Synthesis of Salicylic Acid

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

Salicylic acid production by chemical means is energy intensive and generates many by-products. Designing a bacterium that will generate an enzyme for a pathway to produce salicylic acid would be an effective solution that would resolve many complications connected to chemical synthesis of salicylic acid. To begin the process, we identified the sequence for Isochorismate Pyruvate Lyase (IPL), which was extrapolated from a plant pathway and transformed a bacterium with the DNA by a vector. Successful transformation of a bacterium will set groundwork for further experimentation in the biological synthesis of salicylic acid.

Keywords: Isochorismate Synthase, Salicylic Acid

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Salicylic acid plays an important role in medicine and everyday products in today's world and is a naturally occurring compound found in the bark of willow trees as well as in smaller amounts in various other plants (Anastas, 2000). The acid acts as a defense barrier for the species of plants that produce it because of its properties as an antiseptic, antimicrobial, and its antifungal properties (Household, 2016). However, as useful as this acid is, there are certain aspects about obtaining the acid that pose a problem. Extracting the acid from plants can be done but the methods to do so are time consuming and tedious (Kaur, 2017). Other chemical methods have been developed (such as the Kolbe Schmitt process) but these means of production are energy intensive, and generate large amounts of waste product (Anastas, 2000). The goal of this project is to introduce a plasmid to produce an enzyme (Isochorismate Pyruvate Lyase) for a specific plant pathway that produces salicylic acid. The bacteria can then be grown up on plates; by doing so the process for production of salicylic acid will be more cost and time effective; this method will also reduce waste products (Anastas, 2000). We first use PCR to amplify the gene fragment that we had synthetically produced. Then we used restriction enzyme digest to cut the ends of the DNA. This allowed us to ligate the new DNA to the bacterial plasmid. After the ligation reaction, we ran gel electrophoresis. This was to verify that the reaction produced recombinant DNA that was the length of the sum of the plasmid and insert DNA. A purification kit was then used to extract the DNA out of the gel bed so we could continue with the process of bacterial transformation. After transformation, individual colonies were grown in broth and the process of protein electrophoresis performed. The results from this procedure indicate that the enzyme required to produce salicylic acid has been synthesized.

Materials and Methods

DNA Design

The first step was to design an "insert" DNA fragment containing the nucleotide code for the protein we wanted to produce. This DNA insert would be ligated to a plasmid for insertion into the *Escherichia coli* bacteria. To design this DNA insert, we must look at the main components of an operable gene fragment; first there is the promoter. In our gene fragment we used a common promoter, holoenzyme sigma 70. This promoter initiates RNA synthesis specifically in bacteria and phages. The

next component that we required was the ribosome binding site (RBS), which ultimately determines how quickly ribosomes are being initiated to start translation. After the RBS is the sequence for the IPL protein, which is rather small at only 340 bp. After the sequence for IPL, we included a stop codon. After having all of the essential parts of our fragment, we then included the EcoR1 restriction site at the beginning and end of the sequence. This will serve as the means to combine the gene fragment to the plasmid through ligation. We will insert this recombinant plasmid into E.coli bacteria (through bacterial transformation) to produce our prototype, which in theory, will synthesize IPL for our process of creating salicylic acid. The overall size of our gene fragment was 499 bp.The next step in our process is to amplify the amount of our DNA using polymerase chain reaction (PCR, New England Biolabs Protocol). We designed a set of forward and reverse primers specific to our strand of DNA.

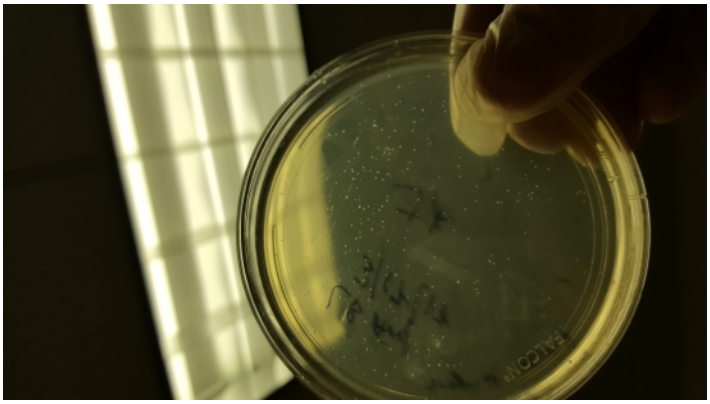
Plasmid Construction, Transformation, and Confirmation

After having amplified the volume of our DNA, a restriction enzyme digest protocol (Addgene Restriction Enzyme Protocol) was performed to "eat back" the ends of our DNA insert. After this process, the DNA insert is ready for ligation with the linearized vector (bacterial plasmid) (Biolabs, 2016). Once the recombinant plasmid was formed in the ligation reaction (NEB protocol), bacterial transformation was utilized to introduce the plasmid into the bacteria. The plasmid contained a resistance gene for ampicillin, which was used to identify the success of the transformation. The final step in our process is running protein electrophoresis with the lysed *E.coli* cells to verify whether the bacteria had produced our IPL enzyme or not.

Results

Once the gene fragment was obtained, ligation was the first step of our process. Once the ligation reaction was over we verified that the insert DNA had combined with the bacterial plasmid. Using gel electrophoresis, we identified the DNA segment that was close to 3500 bp. This was important because it showed that the 500 bp segment of DNA insert had combined with the 3000 bp plasmid. However, because the restriction sites we used were at both the beginning and end of the segment and the plasmid, there are several possibilities as to how the fragments could have ligated together. Several of the insert DNA strands may have combined with each other, the plasmids may have bound to each other or themselves individually, several insert segments may have bound to one plasmid, or none had combined to any segment or plasmid. Knowing that there was a plasmid that had bound to an insert strand or that there were a series of insert segments bound together posed a problem. However, resolution came after we had purified the section of the gel bed containing our desired recombinant DNA. After purification of the gel bed with our desired recombinant plasmid, we ran a transformation procedure. Ampicillin resistance was already contained within the plasmid that had been ligated to the insert DNA, therefore we made agar plates for bacteria with ampicillin present. This way only the bacteria that had integrated the plasmid into their cells will be able to survive in the ampicillin.

A



B

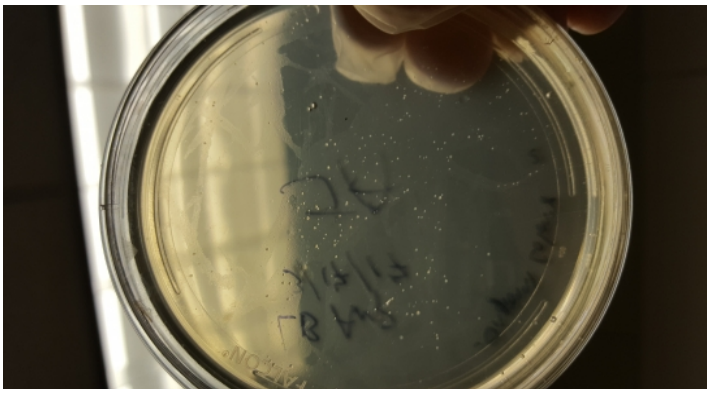


Figure 1. First (A) and second (B) ampicillin plates containing colonies of transformed E.coli bacteria.

These plates contain a plethora of small colonies of transformed bacteria (Figure 1). The next step in our process was protein electrophoresis. To begin this step, three colonies from each plate were harvested with sterile loops, and placed into their own polypropylene tube. They were incubated in broth for 24 h at 37°C on a rocking bed. Once incubation was complete, the samples were transferred into microcentrifuge tubes. Then, 100 µL aliquots were transferred by micropipettes into their own tubes and centrifuged at 12,000 RPM for 1.5 minutes. The first time this step was attempted, there was insufficient bacterial growth to perform protein electrophoresis. Possible explanations could include that when the broth was made up for the incubation, too much water was added in to compensate for what would be lost to the boiling the mixture. Another explanation could be that the particular samples did not survive or may have been very small droplets of water. However, when the plates were incubating they were upside down for this very reason so as not to obtain water droplets. Since there was no growth, more samples were incubated with the same previous procedure and spun down in the centrifuge. The second attempt yielded reasonably sized bacteria pellets (Figure 2).

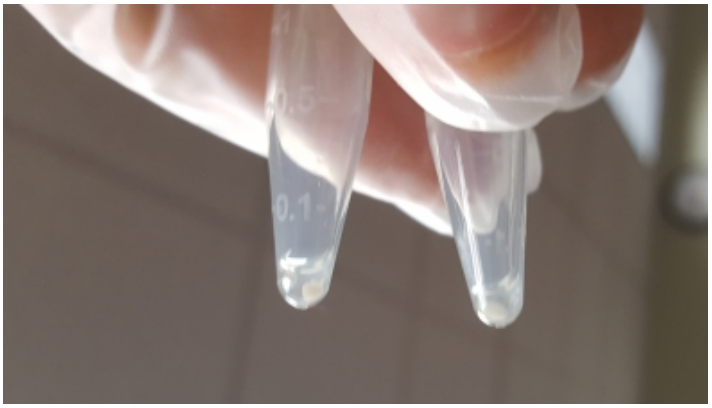


Figure 2. Centrifuged pellets of transformed bacteria to be lysed for protein electrophoresis.

After centrifuging, the cells were lysed in TE buffer by lysozyme and frozen to break the cells apart. After freezing for 24 h, the product was thawed and finally ran through a protein electrophoresis procedure. After running and analyzing the gel bed (Figure 3), we concluded for the first trial that there was no IPL present. The justification behind this reasoning is that when a cell is lysed or broken apart, all of the proteins in the cell are exposed to be run through the gel stopping at their individual molecular size variations in kDa. Seeing as there is only one band (a rather thick one as well) and no streaking on the gel bed, there is evidence the cells may not have completely lysed.

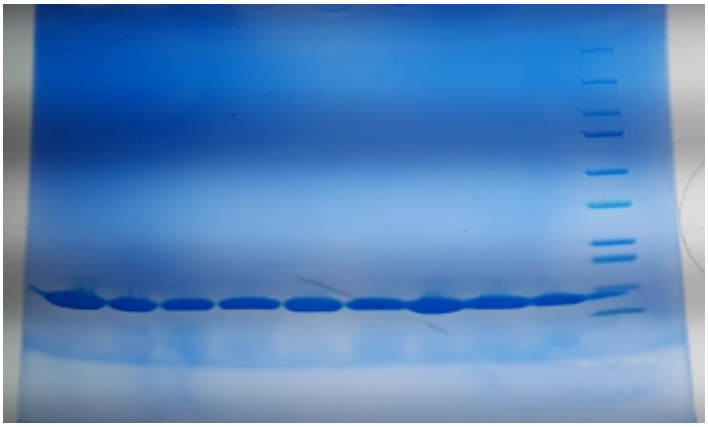


Figure 3 - Unsuccessful protein electrophoresis trial. There is no lane streaking.

Another more feasible explanation is that the dithiothreitol added to the samples before being run was in the wrong concentration and all of the samples individual proteins clumped together. Another possible cause may have been the SDS PAGE loading sample buffer we used. Seeing as this is a detergent and detergents break proteins down, they may have all just been broken apart into many small pieces. Either way the results for the first trial are inconclusive. Another trial for protein electrophoresis was performed. We took samples of broth with bacteria grown from five of the original tubes with the individual colonies from transformation. We centrifuged at 12,000 RPM for 1.5 minutes,

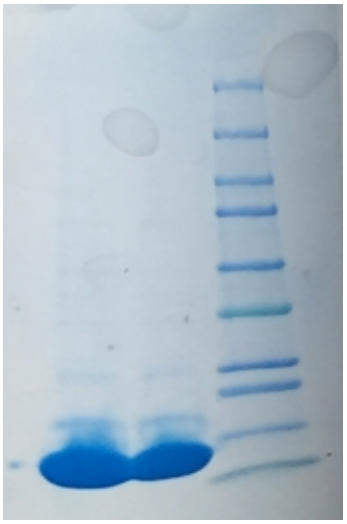


Figure 4. Proteins from lysed bacterial cells between 10 and 15 kDa size as compared to protein ladder.

however this time we used only the samples from the third transformation plate and only colonies 3.3 and 3.2 seeing as centrifuging them revealed the best pellet at the bottom of the microcentrifuge tube. After having run protein electrophoresis for a second time, the gel bed yielded the same results as previously, a very thick band in the size range that indicates the presence of IPL (Figure 4). However, this time there was streaking and multiple bands in the lanes of the samples that had been run. This indicates that the cell had produced our desired protein and been completely lysed.

Discussions

Overall, our experiment worked very well. The band on the protein gel electrophoresis was visible at 11.4 KDa, which is the size of our protein, IPL. The next step in the process would be to put the IPL enzyme in the presence of isochorismate. The isochorismate would act as the substrate with the IPL as the enzyme. This combination would make the desired salicylic acid.

Acknowledgements

The experiment was possible because of the knowledge and assistance from Dr. Kevin McCormick and Ms. Peggy Hinzman. The funding for our project was provided by Summit Technology Academy.

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