STAIN REMOVER

CREATING AN ENVIRONMENTALLY FRIENDLY ALTERNATIVE

WALTHAM BIOHAWKS

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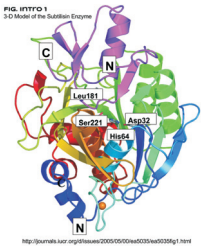
Introduction

FIG. INTRO 1
3-D Model of the Subtilisin Enzyme



http://journals.iucr.org/d/issues/2005/05/00/ea5035/ea5035fig1.html

Laundry detergents can cause rashes, red and blistered skin, sun sensitivity, sneezing, and itchy or watery eyes. So, our project was to create an environmentally friendly, safe alternative to the harsh chemicals in modern detergents.

During our research we discovered a protease enzyme called subtilisin that is naturally found in the genus of bacteria *Bacillus*. Subtilisin is made up of about 269-275 amino acids in a typical globular shape. The active site of subtilisin is made from the resulting 3D shape of amino acids Asp-32, His-64, and most importantly Ser-221; which cleaves peptide bonds with its partially-negative oxygen. We decided to transform the subtilisin gene into *E. coli* to create bacteria that could rapidly produce many of these environmentally friendly, protein-degrading enzymes.

FIG. INTRO 2

Design of the Experiment

Our stain remover device is built of four main components: a promoter, a ribosome binding site (RBS), the subtilisin enzyme and a terminator. The promoter and RBS are taken from the distribution kit part BBa_J04500. We had the subtilisin enzyme and terminator synthesized by the company BioBasic, which included a BioBrick prefix and suffix to make it 3A compatible.

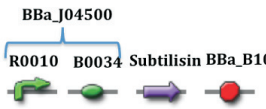
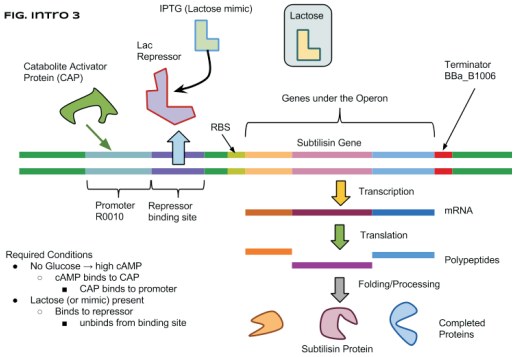


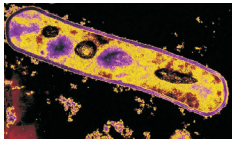
FIG. INTRO 3



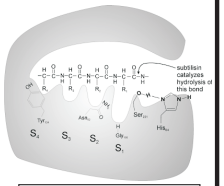
Human Practices

The enzyme subtilisin is produced by the bacteria *Bacillus subtilis*. It has the ability to hydrolyze peptide bonds while being environmentally friendly, not producing any harmful side effects that harsh chemicals in laundry detergent can cause. There are no major health concerns for its use in cleaning products due to the low concentrations used in the detergent. This makes it not a major concern for skin or eye irritation.

Blood coagulation is the process in which mature blood platelets and fibrin combine to form a liquid gel, which forms blood. Because it uses nucleophilic attacks to hydrolyze peptide bonds, subtilisin has the ability to break down potentially harmful blood clots. Ser-221 is the part of the subtilisin enzyme that blocks the active site in blood clots, preventing the substrate from binding and blocking further clotting. Many potential deaths from blood clots could be prevented if the blood clots could be degraded by subtilisin.



Bacillus subtilis SEM



Shown: Ser-221 cleaving the peptide bond of proteins with its partially negative oxygen atom

Building the Stain Remover

There are 4 fundamental steps to the process:
1. Digestion of the component parts, BBa_J04500 and the synthesized subtilisin DNA using restriction enzymes.
   - Our subtilisin gene and terminator were synthesized by BioBasics.
   - They were delivered in a plasmid with compatible BioBrick restriction sites.
2. Running a gel electrophoresis prior to ligation to verify the fragment sizes match the calculated predictions.
3. Ligation of the two parts into a plasmid.
4. Transformation of the *E.coli* with the plasmid.
   - The engineered bacteria can then be incubated, allowing it to secrete subtilisin.
   - With enough time, the subtilisin can then be extracted for potential stain removal.

1: Restriction Digest

As seen in Fig. Pro 1, we first digested BBa_J04500, subtilisin, pSB1C3 and RFP control with their respective restriction enzymes. Each tube had approximately 50 ul of solution total and was incubated in a water bath.

FIG. PRO 2

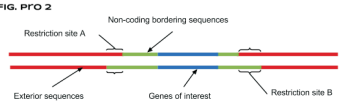
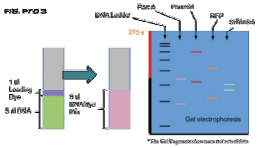


FIG. PRO 3



*The Gel Electrophoresis was carried out for 60 minutes

2: Running the Gel

After performing the restriction digest a gel was run to identify the fragment sizes and verify the digest occurred properly. The gel lanes were loaded using the DNA and loading dye, totaling 5 ul. The gel ran for 6 minutes under 275v.

If the digestion worked correctly, and the gel supplied evidence to support it, then the transformation could continue. However, after running the gel electrophoresis of all digested sequences and even repeating the digest, no visible results were found.

Since the DNA ladder was present and its fragments were correctly distributed, the lack of data from the samples inferred a digestion-based error of some kind.

To test for this error, we digested distribution kit DNA with restriction enzymes. We ran the results on a gel, and the DNA did not appear to be digested, implying that the restriction enzymes were nonfunctional.

Without the vector, the experimental *E. coli* could not be grown. Without enough DNA, we were unable to continue our transformation.

Restriction Digest 1

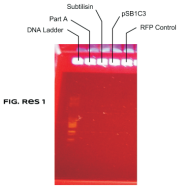


FIG. RES 1

The ladder is visible, but the digested DNA is not, indicating an error in our experiment.

Restriction Digest 2
DNA from the Distribution Kit

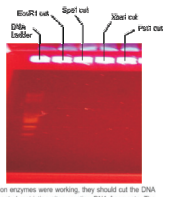
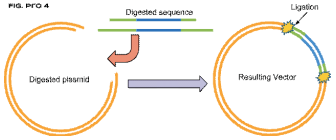


FIG. RES 2

If the restriction enzymes were working, they should cut the DNA at their designated restriction sites creating DNA fragments. The results show none appearing on the gel, meaning that the DNA was never digested, and therefore either the restriction enzymes are nonfunctional or our procedure was erroneous.

FIG. PRO 4



3: Ligation of Plasmid

If digestion worked properly, the parts would be combined in solution and ligated, as seen in Fig. Pro 4. Although some variations of ligation would be arranged incorrectly, there should be sufficient correct vectors that could be inserted into *E. coli*.

4: Prokaryotic Transformation

After the competent *E.coli* are incubated on ice, the vectors would be forced in by heating the cells in a waterbath. Fig. Pro 5 shows the basic process. The bacteria are then put back on ice, and then grown at 37 degrees Celsius in an incubator for 24 hours on agar plates and 24-hours growing at room temperature.

An antibiotic would be applied, and all bacteria not properly transformed would not grow, leaving only the subtilisin-producing colonies. The colonies are then placed in 5ml tubes, where the subtilisin will be secreted into solution and then extracted from the cells.

FIG. PRO 5

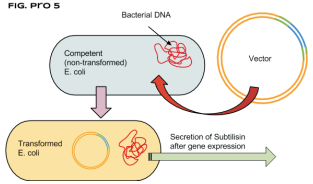
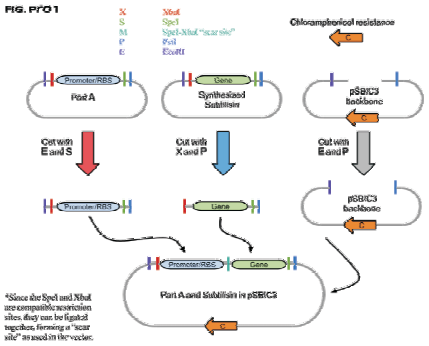


FIG. PRO 1



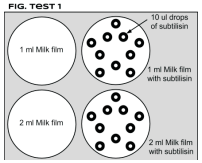
Testing the Stain Remover

Even though our transformation of *E.coli* failed, we still wanted to simulate how our project idea would have worked if the experiment was successful. Since our goal was to transform the subtilisin gene into *E.coli* to produce enzymes that digest proteins, we re-hydrated subtilisin enzyme powder and tested its effectiveness on milk. We chose skim milk because it had a high concentration of proteins.

	IPTG -	IPTG + (0.5 mM)	IPTG + (1.25 mM)	Just growth media
Subtilisin *E.coli*	X	X	X	
E. coli (wild)	X	X	X	
Subtilisin enzyme	X	X	X	
Water	X	X	X	

Conditions Tested | Not Tested

FIG. TEST 1



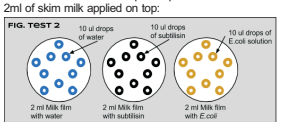
1: Initial Subtilisin Control Test

We added 1ml and 2ml of skim milk to agar plates, and placed 10 drops of 10ul of subtilisin enzyme onto each experimental dish as seen in Fig. Test 1, and documented below. Subtilisin enzymatic decomposition was successfully observed over 60 minutes, and control plates with only milk were relatively unchanged. This experiment supported the functionality of subtilisin in the decomposition of the proteins.

2: Complete Biological Control Test

Following the first test, we wanted to confirm that subtilisin exclusively had protein-degrading capabilities, not *E. coli*. We also wanted to eliminate liquid dispersion as a source of error. So, we repeated the experiment with three plates, each with 2ml of skim milk applied on top:

FIG. TEST 2



1. Negative control plate with 10 drops of 10ul water
2. Positive control with 10 drops of 10ul subtilisin solution
3. Negative control with 10 drops of 10ul *E. coli solution*, which was grown in LB overnight.

Similar to the previous experiment with only milk, the control with water was unchanged and the water diffused into the milk without modifying it. When *E. coli* was applied to the milk, there were also no significant changes. Similar to the last experiment, milk treated with subtilisin was degraded. This proved that subtilisin was solely responsible for protein degradation.