Synthesis of Methyl Salicylate

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Reviewed on 29 April 2017; Accepted on 19 June 2017; Published on 10 November 2017

The purpose of this experiment was to make methyl salicylate synthetically using gBlocks DNA coding sequence. Methyl salicylate is an organic ester naturally produced by many species of plants, particularly wintergreens. Aromatherapy using essential oils like peppermint has had effects in studies to lower blood pressure and increase clarity. Our goal was to make this methyl salicylate synthetically, to decrease cost instead of making it chemically, and so that we could use it as an aid to students with test anxiety. We researched aromatherapy and conducted a small test for association between methyl salicylate and memory. We researched protocols for how to digest, ligate, and transform our gBlock cells onto an Escherichia coli cultured plate. We designed our DNA sequence and found suitable restriction enzymes as well as ordered the correct gBlock sequence. After ligating the gBlock cells, we verified to see a plasmid by running a gel electrophoresis bed. After running the gel bed, we found a fragment of cells that we then cut back out of the gel, and then used a kit to purify the cells back down. We are then going to make ampicillin resistant LB agar plates to transform our bacteria. We are testing our results by the presence of a mint smell – if it smells like mint, then we know that methyl salicylate has been made and transformed.

Keywords: Methyl salicylate, salicylic acid, benzoic acid/salicylic acid carboxyl methyltransferase I

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Background

Anxiety is an emotion characterized by feelings of tension, worried thoughts and physical changes such as increased blood pressure (Kazdin, 2000). Test anxiety is a more specific type of anxiety; it is the fear of failing on an important exam or test. Causes include the fear of failure, lack of preparation, and poor testing experiences. The symptoms can be physical, with headaches, nausea, and diarrhea, and all this can lead to a panic attack, which has complications of its own. Emotional and cognitive symptoms are also evident, making taking the test very hard and frustrating. Many attempts have been made to solve this problem. Some have included the use of aromatherapy to limit test anxiety or possibly relieve the effects. According to Cho et al. (2013), aromatherapy essential oils were blended with lavender, roman chamomile, and neroli and given as inhalations before and after percutaneous coronary intervention (PCI), and this condition had significantly lower anxiety levels than the control group. Raudenbush et al. (2001) have shown that the smell of peppermint can significantly raise levels of oxygen to the brain and lower blood pressure levels, leading to enhanced concentration. The study found that drivers who were exposed to a peppermint aroma whilst driving experienced less frustration, less anxiety, and felt less fatigued for longer periods of time. Menthol can stimulate the hippocampus – the area of the brain that controls "mental clarity and memory" (Sweitzer, 2015). Methyl salicylate, the active compound in wintergreen oils, can be found naturally and is also synthetically produced. "It's not clear exactly how many students have it, but severe test anxiety could afflict as much as 20 percent of the school-going population, according to the American Test Anxiety Association, and another 18 percent may have a moderate form of the condition" (Strauss, 2013). Test anxiety is an issue in schools, and it can hinder students' abilities, even to take tests that they have the knowledge to complete. But the anxiety arising from the fear of failing or of repeating a bad experience can prevent students from reaching their full potential. If methyl salicylate is present in the test environment, it may be able to relieve stress. Methyl salicylate, an organic ester, is naturally produced by wintergreens, among other plants (ChEBI, 2017). This methyl salicylate is the focus of this project; it is what we were going to make originally from salicylic acid.

Materials and Methods

Experimental Overview

We began by resuspending the DNA gBlocks (Integrated DNA Technologies; IDT) fragment using the DNA resuspension procedure described below. When not using the DNA gBlocks fragment, it remained on ice. The gBlocks DNA and plasmid DNA were digested with EcoRI (New England Biolabs; NEB) and KpnI (NEB) as detailed in the DNA Digests section. Ligation the digested DNA segments followed the DNA Ligation method. We prepared and ran an agarose gel (as described below) to verify that the gBlocks and plasmid DNA had successfully ligated together. After verifying that there is a DNA sequence on the gel bed, we extracted the DNA and put it into a new microcentrifuge tube using the DNA Gel Extraction procedure. Finally, E. coli were transformed with the plasmid and plated onto LB agar as described in the Bacterial Transformation section.

DNA Resuspension

We centrifuged 1000 ng of the gBlock DNA fragment for 5 s at 6,600 RPM and then added 100 µL TE Buffer, giving us a final DNA concentration of 10 ng/µL. After vortexing the tube for 10 seconds, we incubated it at 50°C for 20 min. Finally, we vortexed the tube for 10 s and centrifuge for 5 s.

DNA Digest

We combine 10 µL 10 ng/µL DNA solution, 3 µL 10X CutSmart Buffer (NEB), 1 µL EcoRI, and 16 µL nuclease-free water in a microcentrifuge tube labeled "gBlocks". The tube was incubated at 37°C for 1 h prior to using the cleanup method described below. We purified the DNA at 65°C for 20 min. In the same microcentrifuge tube, added 3 µL10X NEBuffer 1.1 (NEB), 1 µL KpnI, and 16 µL nuclease-free water and incubated at 37°C for 1 h. We repeated the cleanup method and then performed the plasmid miniprep procedure, also described below. In a separate microcentrifuge tube, labeled "vector", we combined 25 µL vector DNA, 3 µL 10X CutSmart Buffer, 1 µL EcoRI, and 16 µL nuclease-free water and incubated at 37°C for 1 h. Once again, we followed the cleanup procedure and purified the DNA at at 65°C for 20 min. In the same vector microcentrifuge tube, we added 3 µL10X NEBuffer 1.1, 1 µL KpnI, and 16 µL nuclease-free water, incubated the tube at 37°C for 1 h and reapplied the cleanup procedure.

DNA Ligation

Using NEB Cloner Ligation, we began by obtaining a microcentrifuge tube and putting it on ice. Next, we retrieved the microcentrifuge tubes labeled "vector" and "gBlocks". We added 6.7 µL gBlocks solution, 2.08 µL vector solution, and 11.22 µL water to a tube containing premade DNA ligase buffer and gently mixed by pipetting up and down. After spinning in microcentrifuge for 10 s, we let it sit at room temperature for 10 min. We chilled the tube on ice or immediately transform.

Gel Electrophoresis

We prepared the gel by mixing 1 g agarose with 100 mL 1xTAE in a flask and mircowaved in four 30 s intervals, swirling the flask in between each interval. We let the solution cool for 5 min before slowly pouring the molten agarose into a gel bed and adding a comb. The gel solidified after sitting at room temperature for 30 min. Next, we added 150 µL 1XTAE into the gel bed and dispensed 20 µL DNA ladder into the first well (lane 1) of the gel. We combined 2 µL of loading dye and 10 µL of ligated DNA in a microcentrifuge tube and spun briefly before loading the contents into lane 2 of the gel bed. Finally, we covered the gel bed and turn voltage to 120 V for 1 h.

DNA Cleanup

Using the Monarch® PCR & DNA Cleanup Kit (NEB), we began by adding 150 µL DNA Cleanup Binding Buffer to both the plasmid and the DNA sequence solutions and mixing well by pipetting up and down. Next, we loaded the sample onto a column and closed the cap. After spinning the column for 1 min, we discarded the flow through. We re-inserted column into collection tube and then added 200 µL DNA Wash Buffer before spinning for 1 min and discarding the flow-through. After repeating the wash step a second time, we transferred the column into a clean 1.5 mL microfuge tube, added 10 µL DNA Elution Buffer to the center of the matrix, waited 1 min, and spun the column for 1 min to elute the DNA.

Plasmid Miniprep

Using the Monarch® Plasmid Miniprep Kit (NEB), we added 2 mL of bacterial culture to a microcentrifuge tube and centrifuge for 30 seconds, discarding the supernatant. Resuspending the pellet in 200 µL Plasmid Resuspension Buffer we vortexed the tube to ensure the cells are completely resuspended. Next, we added 200 µL Plasmid Lysis Buffer, gently inverting tube 5-6 times, and incubating at room temperature for 1 min. We then added 400 µL of Plasmid Neutralization Buffer, gently inverting tube until neutralized, and incubating at room temperature for 2 minutes. We centrifuged the lysate for 5 min and carefully transfer the supernatant to the spin column, centrifuging for another minute. We discarded the flow-through, re-insert the column in the collection tube and add 200 µL Plasmid Wash Buffer 1, centrifuging for 1 minute. After discarding the flow-through, we added an additional 400 µL of Plasmid Wash Buffer 2 and centrifuge for another minute. Finally, we transferred the column into a 1.5 mL microcentrifuge tube, added 50 µL DNA Elution Buffer to the center of the matrix, waited for 1 min, and centrifuged for 1 min to elute the DNA.

DNA Gel Extraction

Using the Monarch® DNA Gel Extraction Kit. (NEB), we carefully cut the DNA fragment out of the stained gel bed. We weigh out the DNA fragment: 0.0783 g (78.3 mg), transfer the DNA fragment to a 1.5 mL microfuge tube, and added 315 µL Gel Dissolving Buffer to the tube. Next, we incubated the tube at 50°C, briefly vortexing every minute, until the DNA fragment we completely dissolved. We loaded the dissolved sample into a column, closed the cap and spun for 1 min before discarding the flow-through. We re-inserted column into a collection tube, added 200 µL DNA Wash Buffer and spun for 1 min before discarding the flow-through. After washing the sample twice, we transferred the column into a clean 1.5 mL microfuge tube and eluted the DNA.

Kit Used: (Monarch DNA Gel Extraction Kit)

Bacterial Transformation

We began by adding 50 mL distilled water and 1 g LB broth powder to the beaker, boiled for 20 min and autoclaved for for 1 h. Combining 3 mL sterile broth and one E. coli bead in a large tube, we incubated the mixture at 37°C for 24 h while on a rocking bed. After 24 h, we transferred 1 mL of bacteria broth from the incubator into a clean microcentrifuge tube and centrifuged for 1 min at 8,000 RPM, before discarding the supernatant. We resuspended the pellet in 250 µL CaCl and added 2 µL of the ligation reaction, incubating on ice for 30 min. We placed the tube in a dry bath at 42°C for 45 s before incubating on ice for 2 min and plating a 250 µL aliquot onto LB+amp plate. The plate was incubated at 37°C for 24 h.

Results

Developing a viable design for creating the protein took a considerable amount of time. The first thing that we had to do was find the DNA sequence that codes for methyl salicylate. Following this, we needed to find suitable restriction enzymes for each end of the plasmid and gBlocks sequence. We knew we wanted the restriction enzymes on our plasmid to be EcoRI and KpnI because their restriction sites (EcoRI- GAATTC, KpnI- GTTACC) did not repeat anywhere else in the gBlocks DNA sequence. We added these restriction enzymes' sequences to each tail of the gBlocks sequence. We also added junk DNA to each end (TATAT). Then, we ordered this sequence from IDT and it came in a tube with 1000 ng DNA; this was now our gBlocks sequence.

While the DNA sequence was on its way, we researched how to digest, ligate, and transform cells onto an E. coli cultured plate. We came across a few very helpful resources in the process. But in preliminary steps, we ran into a couple of problems. We needed to order the 10X NEB Buffer 1.1, a DNA cleanup kit, and a Plasmid Miniprep Kit. When we realized we did not have this in the classroom, we quickly ordered it and awaited its arrival. Then, we created a procedure based on the research on the two sites that we saw.

Based on a few different sources, we decided to combine the two designs and make our own procedure. For the digestion step, we had to do a sequential digestion because our enzymes were not compatible with each other. Our first source (NebCloner Sequential Digestion) had us combine Reaction A reagents (Table 1) together in a microcentrifuge tube, incubate at 37°C for 10 minutes, use a cleanup kit, inactivate the EcoRI at 65°C for 20 min. Next these same steps were to be performed with Reaction B. Our second source (GBlocks) had different component amounts for both reactions (also shown in Table 1).

We found that by replacing the component amounts from our first source with the amounts from our second source, our procedure used less components. We stuck with the protocol on the first source, finding it more precise than the second. Our full procedure is outlined below

Table 1. Comparing NebCloner and Gblocks Recommended Reactions

	NebCloner Amounts	gBlock Amounts
Reaction A		
DNA	1 µg	100 ng (we estimated it to be 10 µL)
10X EcoRI Buffer	5 µL	3 µL
EcoRI	1 µL	1 µL
Nuclease-free Water	43 µL	16 µL
Total	50 µL	30 µL
Reaction B		
DNA	1 µg	100 ng (we estimated it to be 10 µL)
10X NEBuffer 1.1	5 µL	3 µL
KpnI	1 µL	1 µL
Nuclease-free Water	43 µL	16 µL
Total	50 µL	30 µL

During the testing stage of the experiment, which was after we had already digested and ligated our gene, we used gel electrophoresis to verify that we had a fragment (Figure 1). A small fragment was found. The fragment was then cut out of the gel, extracted from the gel, and then prepared for transformation. Two transformations were recorded. The first time the transformation was run, the mixture was split into two pieces and spread on two LB agar plates: one ampicillin and one without.

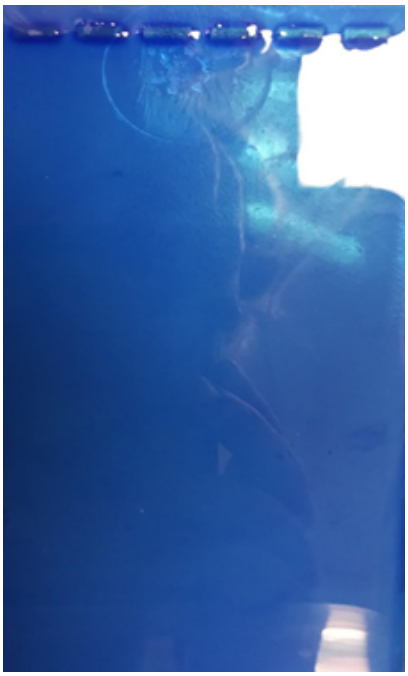

Figure 1. Gel electrophoresis results.

The plates were inverted and set into the incubator at 37°C and left for 24 h. The first attempt did not have any methyl salicylate production (see Figure 2 and Table 2). A second transformation was attempted using newly made E. coli broth, and the whole volume of the mixture was put onto one LB with ampicillin agar plate. The same process was completed as before and it was left inverted in the incubator for 24 h and checked the next day. There was still no methyl salicylate production, and because time was limited, the experiment was ended and

no more transformations were attempted. Since it was decided that qualitative research was the best way to analyze our results, there was no mathematical data to represent the results. The smell of wintergreen was supposed to indicate whether methyl salicylate was grown or not. The only way to analyze this data is by looking back and reflecting on what was done wrong and what could have possibly been the causal factor in having no growth.

Table 2. Methyl Salicylate Production Results

Media	Methyl Salicylate Production?
LB with ampicillin plate #1 (Attempt #1)	No
LB without ampicillin plate #2 (Attempt #1)	No
LB with ampicillin plate #3 (Attempt #2)	No

Figure 2. LB agar plate after first transformation – no methyl salicylate produced

Discussions

The very first plan was to create methyl salicylate from phenylalanine ammonia lyase (PAL), but we were missing a key amino acid in the whole pathway which is not known yet. We then considered getting methyl salicylate from this salicylic acid, but decided against this route due to lack of time. We finally decided to create the methyl salicylate synthetically, skipping the pathway to save time.

When we initially began, we had foreseen a few obstacles. Firstly, when we were undergoing pre-lab, we had to complete calculations that we had not done in class. We had to consult with our teacher to find a proper formula for the computations. Overcoming this initial obstacle helped us complete the experiment protocol faster as we could write the procedure beforehand with each material and amount needed. Another obstacle that we knew we would face was time. We knew that we had to complete the lab work in 4 weeks. With our 90-minute class periods, the digestion was a problem. We not only had to digest one enzyme, but two, and this would take many days to complete. In the end, we found that we would just have to get through the digestion step as quickly as possible, although we had other lab work to complete in the time that we were digesting the DNA fragments (such as making agar plates).

When designing the experiment, we chose the restriction enzymes, EcoRI and KpnI because our school had those restriction enzymes on hand. In addition, we chose to find a cohesive end cloning protocol for digestion, ligation, and transformation for our reaction rather than follow the Gibson Assembly method, which we would then use with multiple smaller gBlocks fragments. If we did use the Gibson Assembly method, we could also save money in ordering our gBlocks fragments. Alas, we chose to undergo the cohesive-end protocol and adapted a few source materials to come to our final design.

In the lab, we used a multitude of techniques that were exclusively taught during our year of Medical Interventions/ Biomedical Innovations. We had to remember to balance the tube correctly, by placing another tube in the opposite side of the centrifuge machine. We also had to remember to adjust the RPM accordingly. Some of our protocols advised us to spin with g force, so we had to convert to RPM according to the arm length of the centrifuge. Calculations were another large part of the experiment. In addition to converting g force to RPM, we had to convert units such as µg to µL, or pmol (picomole) to µL. These are just a few of the many techniques and skills we utilized from the year of MI/BI.

Throughout the beginning experiment, our Medical Interventions/Biomedical Innovations teacher helped point us in the right direction. He helped us choose viable protocols and assisted in ordering the necessary kits and fragments.

To start, our first step was to create a protein using a DNA sequence that codes for methyl salicylate and design this sequence with two restriction enzymes. The reason we used two different restriction enzymes (KpnI and EcoRI) was to remove the risk of binding the sequence to itself by accident when inserting the plasmid. If we had used the same restriction enzymes on both sides, it could have caused the sequence to bind to itself and reject the plasmid. We used the plasmid pUC19 because that allowed us to use KpnI and EcoRI in the vector without having any DNA base pair matches anywhere else in the sequence (KpnI-GTTACC; EcoRI-GAATTC). If we were to complete a second attempt at this, then I believe we would have tried to use a different plasmid with different restriction enzymes so that we could use the Gibson Assembly Method rather than do the two separate methods in one, saving us time and ultimately being more effective. We had to push together two methods from two sources and change things accordingly. Because of the incompatibility of the two restriction enzymes, we had to do a sequential digestion.

Once again, this could have been avoided if we had used the Gibson Assembly Method rather than combining these two methods. This was our most prevalent confounding variable just

because since we had a very long methodology, it left a lot of room for simple error all the way throughout the experiment. Another major issue is that when ligating, it is advisable to investigate or look into the possibility of increasing the volumes of items used; when moving on to verify the fragment, we had an unexpectedly low volume of our ligated reaction left to run on the gel bed. Another big confounding variable would be to make sure that the microcentrifuge is calibrated correctly and balanced it, because on a number of occasions we stopped our spin and got an error code and it spun for longer than necessary. A helpful tip is to do everything you possibly can in a practice attempt. We practiced laying the gel bed down and inserting the ladder with our reaction. We also practiced pipetting up and down to resuspend to avoid making bubbles in the microcentrifuge tubes. Throughout the experiment there were instances where we had to transfer and pipet very small volumes of substance, which occasionally made it hard to transfer 1 µL of something into another tube without getting the substance stuck on the wall. A solution to this, however, would to be to centrifuge it briefly to bring the substance down to the bottom of the tube.

On reflection, we have plenty of ideas on how to make our process better, or how to change it to be more efficient and successful. We acknowledge the mistakes we've made and have thought of ways to solve these problems for future experiments, as mentioned above. If we could start from the beginning again and choose the Gibson Assembly Method to digest and ligate at one time instead of doing two separate processes due to the incompatible restriction enzymes we chose. I would advise future students attempting to run this experiment to start sooner and to make sure they use a compatible set of restriction enzymes with a plasmid that is viable under the Gibson Method.

Acknowledgements

When designing, running, and completing this experiment we were under the supervision of Dr. Kevin McCormick, our teacher here at Summit Technology who also worked with us outside of school at our club, BioBuilders, which is ultimately supervised by qualified sources at MIT. We started designing back in November and tried to come up first with an overarching problem and a solution to said problem. Dr. McCormick supervised our idea at first to create methyl salicylate starting with (PAL) via the pathway. The more research we did, the more we realized we would not be able to start from PAL. Due to time constraints and after consulting with Dr. McCormick about still having to design the sequence we needed, and wait for the sequence to arrive after being synthesized, we decided to just create methyl salicylate instead of using the much more time consuming pathway.

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