Utilization of Intestinal Probiotics to Improve the Degradation and Absorption of Food and Drug Homologous Flavonoids

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Keywords: Flavone, E. Coli, Degradation Rate, PCR, Deamination Tyrosine, FLR.

Abstract: Under the condition that the slow degradation rate of flavone in food in the gastrointestinal tract of the human body has long been a problem for researchers, we constructed and justified a protein to improve the degradation and absorption rate of flavone. The paper, through methods including polymerase chain reaction and gel electrophoresis, explores the possible solution for increasing degradation rate with a flr gene. The FLR enzyme produced throughout the experiment would successfully degrade flavonoids into deamination tyrosine (DAT) to achieve the goal of having anti-inflammatory function. The paper concludes that with E. Coli carrying Pet28a-flr-chi-enoR-phy, flavonoids tested can be dissolved and decomposed up to 95% within 6 hours.

1 INTRODUCTION

In modern life, people often have poor resistance and get sick easily. Due to the situation, flavonoids should be a good choice as flavones in fruits and vegetables have been found to help fight cancer and bacteria. They have antioxidant, hypocholesterolemic, and anti-inflammatory properties, as well as the ability to modulate cell signaling and gene expression, which are linked to disease development. (Thilakarathna S H, 2013) Nevertheless, the low apparent availability of flavonoids is currently considered as a problem as the benefits of flavones cannot be expressed fully. The enzyme in the human intestinal flora was unable to practice degrading and absorbing flavonoids well. (Ravishankar D, 2013) Therefore, attempts to improve their bioavailability in order to improve the efficacy of flavonoids are always being made and studied. (Yang, 2021) Research on flavonoid degradation has been ongoing, but the way to improve the absorption of flavonoids in the gastrointestinal tract of the human body is still to be found. The situation stayed still until 2020, when Nature Communication reported a key enzyme FLR that would initialize the degradation of the flavone. In this case, flavone functions and can help the human body absorb various types of flavonoids.

Flavones are largely found in plants. (Leonard, 2006) The research on flavonoids in recent years is extensive, such as Soybeans flavone, Baicalensis flavone, and Epimedium flavone. (Geng, 2003) They are pharmacodynamic compounds and have been widely used in the treatment of cancer and various diseases. (Yao, 2004) However, it is unfortunately that some people do not have the ability to digest certain groups of flavones, which become detrimental to their health in this way. During this experiment, we made E. coli carry the flr gene to more efficiently produce the FLR enzyme. This manufactured bacteria can now express the FLR gene well, increase the degradation rate of flavonoids, and furtherly generate DAT, which would activate the immune system of the human body so as to achieve anti-inflammatory, antibacterial, anti-cancer and other purposes, at the same time helping reduce clinical treatment costs. (Ashrafizadeh, 2020)

2 CONCEPTS

Flavonoids belong to a large class of significant secondary metabolites of plants, which have good pharmacological activities and important nutrition. The metabolism, absorption, and excretion of it are accomplished in the gastrointestinal tract of the human body. (Chen, 2021) It acts as a role in the gastrointestinal tract in a physiological way, leading to the functions of antioxidant, anti-inflammatory, and anti-cancer as shown in Figure 1.

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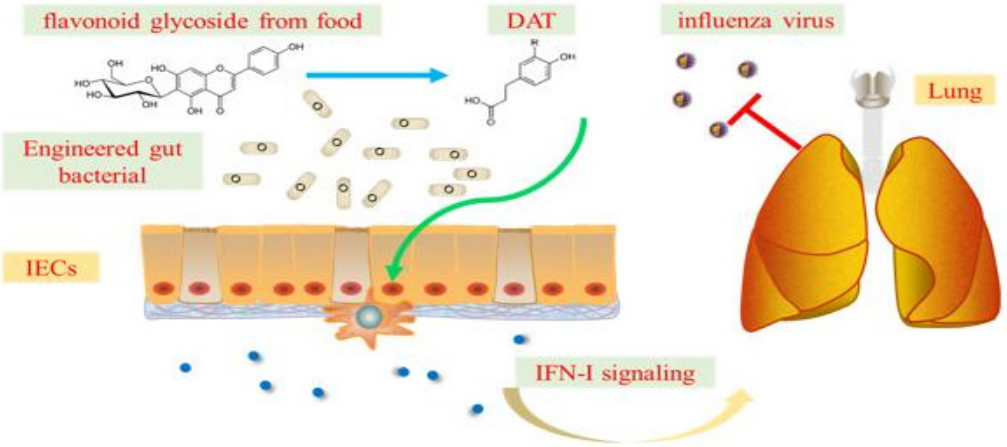

Figure 1: Multiply diagrams to illustrate the benefit brought by flavonoids (Jeon, 2009).

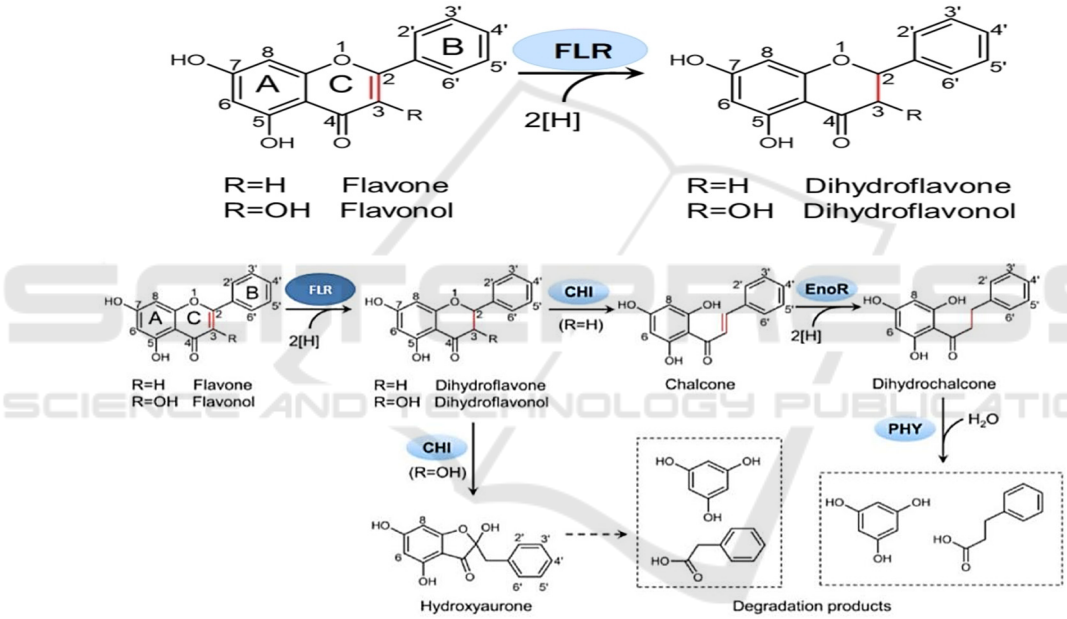

Figure 2: Chemical process of FLR participating in changing flavone into DAT (Jeon, 2009).

In the past ten years, a large number of studies have been carried out to discover the digestion and absorption of flavonoids, especially in the digestive tract of the human body, with the aim of applying the functions to foods, drugs, or even synthetic products to treat diseases. Along with this goal, we managed to build intestinal engineering probiotics to increase the degradation of food and drug homologous flavonoids.

Inspired by a paper from Nature Communication written by Weihong Jiang Research Group, CAS, which demonstrated the newly discovered alkene reductase-flavone reductase (FLR), we started our project. FLR enzyme not only initiates flavonoid absorption and degradation in the human body, but it also has similar effects and functions for another type of flavonoid, flavonol. According to the paper, the enzyme comes from intestinal bacteria, so this experiment should have an outcome that is highly practicable.

In order to increase the practicability and improve the feasibility of our experiment, we chose apigenin, chrysin, luteolin, and diosmetin as four samples for function tests based on the substrate spectrum analysis since chronic diseases can be cured or alleviated by intaking them (see figure 3). (Tang, 2017).

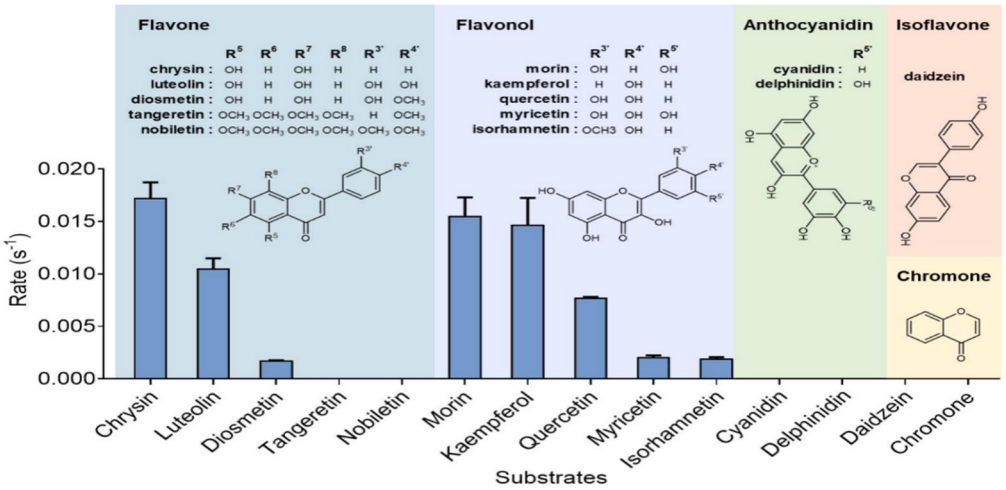

Figure 3: Demonstration of part of the substrate spectrum of flavonoids.

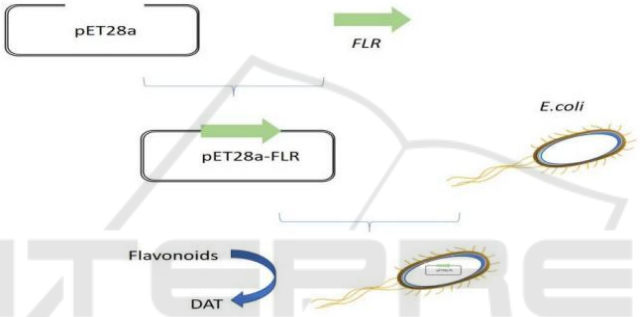

Figure 4: Flow chart representing the principle of E. coli carrying pET28a-FLR.

3 DESIGN

By starting with the pET28a vector, we constructed the pET28a-flr plasmid. After that, we insert it into the carrier, which is the E. coli, to produce the engineered strain for realization of the concept of our product. In the ATLATL lab in Shanghai, we successfully built the pET28a-flr plasmid and proved it with methods like polymerase chain reaction and sequencing.

Next, with the aim of determining the performance of the E. coli carrying pET28a-flr, we designed and used two experiments with the four types of common flavonoids mentioned before, which include apigenin, chrysin, luteolin, and diosmetin. With the initial concentration set at 10mg/L, we measured and recorded the concentration of flavonoids after 2 hours and 6 hours. We repeat the enzyme activity test for each flavonoid three times, to guarantee the credibility of the outcome.

4 RESULT

After we purified the protein, which is the FLR enzyme, an enzyme activity test to evaluate the functions of the FLR enzyme was carried out by us. The same four kinds of flavonoids were used as samples: apigenin, chrysin, luteolin, and diosmetin. Starting with an initial flavonoids concentration of 100.05 mg per liter, an OD600 as 1.0, and an FLR enzyme concentration as 1 mM per liter, we conducted the tests. Every test is repeated three times to reduce the errors and limit the uncertainty to the best of our ability.

The results are recorded for 0h, 2h, and 6h to demonstrate the changes:

Table 1: Data of concentrations of flavonoids.

Concentration (mg/L)	0h	2h	6h
Apigenin	9.96	2.16	0.03
Chrysin	9.97	2.79	0.12
Luteolin	9.96	2.09	0.11
Diosmetin	10.05	5.15	0.53

The results are also shown in the following bar graphs:

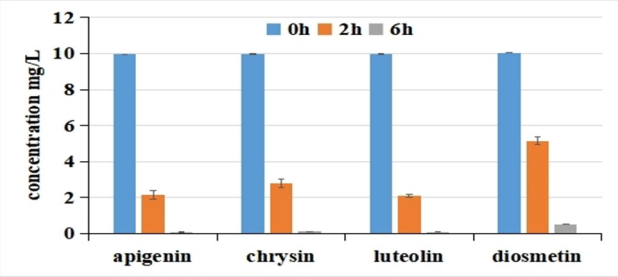

Figure 5: Bar graph of the concentration of flavonoids after 2 hours and 6 hours.

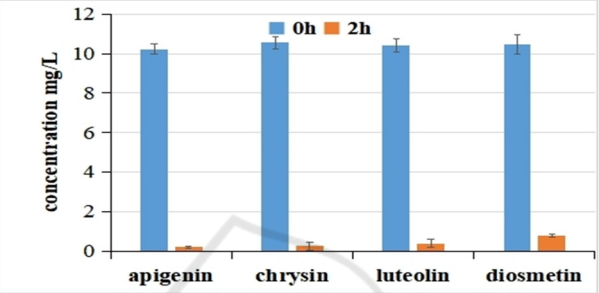

Figure 6: Bar graph of the concentration of flavonoids after 2 hours.

As shown in Figure 5, the concentration of the four types of flavonoids significantly decreased after 6 hours. Although after 6 hours, approximately 0.3-5% of flavonoids are still left, it can be used to indicate that the enzyme we made has high activity in degrading and certain universality in degrading different flavonoids.

Similarly, in Figure 6, it can be inferred that all the tested flavonoids have only 1-7% left, which is near the stage of full degradation. The strong and undoubtable ability of our E. coli to degrade flavonoids is shown with the data and visualized graphs in the way we expected.

To sum up, the E. coli carrying the FLR enzyme has the ability to degrade various common flavonoids with certain practicability. In the comparison of the degradation rates of the tested flavonoids, they should be ranked from least to greatest: diosmetin < luteolin < chrysin < apigenin.

5 MODEL

Besides tables and graphs, we also designed models to represent and forecast the degradation rate of our FLR enzyme using the data we got from the testing. In considering about the quantity of our data, we chose MATLAB as the tool to visualize our results in the form of model.

Since the data follows a declining trend, we chose to use the following negative exponential function to prevent the appearance of negative values in the y-axis:

$$\frac{dy}{dx} = a(y - x)$$

where dy represents the change in y, dx represents the change in x, and a represents the initial concentration of the four flavonoids.

In this case, the analytic expression will be:

$$y = be^{ax} + c$$

where a, b, c are constant resulted from the line of best fit in the models.

According to the modeling results for each samples showing below, the fitting degrees are the same.

As shown by Figure 8, these four curves have pretty similar trends. The functions can be used as a reference to predict the time needed for our E. coli carrying pET28a-flr to achieve its efficiency. For example, it may take 0.8 hours for the bacteria to degrade half of the apigenin, and it may take 3.9 hours to degrade chrysin to 10% of the original amount.

Furthermore, the derivative of these curves is also calculated and visualized by us to directly see their degradation rate in certain hours:

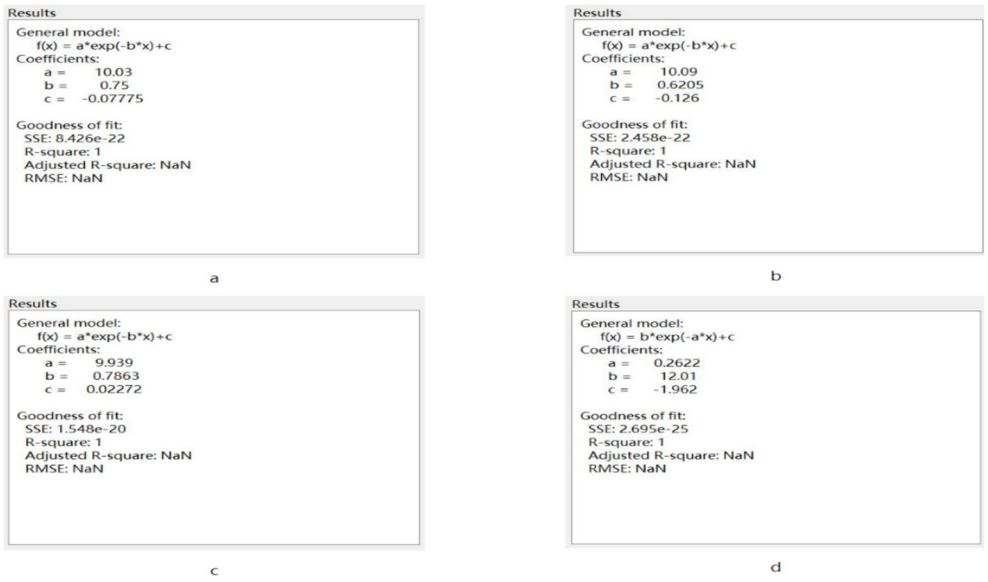

Figure 7: Results of apigenin(a), chrysin(b), luteolin(c), diosmetin(d) is used for modeling.

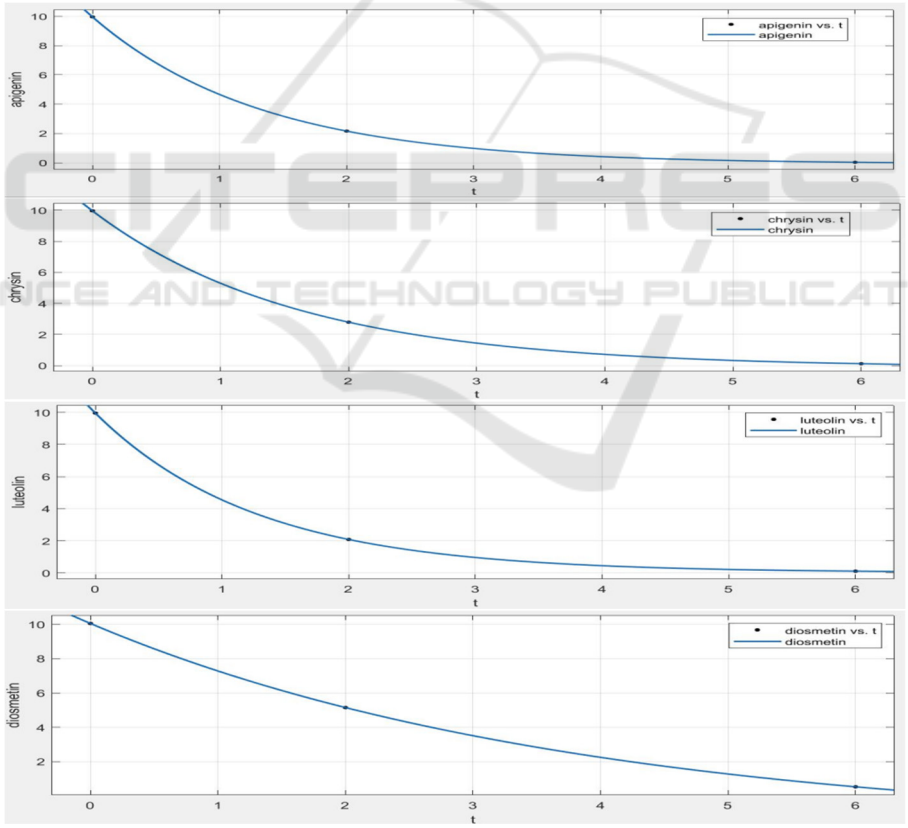

Figure 8: Line of best fit in exponential mode to represent the curves of degradation.

In Figure 9, though the degradation rate of diosmetin is much slower than the others during the first 2 hours of degradation, the rate increases and turns to be the fastest in the next four hours. Because Figure 9 shows that the lower the concentration of flavonoids, the faster the degradation rate, we can conclude that FLR enzyme should be suitable for degrading flavonoids in large quantities.

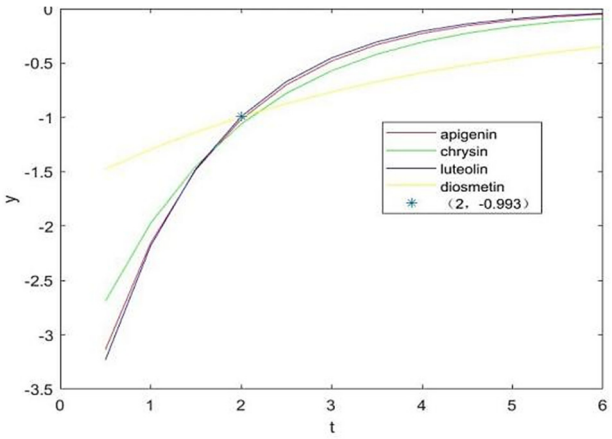

Figure 9: Hours vs. degradation rate graph for four tested flavonoids.

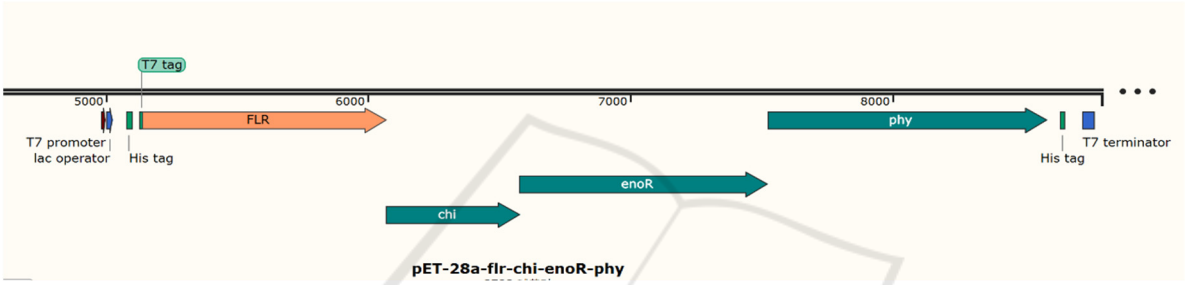

Figure 10: Illustration of pET28a-flr-chi-enoR-phy enzyme.

6 PROTEIN CONSTRUCTION

The target fragment for protein expression, BBa_K3998004, is built into the vector of pET28a. This fragment, transcribed by the T7 promoter and ended by the T7 terminator, contains a His tag for protein purification. Molecular biology experiments have been carried out by us, in which we successfully made the fragment in the vector of E. coli BL21.

Detailed descriptions of all component parts are listed:

6.1 BBa_K3998000

The flr gene, a 6275bp long gene that increases the rate of flavonoid degradation, is used to create a strain that secretes enzyme more efficiently. It can assist in better producing DAT, activate the immune system of the human body, and achieve the functions of flavonoids, including anti-inflammatory, anti-cancer, and antibacterial. Also, treatment costs would be largely reduced with the participation of this gene.

6.2 BBa_K3998001

Chi, a 510bp long gene used to improve the rate of flavonoid degradation, is used to create a strain capable of secreting enzyme more efficiently. With chi gene's function of converting dihydroflavone into chalcone, it can improve degradation rate, DAT production, and activate the human immune system to protect the human body from serious diseases like cancer, inflammation, and tumors. Improving immunity of the human body would also make clinical treatment cost less.

6.3 BBa_K3998002

EnoR, a 948bp long gene that increases the degradation rate of flavonoids, is used to create a strain that secretes enzyme more efficiently. It has almost the same function as the chi gene does. With the enoR gene's function of converting dihydroflavone into chalcone, it can improve degradation rate, DAT production, and activate the human immune system to protect the human body from serious diseases like cancer, inflammation, and tumors. Improving immunity of the human body would also make clinical treatment cost less.

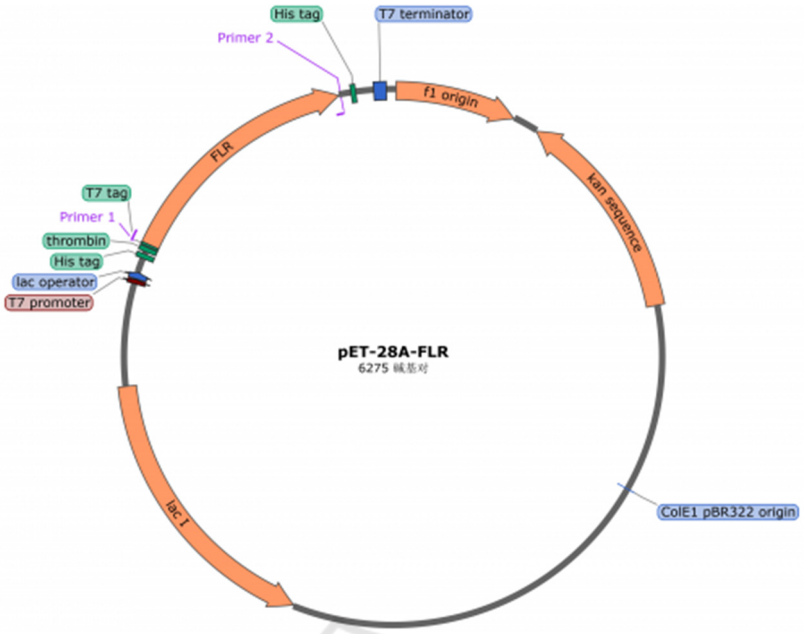

Figure 11: DNA map of plasmids expressing FLR.

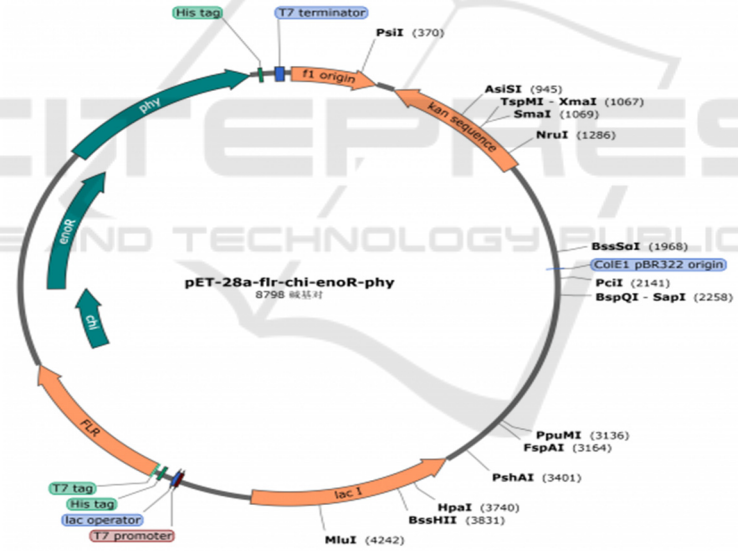

Figure 12: DNA map of pET28a and location of flr/chi/enoR/phy on plasmids.

6.4 BBa_K3998003

Phy, a 1068bp long gene that improves flavonoid absorption, is used to create a strain that secretes enzyme more efficiently. It has similar functions to the chi gene and the enoR gene. With phy gene's function of converting dihydroflavone into certain degradation products, it can improve degradation rate, DAT production, and activate the human immune system to protect the human body from serious diseases like cancer, inflammation, and tumors. Improving immunity of the human body would also make clinical treatment cost less.

7 EXPERIMENTAL APPROACH

During the experiment, we managed to build the plasmid and prove it with methods like polymerase chain reaction and gel electrophoresis.

The pET28a vector is already in the plasmid library, so we just needed to obtain it from there.

Acquisition of Inserts: introduce homologous sequences of pET28a vector into the 5'-end of forward and reverse primers, to make the ends identical to each other.

Table 2: Acquisition of inserts.

Primer	Sequence
Catalase-F-NheI:	atggctagcatgagttcaaataaactgacaact
Catalase-R-XhoI:	gtgctcgagttaagaatctttttaatcggcaa

Recombination: use formula to calculate the amount of DNA needed for recombination and diluted pET28a vector and inserts earlier to ensure the accuracy of loading.

Table 3: Recombination.

Components	Recombination
pET28a	107.3ng, convert to volume
flr/chi/enoR/phy	37.3-42.72ng, convert to volume
Buffer	4ul
Exnase II	2ul
ddH2O	To 20ul

8 CONCLUSION

In conclusion, by introducing the pET28a-flr-chi-enoR-phy into the E. coli BL21, we successfully increased the degradation rate of the medicine and food homologous flavonoids. As results showed that more than 95% of the tested flavonoids were degraded after 6 hours of time. In this case, the targeted anti-inflammatory, antibacterial, and anti-cancer functions of flavonoids would work as intended. When we were doing the experiment, the bacteria in the culture medium did not grow at a fast rate, which limited us to repeating it for 3 times. Possible errors that may be accidentally included in the data are not totally eliminated. In the future, since the FLR enzyme appears to have an incredible capacity to break down flavonoids, we are looking forward to testing how the human body would perform with and without the E. coli with pET28a-flr. What's more, in the event that the further experiment succeeds, we are going to likely attempt to include the FLR-built microscopic organisms into yogurt. Yogurt contains a huge number of lactic-corrosive microscopic organisms. By including E. coli with pET28a-flr, we may make a health-care yogurt to be consumed after meals, which is beneficial for human well-being advancement.

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