INTRODUCTION

Crohn's disease is a type of inflammatory bowel disease. The exact etiology is still unknown, but it seems, it can be caused by foul ways of living, smoking and genetics can also play a big role in it. It is scientifically proven, that N-acetylglucosamine (NAG) has tissue repairing effects [1]. The human digestive tract cells produce an enzyme called AMCase, which breaks down chitin into its oligomers, but this enzyme is only produced intracellularly, so the oligomers do not leave the cells. There are other extracellular enzymes which break down chitin oligomers into NAG. So we searched for an extracellular enzyme that can break down chitin into oligomers. We found a former iGEM team (Kyoto 2011), that designed a BioBrick (BBa_K622006), that is very similar to our needs, but unfortunately their chitinase gene did not work. We looked then for other iGEM teams which were concerned with Crohn's disease (http://2008.igem.org/Team:KULeuven/Project http://2011.igem.org/Team:UT_Dallas), but we didn't find anything useful on their websites.

Then we have discovered in a Brazilian article [2] which handled with the expression and efficient secretion of a functional chitinase from *Chromobacterium violaceum* in Escherichia coli. *Chromobacterium violaceum (Ch. v.)* has a gene that encodes an extracellular enzyme (CV2935), which converts chitin into its oligomers. This chitinase catalyzes the hydrolytic cleavage of the b(1-4)-glycoside bonds present in biopolymers of N-acetylglucosamine. Our aim is to use a genetically modified probiotic bacteria transformed with this chitinase gene to break down chitin into oligomers. These oligomers are expected to be digested further to NAG by the enzymes of intestinal tract cells as mRNA studies shows these cells produce NAG-ases in huge amount.

PROCEDURE

PCR: Firstly we were going to have the chitinase gene from *Chromobacterium violaceum* (*Ch. v.*) synthesized, but our request could not be carried out. Then we ordered the whole *Ch. v.* genome from the German company, DSMZ. We amplified the chitinase gene from the genome with PCR, at BRC (Biological Research Institute of Szeged) and in our school lab, as well, using two different primer-pairs. One pair was designed for a plasmid called pZA31. This plasmid can be easily transformed into *E. coli Nissle* strain bacteria. The other pair of primers were designed for the pSB1C3 plasmid, which is the standard plasmid backbone for iGEM BioBrick parts.

Purification: After the PCR we purified the DNA using a GeneJET PCR Purification Kit at our school. The DNA, amplificated at BRC was electrophoresed, isolated and purified with Promega Wizard SV Gel and PCR Clean-Up System kits.

Ligation: The predigested chitinase gene was ligated into the predigested (both with EcoRI and PstI) plasmids pSB1C3 and pZA31 at the school and at BRC, respectively.

Transformation: We transformed the gene with the primer for pZA31 into *Nissle* strain *E. coli* bacteria using electrophoresis and the gene with the primer for the pSB1C3 into *DH5 alpha E. coli* bacteria using heat-shock.

Chitinase-Assay: We did three experiments with the media in which we mixed colloidal chitin.

In the first, we verified the chitinase-assay by inoculating *Bacillus thuringiensis*, which we ordered from the National Collection of Agricultural and Industrial Microorganisms (NCAIM) onto the chitin containing medium.

In the second experiment we collected soil samples from five different environments. After that we diluted the samples in distilled water and inoculated them onto the media. Then we inspected which samples contained bacteria that can break down chitin.

In the third experiment we inoculated the *E. coli* bacteria, containing the chitinase gene, CV2935, onto these media and checked if the transformation was successful by observing the clearing zones on the media and their appearance on chloramphenicol containing media.

The Chitinase Assay Kit, which we ordered from Sigma-Aldrich, provides all the reagents required for efficient detection of chitinase activity in fungal and bacterial growth media, macrophage lysates, and purified enzyme preparations. In addition, the kit provides three different substrates for the detection of the various types of the chitinolytic activity. The used substrates were: *4-Nitrophenyl N-acetyl-b-D-glucosaminide* (a substrate suitable for exochitinase activity detection), and *4-Nitrophenyl b-D-N,N¢,N¢¢-triacetylchitotriose* (a substrate suitable for endochitinase activity detection).

We grew two strains of *Bacillus thuringiensis*, which we have acquired from NCAIM in fluid media containing chitin. We also acquired homogenized rat intestines from BRC. We centrifuged the intestine and bacterial solutions and took samples from the overflow. Then we mixed the two samples and measured the enzyme activity with a microplate reader. By adding intestine extractions to the chitinase-enzyme solutions, we examined whether the gut system and its enzymes have an enhancing effect on the chitinolytic reactions or not.

SDS-PAGE: Finally we planned to make an SDS-PAGE to prove that the chitinase enzyme is produced by the bacteria.

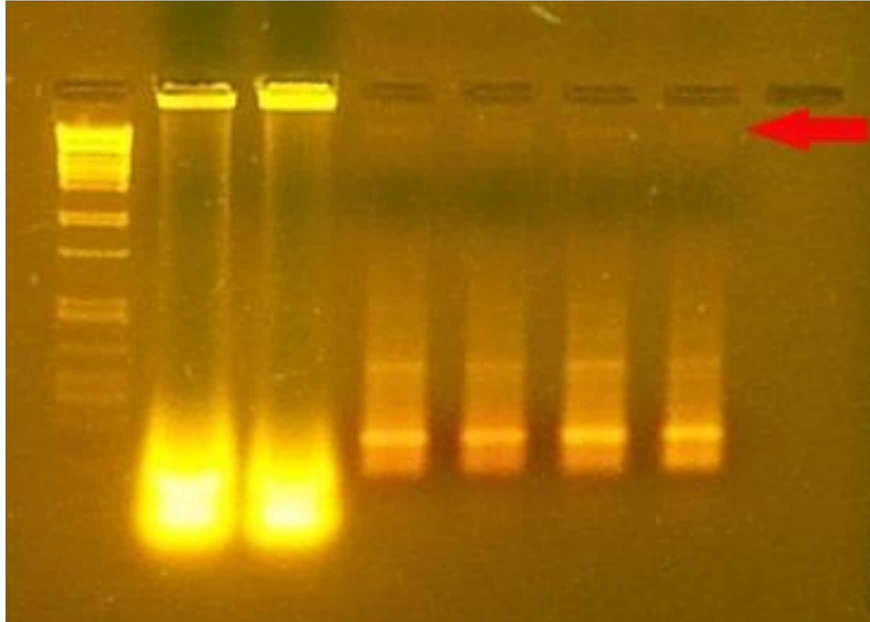
METHODS

Methods and protocols can be seen on Methods page of our homepage.

RESULTS

PCR: One of the PCRs was done to extract and amplify the gene demanded, with the primers for pSB1C3 plasmid and the genome of *Ch. v.* This was done in our own laboratory.

After the first PCR reaction we have run a gel electrophoresis with the product of the program, but the school-safe staining method was not enough sensitive to show little amount of DNA. In this second reaction we additionally did a so-called colonial PCR. Afterwards, we ran a gel electrophoresis, we filled the product of the colonial PCR and the products of the first PCR into the wells. The result can be seen on the picture below.



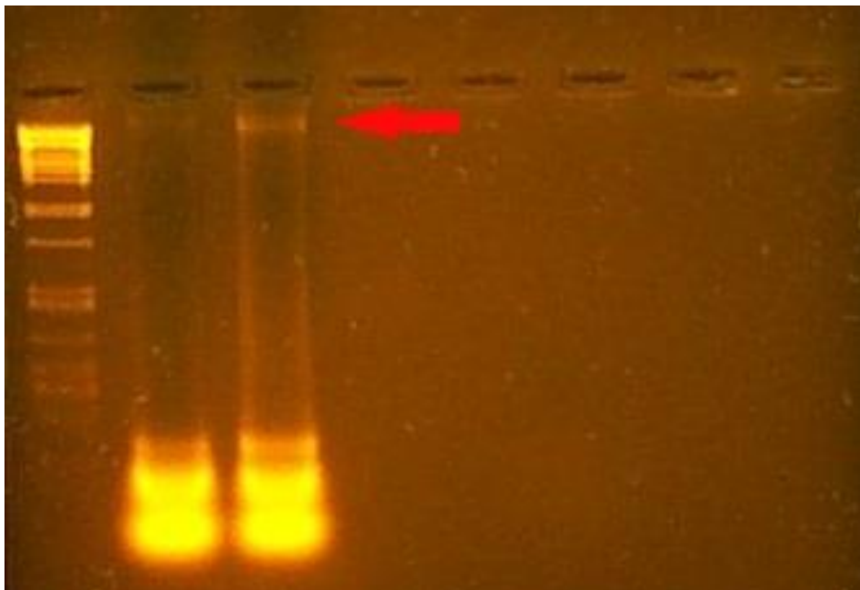
The result of the first and second PCR

1st well: size ladder (Lambda phage DNA digested with PstI - Thermo Scientific)

2nd - 3rd wells: colonial PCR from the transformed and chloramphenicol resistant *E.coli (DH5 alpha)*

4th-7th wells: products of first PCR reaction with iGEM prefix and suffix primer (The demanded gene is slightly visible in the line of the red arrow in wells #4-7.)

This PCR procedure, with the *Ch. v*. genomic DNA and the primers for pSB1C3 plasmid, was repeated for a third time. Now we ran a gel electrophoresis immediately. The born results are visible in the picture below.

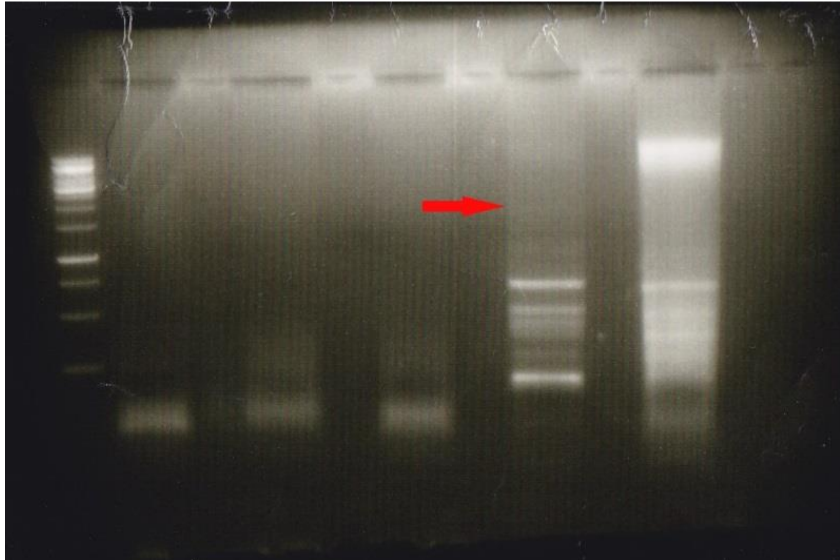


The result of the third PCR

1st well: 1st well: Pst I size ladder (Lambda phage DNA digested with PstI - Thermo Scientific)

2nd - 3rd wells: PCR-check (2 samples) – The red arrow indicates the weak lines of DNA at 2 kbp.

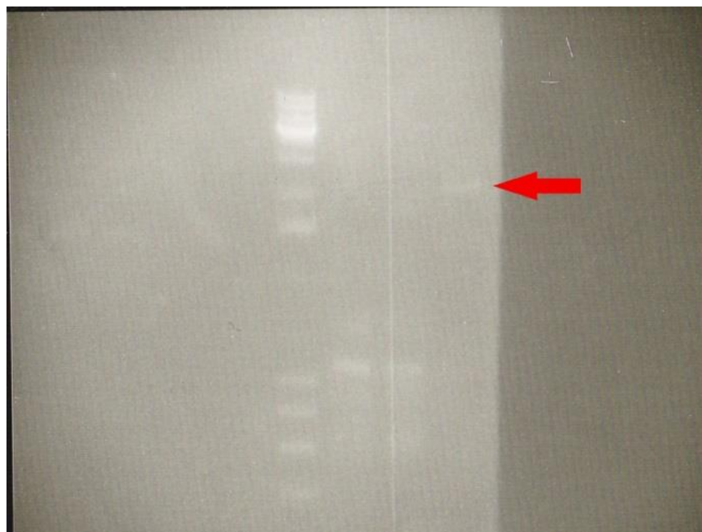
Simultaneously, also for extraction and amplification, another series of PCR reaction was done, with the *Ch. v.* genome and the primers for pZA31 plasmid. This was done in BRC. There were three PCR reactions and these were followed by gel electrophoresis. Results are visible in the next pictures.



Results of the first PCR of gene CV2935

1st well: size ladder

2nd-6th well: Samples treated at different annealing temperatures. The red arrow indicates the slightly visible line of DNA under the 5th well. During the PCR of the DNA of the 5th well, the annealing temperature was set to 58C.

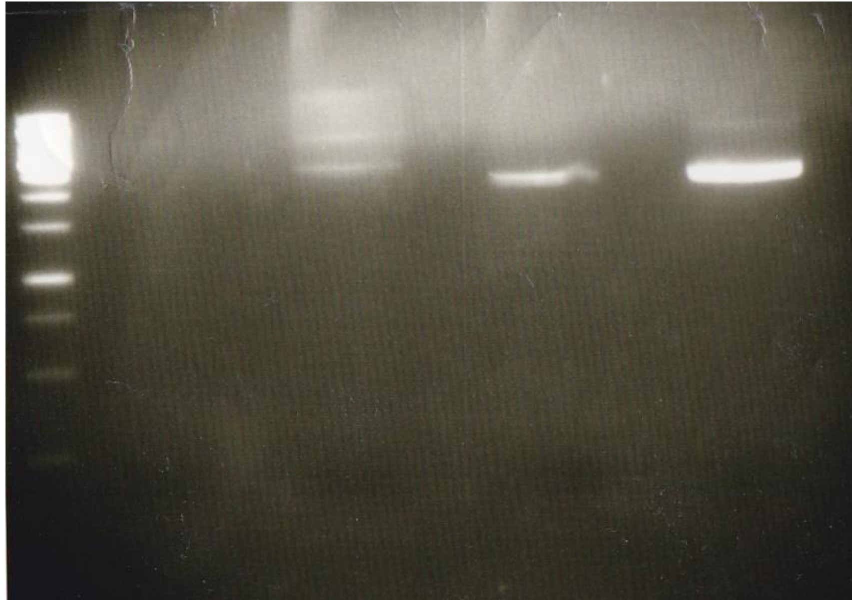


Results of the second PCR of gene CV2935

1st well: size ladder

2nd-4th well: Samples treated with different polymerases: we treated the DNA in the second and third well with the standard Taq polymerase. We added Phusion High-Fidelity polymerase to the DNA, that can be found in the 4th well. The red arrow shows the line of chitinase gene at 2kbps.

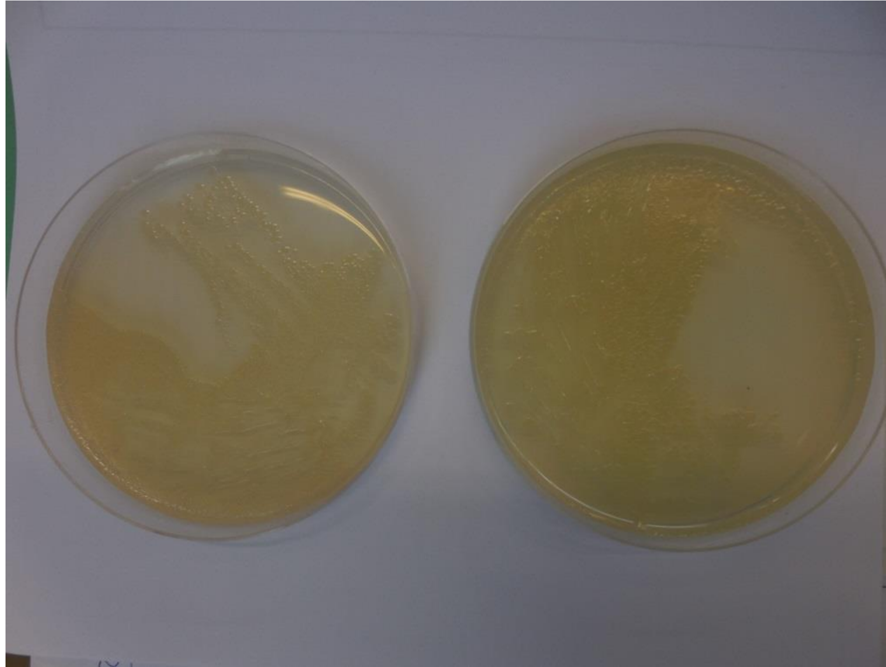
After the amplification of the pZA31 plasmid, we carried out an electrophoresis. Result of the PCR reactions are depicted on the picture below:



1st well: size ladder

2nd-4th well: Backbone samples from PCR reactions with different buffers. The best result is can be seen under the 4th well.

Transformation: After the PCR reaction (done in our own laboratory), the product DNAs were purified and then digested and ligated into pSB1C3 plasmid (iGEM standard). Then we transformed it into *E.coli DH5* alpha strain by the means of heat-shock (in BRC). Afterwards the *E.coli* bacteria were inoculated onto different Agar-plates: ones which contain colloidal chitin, ones which contain only LB and ones which contain LB and Chloramphenicol in the medium. We detected no growth on the medium containing colloidal chitin, but we saw significant growth on the LB medium. Just as on the media containing Chloramphenicol. This proves that at least the plasmid backbone (pSB1C3) had been successfully transformed into the *E.coli*. You can see these Petri-dishes on the following picture.

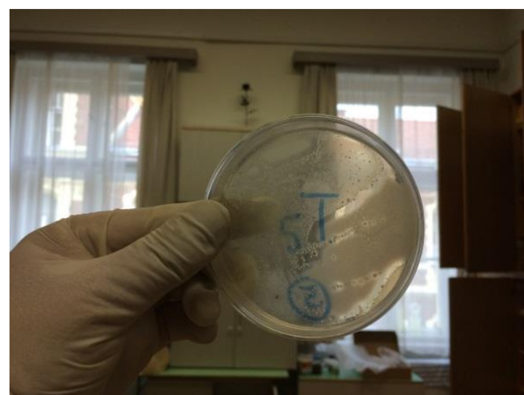


CV2935gene in pSB1C3 plasmid transformed into E.coli

Measuring Chitinase Activity on Colloidal Chitin Media: The *Bacillus thuringiensis* strain, inoculated onto Colloidal Chitin Media, has grown out, causing clearing zones on the media.

The collected soil samples, which were inoculated onto Colloidal Chitin Media, have also grown out, also causing clearing zones on the media. This growth and clearing was only visible on plates #3 and #5. Sample 3 is from the bank of river Tisza, sample 5 is from a flowerbed of the Main Square. For more details see the map. This growth proves the natural chitinase activity in soil.

In the third experiment, where the CV2935 gene had been transformed into *E.coli* and then inoculated onto Colloidal Chitin Media we haven't seen any growth. However this was expected (see discussion!).



The visible clearing zones of soil samples #3 and #5

Chitinase-Assay: In the Methods part we have written the details of the used Sigma-Aldrich Chitinase Assay Kit (SACAK). We have arranged two plates for measuring different types of natural and transformed microorganisms' chitinase activity on two consecutive days. According to the measurement data we have received the following results.

Our first day's measurement data are depicted on the following figure. Concerning the different types of solutions in the wells you are kindly asked to see the Methods part of this study. Our findings were as follows.

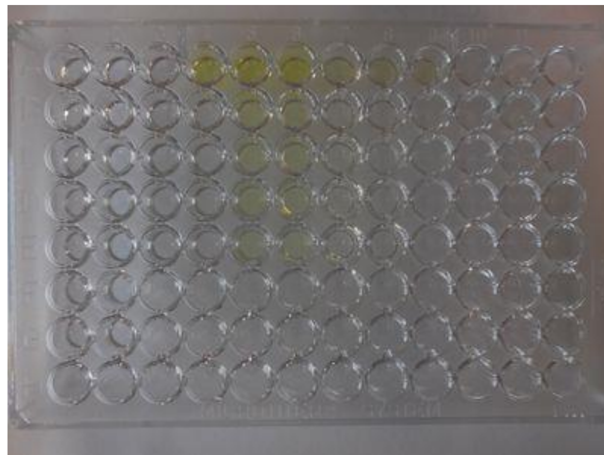
Microplate Manager Bio-Rad Laboratories, Inc.
Raw Data Report

Reader Type : Benchmark   Plate File : Plate5
Date : 2014.19.06 16:57

Measurement Wavelength: 405nm
Incubator Temperature: 29.0 °C
Reading Type: Endpoint
Mix Time: 0 sec

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.009	0.009	0.007	2.896	2.766	2.650	0.445	0.454	0.450	0.071	0.067	0.060
B	-0.013	-0.045	-0.013	0.004	0.638	0.430	-0.011	-0.018	0.082	0.077	0.079	0.078
C	0.018	-0.001	0.020	0.034	0.681	0.469	0.023	0.014	0.085	0.074	0.063	0.060
D	0.039	0.041	0.049	0.035	0.684	0.457	0.051	0.056	0.072	0.087	0.078	0.073
E	0.070	0.017	0.026	0.022	0.650	0.421	0.045	0.061	0.094	0.089	0.099	0.096
F	0.035	0.024	0.092	0.075	0.067	0.075	0.090	0.070	0.077	0.075	0.055	0.072
G	0.023	0.011	0.076	0.085	0.084	0.065	0.077	0.072	0.078	0.078	0.071	0.069
H	0.094	0.091	0.091	0.081	0.069	0.082	0.104	0.094	0.083	0.074	0.077	0.068

1. The SACAK was appropriate for measuring natural chitinase activity. We could see strong color changes at the kit's own chitinase enzyme.



Sigma-Aldrich Chitinase Assay Kit in action

2. The chitinase activity of Bacillus thuringiensis bacteria was the highest if it was cultured in shaking plate. The presence of chitin didn't have any effect on chitinase activity as it was expected, because the chitinase gene was found to be a part of a constitutive operon by former research.

3. The chitinase activity of the natural chitinase of Bacillus thuringiensis liquid media showed 4.19 U/ml activity according to the given formule by the SACAK provider.

4. The large intestine extract of the rat didn't show any chitinase activity itself.

5. The large intestine extract didn't have any enhancing or inhibiting effect on natural chitinase activity.

The second day we planned to answer some questions on the different types of activity. Concerning the different types of solutions in the wells you are kindly asked to see the Methods part of this study.

Microplate Manager Bio-Rad Laboratories, Inc.
Raw Data Report

Reader Type : Benchmark   Plate File : Plate2
Date : 2014. 20. 06 10:04

Measurement Wavelength: 405nm
Incubator Temperature: 30.0 °C
Reading Type: Endpoint
Mix Time: 0 sec

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.009	0.011	0.013	2.822	0.016	3.236	0.442	0.451	0.451	0.072	0.061	0.059
B	-0.021	0.109	-0.029	0.012	0.010	0.027	0.040	-0.003	0.093	0.061	0.079	0.062
C	0.015	0.010	0.020	0.043	0.043	0.044	0.041	0.025	0.075	0.070	0.064	0.064
D	1.491	0.184	1.025	0.118	0.085	0.080	0.076	0.073	0.082	0.080	0.076	0.061
E	0.087	0.082	0.093	0.094	0.100	0.102	0.100	0.080	0.080	0.092	0.086	0.061
F	0.070	0.066	0.074	0.069	0.062	0.064	0.068	0.070	0.053	0.074	0.058	0.065
G	0.088	0.093	0.082	0.080	0.078	0.074	0.071	0.071	0.085	0.067	0.062	0.070
H	0.081	0.099	0.072	0.065	0.059	0.064	0.079	0.067	0.078	0.064	0.067	0.067

Based on these data we found that:

1. Natural chitinase activity of Bacillus thuringiensis is mainly exochitinase activity rather than endochitinase activity.

2. The transformed E.coli cells didn't show enough chitinase activity after 12 hrs of cultivation, nor the formerly chitinase producing soil bacteria.

DISCUSSION

Cloning with primers for pSB1C3 (creating the new Biobrick)

Our main aim was to create two types of genetic constructs: an iGEM biobrick and also a plasmid with which our targeted probiotic bacteria can be transformed successfully.

According to the result of the electrophoresis, in which the DNA from the colonial PCR was run, the quantity of the primers were too high and some genomic DNA must have been left in the solution. These can be concluded from the presence of highly luminous spots (that could be primer dimers) and the lines of DNA positioned close to the wells, respectively.

The products of the first PCR in which we used the iGEM prefix and suffix primers, were run on the same gel with colonial DNA. The smears visible on the pictures may have been caused by the too little amount of time for elongation time in each cycle for this relatively big gene, but the line of DNA of the chitinase gene can also be seen there at 2kbps, thus this PCR can be considered as a relatively successful one.

Observing the results of the third PCR, we can draw a quite similar conclusion to the previous one: the luminescence on the bottom (which probably indicates the accumulation of primer dimers) could be a result of the high concentration of primer added to the third PCR solution. Although, as the representation of moderate success, a smooth line of DNA appears at 2kbps again.

PCRs with primers for pZA31

Regarding the electrophoresis of the first PCR of chitinase gene with primers for pZA31 we adjusted the appropriate annealing temperature, 58C. Hence the binding affinity of the DNA strands was not high enough, the decrease of this temperature (from left to right on the picture, well 2-6) led to the higher rate of annealing. A hardly visible line of DNA marked with the red line could be an evidence for the presence of the gene.

We used 2 different polymerases during the second PCR of this gene with primers constructed for pZA31. As for these results we had to use Phusion High-Fidelity polymerase to avoid 3'overhangs. The DNA at 2kbps shows that the type of the polymerase also has an effect in the aspect of the prosperity of the PCR.

Transformation of pSB1C3 containing CV2935 into *E.coli DH5*

The presence of colonies which contain chloramphenicol proves that at least the plasmid backbone was transformed into the E.coli.

The absence of bacterial colonies on colloidal chitin containing media shows that this bacteria cannot benefit from either chitin or its derivatives.

The transformation of at BRC was carried out in a different manner: electroporation was used to transform the plasmids. Unluckily there were no colonies on any of the media, onto which the transformed bacteria were inoculated. This could be a result of the lack of time or simply the absence of the successfully transformed bacteria. Unfortunately there was no more time and possibility for our team to repeat the transformation of the targeted Nissle type bacteria.

The Chitinase Activity

We examined the bacterial chitinase activity with two different methods. The first method was studying clearing zones in colloidal chitin plates. Based on this test our results show that the natural (soil) samples and also our Bacillus thuringiensis cultures digested chitin with high activity. The clearing zones in both cases proved that these bacteria synthesize and also export chitinase enzyme into their environment with high efficiency. The lack of time finally kept us from experiencing the same phenomenon with our transformed bacteria.

The second method was Sigma-Aldrich's Chitinase Assay Kit (SACAK). SACAK method was used to verify the results found with colloidal chitin plate through a more exact measurement and also to study the intestinal fluid's effect on bacterial chitinase activity. Our measurements were also used to find out if the cultivation method's influence on chitinase production and to clarify if the studied chitinases are belonging into the endo- or exochitinase family.

With using SACAK we have found that gentle shaking has a positive effect on chitinase activity. This result is really important as in the intestine (where our targeted probiotic bacteria will live) all the materials are in constant motion. We also found that the natural bacteria have mostly exochitinase activity rather than endochitinase activity. This phenomenon can be the basis of the possible cooperation between an appropriate probiotic bacteria and intestinal cells during chitin degradation. Finally it was proved clearly that intestinal cells' extract doesn't have an inhibiting effect on chitinase activity, however agonist effect of intestinal tract can be also excluded. Unfortunately lack of time meant that there was not enough time to grow up our transformed bacterial culture in order to measure it's chitinase activity.

CONCLUSION

During our project we have found out a possible half-natural method for protection of human intestine against inflammatory bowel diseases, such as Crohn's disease. This method means a probiotic bacteria which will synthesize and export natural chitinase into its environment enhancing the continuous NAG production from natural chitin and through this way this bacteria have protective effect on large intestine.

The main part of our wetlab project was to create a genetically modified bacteria with the chitinase gene (Cv2935) of Chromobacterium violaceum. We also aimed to create the Biobrick construction for future iGEM-projects. During the whole project period our main aim remained to synthesize a new genetical construct, we had to prove the high activity of bacterial chitinase with a widely spread biochemical assay kit.

Our results show that Cv2935 gene can be amplified successfully from Chromobacterium violaceum genome and also can be cloned into pZA31 plasmid. Though the Biobrick with Cv2935 could be created, we plan to make it again with higher accuracy in the next few days in order to have a construct for future iGEM teams.

Our chitinase assay proved that natural chitinase can become a highly efficient producer of NAG in the intestine with a possibility to protect the gut system of people affected by Crohn's disease.

References:

[1]: A pilot study of N-acetyl glucosamine, a nutritional substrate for glycosaminoglycan synthesis, in paediatric chronic inflammatory bowel disease

S. Salvatore, R. Heuschkel, S. Tomlin, S. E. Davies, S. Edwards, J. A. Walker-Smith, I. French and S. H. Murch

Alimentary Pharmacology & Therapeutics Alimentary Pharmacology & Therapeutics Volume 14, Issue 12, pages 1567-1579, December 2000 (Article first published online: 2 JAN 2002)

[2]: Expression and efficient secretion of a functional chitinase from Chromobacterium violaceum in Escherichia coli

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