	3.65 mM (highest MIC reported, [7])
4	4.5 mM

1.2. Mechanic emulsification with the Polytron.

Since it was noticed that isoeugenol was not dissolving in the mediums despite the small amounts that were being used, we decided to try to emulsify it with the Polytron homogenizer. For this experiment, the Polytron was cleaned with a 70% (v/v) solution of ethanol, irradiated with UV light in the laminar flow hood, and rotated after every 15 minutes to make sure all sides of the equipment were sterilized. After this, falcon tubes with LB mediums with isoeugenol, prepared according to treatment A, were emulsified for 10 minutes, cleaning the tip of the Polytron with distilled water in between each tube.

1.3. Chemical emulsification with Tween 80.

Another alternative to emulsify the Isoeugenol into the medium was adding Tween 80 emulsifier to liquid culture mediums.Tween 80 was tested at 4%, 6%, and 10% of the total volume of isoeugenol; it was added at the same time as isoeugenol.

1.4. Mechanical emulsification with Vortex and Sonicator.

In order to avoid using other compounds that could have an inhibitory effect on bacterial growth, it was decided to go back to mechanical methods. Therefore, emulsification was carried out, first by vortexing and second, by sonication of the mediums. For this case, the steps followed are presented in Figure 4.

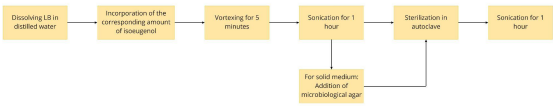

Figure 4. Methodology diagram for medium preparation, with vortexing and sonication.

1.5. Homogeneity analysis in ZetaSizer.

More tests were run and evaluated in order to check out the treatment that could provide a better homogeneity of the mediums at a concentration of 4.5 mM of isoeugenol. The use of Tween 80, the use of the vortex, the resting time of mediums and the effect of sonication time prior and after autoclaving were evaluated through the Zetasizer instrument as shown on Table 2 . Also, based on the results obtained, graphics were performed with ZetaSizer for the evaluation of the peaks that showed greater homogenization

Table 2. Different treatments for 4.5 mM of isoeugenol for its analysis in ZetaSizer.

Sample	Medium		Vortex (5 minutes)	Sonicating		Tween 80 10%
	LB	Water		Prior autoclaving	After autoclaving	
1		✔	✔	60 min	120 min	
2		✔	✔	60 min	120 min	✔
3	✔			60 min	120 min	
4	✔		✔	50 min	120 min	✔
5	✔		✔	50 min	180 min	
6	✔		✔	50 min	180 min	✔
7	✔		✔	50 min	60 min	
8	✔		✔	50 min	60 min	✔
9	✔		✔	50 min	60 min	
10	✔		✔	50 min	60 min	✔
11	✔		✔	50 min	120 min	
12	✔		✔	50 min	120 min	✔

2) Evaluation of the inhibitory effect of isoeugenol on the two most used Escherichia coli strains: DH5α and BL21.

Table 3. Isoeugenol concentrations used for evaluation inhibitory effect

1	0 mM (Negative control)
2	1.8 mM
3	2.5 mM
4	3.65 mM (highest MIC reported, [7])
5	4.5 mM
6	5.5 mM

3) Design of genetic constructs for Isoeugenol Monooxygenease.

As it was said before, isoeugenol molecule can be degraded by the Isoeugenol Monooxygenase and transformed into vainillin.

In order to evaluate the inhibitory antibiotical resistance provided by the Isoeugenol Monooxygenase it was necessary to build two genetic constructs starting from the sequences obtained from this enzyme: one construct directly inducible by isoeugenol (Pp_22: BBa_K4260006) and other regulated by a constitutive promoter (Nc_22: BBa_K4260007); this, in order to test the efficiency of the two systems. In addition, 28 bp were added upstream from the prefix, and 19 bp downstream from the suffix, aiming to allow hybridization of the primers HF_22 and HR_22 designed by TecCEM's 2021 team, for the two sequences.

Pp_22, **(Figure 6)** is a 2807 bp long Biobrick, that includes a bidirectional promoter that regulates the

transcription of Iso (coding gene of *IsoMo*) and a transcriptional activator [17], in the 5' to 3' and 3' to 5' direction, respectively.

Figure 6. Components of Pp_22 (BBa_K4260006). The components of the BioBrick are respectively represented in the scheme.

Nc_22 (**Figure 7**) on its own is 1712 bp long. It is a shorter sequence that shares the Iso gene and rrnB T1 terminator in common with Pp_22. However, this construct includes the strongest constitutive promoter reported:BBa_J23100, as well as a ribosome binding site (RBS: BBa_B0030). For this case, the single appearance of the 4 restriction sites was also ensured, to avoid undesired cuts within the sequence.

Figure 7. Components of Nc_22 (BBa_K4260007). The components of the BioBrick are respectively represented in the scheme. represented in the scheme.

4) Experimental procedures of the constructs performed.

4.1 Ligation

Each tube sequence (IsoMo_Pp22 and IsoMo_Nc22) were resuspended with 40 µL of distilled water sterile, and placed immediately on ice.

For the ligation of the plasmid pJET1.2 + IsoMo_Pp22 were added inside the laminar flow hood the quantity of reagents shown in **Table 4:**

Table 4. Ligation reaction of pJET1.2 + IsoMo_Pp22

Reagents	Quantity
a.Reaction buffer 2x b. H_2O ºBM c. gblock d. Plasmid pJET1.2 e. T4 DNA ligase	10 µL 1 µL 6 µL 2 µL 1 µL
Total of reaction	20 µL

After the addition of reagents the laminar flow hood were incubated for 30 minutes at 22ºC.

For the pJET1.2 + IsoMo_Nc22 ligation it required the quantity of reagents shown in **Table 5.**

Table 5. Ligation reaction of pJET1.2 + IsoMo_Nc22

Reagents	Quantity
a.Reaction buffer 2x b. H_2O ºBM c. gblock d. Plasmid pJET1.2 e. T4 DNA ligase	10 µL 4 µL 4 µL 1 µL 1 µL
Total of reaction	20 µL

Finally, this sample was incubated at 22 ºC for 30 minutes.

4.2 Transformation of chemically competent cells BL21 and DH5∝

The protocol of transformation for chemical competent cell is the following:

First, it is required to streak a strain of the cell in a Petri dish (LB without antibiotic), and let it grow overnight. The next day, inoculate a flask with 300 mL of LB liquid medium and let it grow overnight. This is for each cell of BL21 and DH5∝. The next day the competent cells are prepared following the next steps:

The flask is placed on ice for 10 minutes, then the content is divided into conic tubes of 50 mL each one, and centrifuge these at a rate

of 5000 rpm for 10 minutes. The supernatant was withdrawn and resuspended the pellet with 20 mL of CaCl2 0.1 M (steril).

Afterwards, the tubes were incubated at 4 ºC for 10 minutes, then centrifuged for 10 minutes at a rate of 5000 rpm.

The supernatant was withdrawn and the pellet was resuspended with 5 mL of $CaCl_2$ 0.1 M-glicerol 14%. Finally, aliquots of 200 µL were made and stored at -80 ºC, well labeled.

4.3 Digestions

To carry out the ligation it is necessary to have the cut sequences with the enzymes XbaI and SpeI. These were obtained with the quantity of reagents shown in **Table 6.**

Table 6. Digestions with XbaI and SpeI of ligation previously performed.

Reagents	Quantity
a. H_2O ºBM b. Plasmid c. Buffer 2.1 d. Enzyme XbaI (20,000 U) e. Enzyme SpeI (10,000 U)	10 µL 5 µL 2µL 1 µL 2 µL

Total of reaction	20 µL

4.4 Plasmid extraction

Plasmid extraction was performed through centrifuge purification techniques. The Purification Kit Quick Plasmid Miniprep Kit ® from Invitrogen was used.

4.5 SDS-PAGE

Firstly, the samples for the SDS-PAGE were made. 1 mL was taken from each tube with culture and was poured into microtubes. The tubes were centrifuged for 10 min at 13.4 rpm and the pellet was resuspended in 100 µL with water ºMB. Then 40 µL of laemmli buffer were added to the sample. Samples were heated in the water bath at 90 ºC for 5 minutes. After the samples were incubated on ice for 10 minutes and finally 10 µL of each sample were charged to the SDS-PAGE.

For the SDS-PAGE reagents were required: Acrylamide – Diacylamine 30%, Tris HCl 1.5 M pH 8.8, SDS 10% and PSA 10%.

5. Biosafety

As isoeugenol´s use is new to the laboratory, every residue of it, such as micropipete tips, liquid and solid mediums, were disposed in an special container only for it.

Results

1) *Accomplishment of a homogeneous distribution of isoeugenol culture mediums*

1.1. Ordinary procedure

Given the procedure carried out regarding culture preparations with the isoeugenol integrated (figure 3), after removing the media from the autoclave, droplets of isoeugenol could be seen settling at the bottom of the flasks or falcon tubes (Figure 8). Thus, it was determined that the sterilization process conditions were insufficient to ensure that the isoeugenol was adequately homogenized in the media.

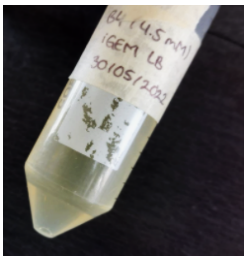

Figure 8. Treatment B, concentration 4, liquid medium. Isoeugenol can be observed at the bottom as a small drop.

1.2. Mechanic emulsification with the Polytron

Regarding the followed procedure to emulsify the isoeugenol with the Polytron, unfortunately, was not effective. Moments after the emulsification, isoeugenol started sedimenting again at the bottom of all falcon tubes. Besides, it was discovered that the sterilization protocol used for the Polytron had not been sufficient for its actual sterilization, since all mediums got contaminated. Therefore, other options needed to be explored.

1.3. Chemical emulsification with Tween 80

What happened with the Tween 80 was not only that no improvement was observed in the homogenization or emulsification of the samples, but also that dirt was present in all the mediums. For this reason, and considering its toxicity for *E. coli*, it was decided that it was better to not use this compound in the medium formulation.

1.4. Mechanical emulsification with Vortex and Sonicator

After testing the new protocol for the preparation of culture mediums by emulsifying the isoeugenol with vortex followed by sonication (mechanical methods), based on the results obtained from the integration of the isoeugenol to the medium, it was concluded that this method was the best implemented so far. Thus, this series of steps were the ones used for the rest of the experiments involving isoeugenol.

1.5. Homogeneity analysis in ZetaSizer.

Despite the good results obtained with the previous protocol, given the run tests to check out the treatment that could provide a better homogeneity of the mediums at a concentration of 4.5 mM of isoeugenol, were perform graphics for the evaluation of the peaks that showed greater homogenization, and the results are shown in **figure 9**, where part A shows a lower polydiversity against part B which has higher polydispersity. Nevertheless, it is recommended to have an absorbance lower than 100 nm and with few peaks as part A.

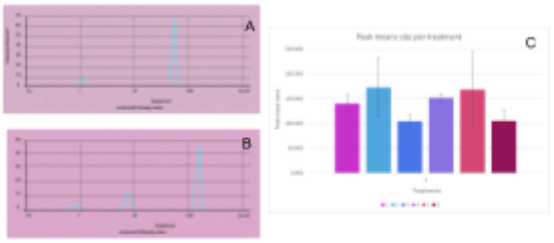

Figure 9. Particle size variation analysis of Isoeugenol in LB medium with zetasizer.a)Lower polydispersity b) Higher

polydispersity c)Particle sizes and polydispersity in different treatments.

1.6. Addition of isoeugenol

Although good results were obtained concerning the homogeneity of the culture mediums, one question remained: At which point of the procedure was it better to add isoeugenol into the mediums? Hence, the efficiency of three different treatments was tested:

A: Addition of non-sterile isoeugenol before sterilization

B: Addition of sterile isoeugenol after sterilization, before plating, similarly to antibiotics

C: Addition of non-sterile isoeugenol after sterilization, before plating, similarly to antibiotics

Serial dilutions $x10^{-4}$ and $x10^{-5}$ of *E. coli* DH5α, were cultured by triplicate at concentrations shown in Table 3.

After analyzing the growth behavior of serial dilutions in each treatment, we determined that results from treatments A and B were very similar, and due to inconsistencies treatment C was discarded. Despite the similarity between treatments A and B it was decided to keep up with treatment A because of the advantage of not having to manipulate the medium anymore after removing it from the sonicator. Thus, the procedure selected for the preparation of mediums for further steps is the one presented in Figure 9.

2) *To evaluate the inhibitory effect of isoeugenol on the two most used Escherichia coli strains: DH5α and BL21.*

2.1 Minimum Inhibitory Concentration in Solid Medium

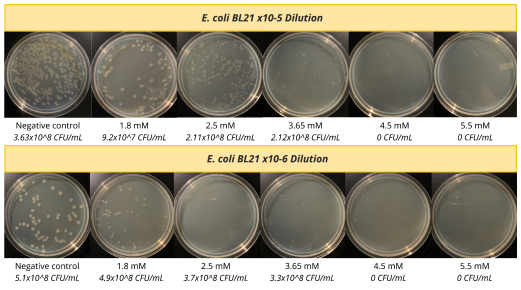

Figure 9. Serial dilutions in BL21 *E.coli* strain

The figure 9 shows the results obtained from *E. coli* BL21 (with no genetic modification) growing in different isoeugenol concentrations (each corresponding concentration is shown in the white section under each image). For this experiment, serial dilutions of *E. coli* BL21 (0.6 of OD 600nm) were carried out until reaching cell concentrations of $x10^{-5}$ and $x10^{-6}$.

As it's shown, both negative controls had grown bacteria in big and thick colonies, as each colony showed a more intense white color, and major thickness, whilst the other plates presented an increase in the number of colonies but with a smaller size and opacity reduction as the isoeugenol concentration increased. These features were quite notable, considering that the size of the colonies was reduced until there was no more growing on the plates with the highest concentration of this antibiotic.

A more concise observation is that in both *E. coli* dilutions ($x10^{-5}$and $x10^{-6}$) spread in the petri dishes with the highest isoeugenol concentrations (4.5 mM and 5.5 mM), no growth was observed, since these increased isoeugenol concentrations avoided any development of the microorganisms, considering that the strain used does not count with a selection marker for this compound in its genome.

In the previous figure it can be observed that there are notable differences between each type of dilutions, as it decreases, the colonies have a notable change with respect to their size and quantity, this can also be attributed in the same way to the concentration in which they are found, as it can be seen in the figure 10.

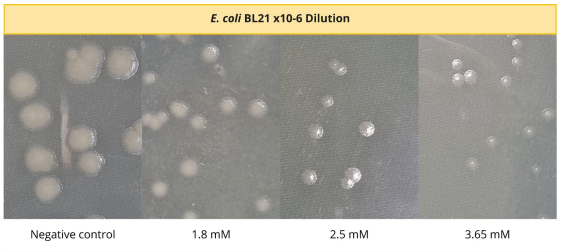

Figure 10. Representation of the effect of different types of concentrations of isoeugenol in BL21 strain.

Taking into account the previously mentioned in figure 10 it can be seen a close-up of the results of the growth of *E. coli* BL21 without any resistance marker, with a 6x resolution. On the negative control plates, the colonies were big, opaque, and of a white/beige color, but as the isoeugenol concentration increased, the number of colonies decreased significantly, they became smaller and transparent, until the isoeugenol concentration became high enough to prevent them from develop and grow correctly.

The same experiment was performed but this time with DH5α strains, nevertheless after repeating them several times, a concise result couldn´t be obtained, each concentrations shown inconsistencies regarding the results obtained with BL21 strains.

2.2 Minimum Inhibitory Concentration in Liquid Medium

On the other hand, with respect to liquid medium, it was decided to use it for the evaluation of the behavior of DH5α. A seed culture of *E. coli*, grown overnight, it was seeded in fresh medium in a ratio of 1:40. Then 200 µl of the corresponding culture was placed in a 96-well plate. The plate was incubated in the Agilent BioTek Synergy H4 Hybrid Microplate Reader at 37°C with 200 rpm shaking, readings at 600 nm were scheduled every 20 minutes for 16 hours.

From the results shown in Figure 11, it was concluded that isoeugenol had a stronger effect when used in liquid medium, than it had when used with agar; this could be attributed to the availability of isoeugenol. In addition, it could be determined that the MIC and MLD values for liquid medium were 1.8 mM and 4.5 mM, respectively.

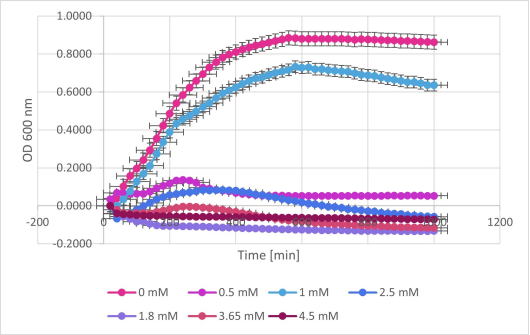

Figure 11. Growth kinetics of *E. coli* DH5α, at different concentrations of isoeugenol, in liquid medium.

3) *Characterization of the behavior of transformed cells with our genetic constructs Pp_22 and Nc_22*

3.1 Molecular docking

To determine that isoeugenol and *IsoMo* would be working together to be used as a selection system, codon optimization and a 3D structure was obtained with ITASSER. With this, static molecular docking was performed in AutodockVina (Figure 12), in order to analyze the interactions between the enzyme and its substrate (isoeugenol), using as a starting point the residues of the active site reported by Ryu, Seo, Park, Ahn, Chong, Sadowsky & Hur (2013): H167, H218, H282, H471, E135, E349 & E413 [15]. A binding energy score of -6.4 and a forming hydrogen bond with Met350.

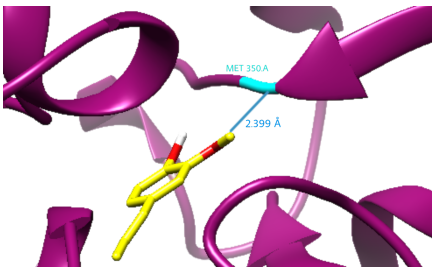

Figure 12. Result of molecular docking of *IsoMo* (purple) and Isoeugenol (yellow). The hydrogen bond with Met350 is highlighted in blue. Own figure.

3.2 Ligation of the genetic constructs into a vector

Genetic constructs were inserted into pJET1.2 vector, in spite of this being a cloning vector and not an expression vector, using the CloneJET PCR Cloning Kit-Thermofisher Scientific (K1231). Both sequences included a promoter and a terminator for each gene. Figures 13 and 14 show the *in silico* ligation of Pp_22 and Nc_22 in the *Eco*RV site of the vector. The selection of this vector resides on its ends facilitates ligation and resistance to Ampicillin.

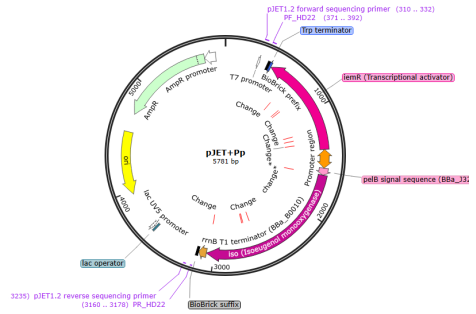

Figure 13. Ligation of Pp_22 (2807 bp) in pJET1.2

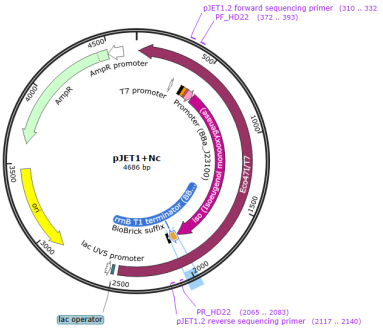

Figure 14. Ligation of Nc_22 (1712 bp) in pJET1.2

3.3 Transformation of chemically competent cells

After this, both vectors were used to transform chemically competent cells DH5α and BL21 *E. coli* cells. Transformed cells were plated on LB with Ampicillin for the assuring the growth of them, then they were incubated at 37°C for 12 hours , the results are reported in Table 7.

Table 7. Growth results of transformed cells, plated on LB+Amp

E. coli strain	Genetic construct inserted	Description
BL21	Pp_22	Only one colony was obtained
	Nc_22	Two colonies were obtained
DH5α	Pp_22	Multiple colonies were obtained, however only 5 were selected for further steps
	Nc_22	Two colonies were obtained

3.4 Plasmid extraction

Once the transformed colonies were obtained, a plasmid extraction was performed with the Quick Plasmid Miniprep Kit from Invitrogen. The results from the run in a 0.8% (w/v) agarose gel electrophoresis, are presented in Figure 15.

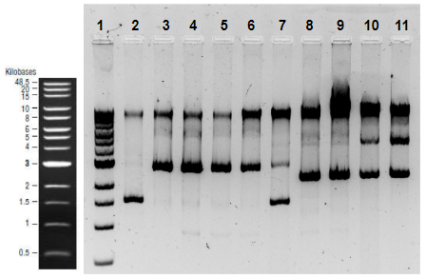

Figure 15. Plasmid extraction of transformed cells. 1) 1 kb DNA Ladder from NEB, **2)** Pp_22 DH5α 1, **3)** Pp_22 DH5α 2, **4)** Pp_22 DH5α 3, **5)** Pp_22 DH5α 4, **6)** Pp_22 DH5α 5, **7)** Pp_22 BL21, **8)** Nc_22 DH5α 1, **9)** Nc_22 DH5α 2, **10)** Nc_22 BL211, and **11)** Nc_22 BL21 2

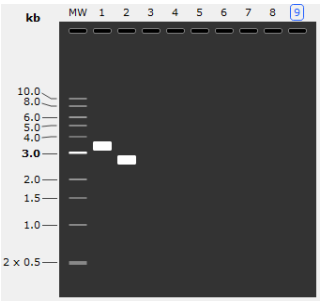

Figure 16. SnapGene Simulation of 1) pJET+Pp_22 and 2) pJET+Nc_22 migration in 0.8% agarose gel. Both lanes show the supercoiled isoform of the plasmids. 1) 5781 bp and 2) 4686 bp.

3.5 Digestions

The next step of the characterization was performing a series of digestion of the 10 plasmid samples; it was carried out with restriction enzymes *Xba*I and *Spe*I. The use of the same enzymes for all samples was possible due to the Biobrick design of both genetic sequences. The digestion product was analyzed with an electrophoresis (Figure 17) and a simulation (Figure 18) was also done in order to establish a comparison between both.

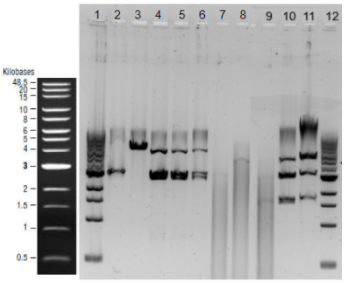

Figure 17. Digestion of plasmids with *Xba*I and *Spe*I. 1) 1 kb DNA Ladder from NEB, 2) Pp_22 DH5α 1, 3) Pp_22 DH5α 2, 4) Pp_22 DH5α 3, 5) Pp_22 DH5α 4, 6) Pp_22 DH5α 5, 7) Pp_22 BL21, 8) Nc_22 DH5α 1, 9) Nc_22 DH5α 2, 10) Nc_22 BL211, and 11) Nc_22 BL21 2

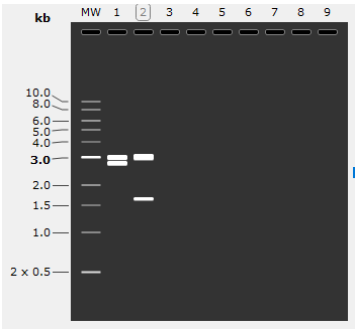

Figure 18. SnapGene Simulation of digestion of pJET+Pp_22 and pJET+Nc_22 migration in agarose gel. 1) pJET+Pp_22 shows two bands, one of 3009 bp and the other of 2728 bp. 2) pJET+Nc_22 shows a band of 3012 bp and another of 1630 bp.

Samples in lanes 7, 8 and 9 were degraded, nevertheless the samples in lanes 4, 5, 6, 10 and 11 showed the correct sizes of the digestion.

3.6 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) was executed in order to observe the amplification of Pp_22 and Nc_22 using PF_HD22 and PR_HD22 primers and be able to confirm their presence in the plasmids extracted from our transformed cells (**Figure 19**). This procedure, as well as the rest, was compared to the *in silico* simulation (**Figure 20**).

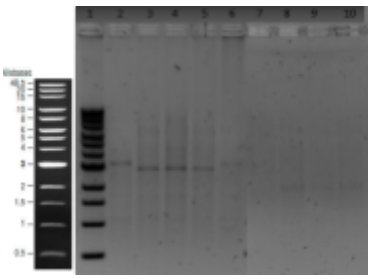

Figure 19. PCR with PF_HD22 and PR_HD22 primers. 1) 1kb DNA Ladder, 2) - 6) Pp_22, 7) - 10

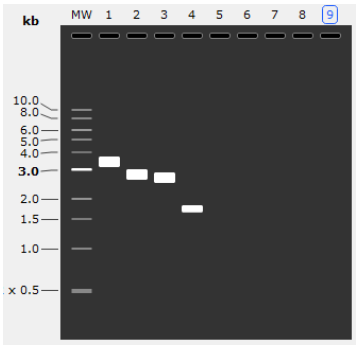

Figure 20. SnapGene Simulation of PCR.
1) pJET+Pp_22, **2)** PCR product of pJET+Pp_22 (2808 bp), **3)** pJET+Nc_22, and **4)** PCR product of pJET+Nc (1712 bp)

Lanes **2, 3, 4, 5** and 6 correspond to Pp_22, amplification products can be observed in all of them. Lanes 7, 8, 9 and 10 correspond to Nc_22, only lanes 8 and 10 show amplification products.

3.7 Isoeugenol monooxygenase expression analysis

After verifying the presence of the Pp_22 and Nc_22 in the transformed cells, the following samples were selected: *E. coli* DH5α transformed with Pp_22 (colonies 3, 4 and 5) and *E. coli* BL21 transformed with Nc_22 (colonies 1 and 2). For preserving the cells and aiming to obtain better molecular results, glycerol aliquots of these were prepared when OD600 was 0.6 for each.

With these aliquotes, the expression of *IsoMo* was evaluated by culturing them in LB medium. The cultures were incubated at 20°C (optimal temperature of *IsoMo*, as reported by Yamada, Okada, Yoshida & Nagasawa [14]) and 200 rpm of agitation for at least 24 hours, after that a total protein extraction was performed, these were the samples used in SDS-PAGE. For controls in this experiment, not transformed DH5α and BL21 *E. coli* strains were also cultured under the same conditions.

Unfortunately, no difference in the band pattern was observed in neither DH5α and DH5α transformed with Pp_22, nor BL21 and BL21 transformed with Nc_22.

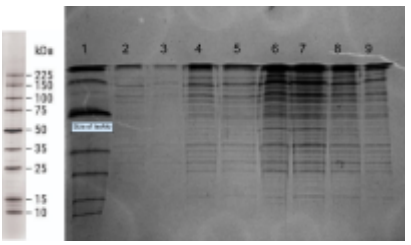

Figure 21. SDS-PAGE of total protein samples.
Transformed cells were cultured in LB with 2.5 mM of isoeugenol, whereas DH5α and BL21 in LB without isoeugenol. No difference was observed in the samples. **1)** Optic protein XL marker. **2)** BL21, **3)** DH5α, **4)** and **5)** DH5α transformed with Pp_22, **6)** and **7)** BL21 transformed with Nc_22

Conclusions

Taking into account all of the previously reviewed, this innovative proposal for the substitution of antibiotics in synthetic biology experimental procedures, could help not only other research teams but also other scientists around the world, to make big contributions for the progress of science in a more secure way and to make a consistent study and evaluation of the real risks of keeping the use of antibiotics in molecular biology procedures, so that the problematic is correctly dimensioned and looked after by the pertinent authorities and for the stakeholders to do an introspection about antibiotics, their use, and determine that a very important task to achieve is relating this topic to Dual use and biosafety & biosecurity.

By analyzing the results obtained, it was possible to understand what measures need to be taken in order to make it work, and these are presented below.

It is necessary to make an evaluation of the effectiveness of the transcription elements of both constructs, Pp_22 and Nc_22. *IsoMo* has an approximate size of 54 kDa thus a band of this size should be observed in all samples. Additionally, in samples corresponding to transformed cells with Pp_22 should also show a band of about 35 kDa that is the transcriptional activator (*IemR*) of *Iso*. Since none of them were observed in the results of the first experiment, it was decided to change a couple of things. As nothing was shown in the gel, indicating that no cell growth was being obtained at 4.5 mM and cells were dying in presence of isoeugenol because they

were not producing *IsoMo* and, therefore, had no way of degrading it.

Despite the advances made, there still are some key milestones in order to achieve a selective system based on isoeugenol, therefore , for this next year, it's proposed the insertion of only *IsoMo's* coding sequence in an expression vector, such as pET17b, the expression of *IsoMo* might indicate whether the problem resides on transcriptional elements or on the coding sequence itself. For this purpose, two primers would be necessary, in order to insert by PCR convenient restriction sites flanking *IsoMo*.

Another step is testing with other Isoeugenol monooxygenases that might be equally or even more efficient for the transformation of isoeugenol to vanillin, since the conversion occurs due to a redox reaction. Perhaps, a monooxygenase from a genetically more similar to *E. coli* microorganism could facilitate its recombinant expression.

It would also be important to seek stakeholders to give professional feedback of the efficiency of the system, this clearly after 1 to 2 years of intense research and experiments that allows the construction of a new plasmid where *IsoMo* acts as a selection marker for recombinant protein production. After accomplishing this, the possibility of shipping kits to synthetic biology teams or laboratories for them to try it grows, giving the chance of exploring entrepreneurial opportunities.

Referencias

[1] Das, A., Thakur, S., Shukla, A., Singh, P., Ansari, J., & Singh, N. P. (2020). Genetic transformation. En Chickpea: Crop Wild Relatives for Enhancing Genetic Gains (pp. 205–224). Elsevier. https://doi.org/10.1016/b978-0-12-818299-4.00008-7

[2] Jones, H. D. (2003). GENETIC MODIFICATION | Transformation, General Principles. En Encyclopedia of Applied Plant Sciences (pp. 377–382). Elsevier. https://doi.org/10.1016/b0-12-227050-9/00197-6

[3] Frieri, M., Kumar, K. & Boutin, A. (2017). Antibiotic resistance. Journal of Infection and Human Health. Vol. 10. Issue 4. https://doi.org/10.1016/j.jiph.2016.08.007

[4] de Kraker, M., Stewardson, A. & Harbarth, S. (2016). Will 10 Million People Die a Year due to Antimicrobial Resistance by 2050? PLoS Med 13(11): e1002184. https://doi.org/10.1371/journal.pmed.100218

[5] Jasovský, D., Littman, J., Zorzet, A. & Cars, O. (2016). Antimicrobial resistance-a threat to the world's sustainable development. Taylor & Francis. https://doi.org/10.1080/03009734.2016.1195900

[6] Schmidt, E., et.al. (2013). Composition and Antioxidant Activities of the Essential Oil of Cinnamon (Cinnamomum zeylanicum Blume) Leaves from Sri Lanka. Journal of Essential Oil Bearing Plants. Vol. 9. Issue 2. https://doi.org/10.1080/0972060X.2006.10643490

[7] Overhage, J., Steinbüchel, Al. & Priefert, H. (2003). Highly Efficient Biotransformation of Eugenol to Ferulic Acid and Further Conversion to Vanillin in Recombinant Strains of *Escherichia coli*. Applied and Environmental Microbiology. https://doi.org/10.1128/AEM.69.11.6569-6576.2003

[8] Hyldgaard, M., Mygind, T., Piotrowska, R., Foss, M. & Meyer, R. (2015). Isoeugenol has a non-disruptive detergent-like mechanism of action. Frontiers in Microbiology, 6. DOI:10.3389/fmicb.2015.00754

[9] Zemek, J., Košíková, B., Augustín, J., & Joniak, D. (1979). Antibiotic properties of lignin components. Folia Microbiologica, 24(6), 483–486. DOI:10.1007/bf02927180

[10] Yamada, M., Okada, Y., Yoshida, T., & Nagasawa, T. (2008). Vanillin production using *Escherichia coli* cells over-expressing isoeugenol monooxygenase of Pseudomonas putida. Biotechnology letters, 30(4), 665-670.