FLAVORATOR :New food preservation method by rose odor *E. coli*

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Abstract

Ideal food preservation is keeping food without causing quality change for longer time with cost effectively. We created a new one satisfying these criteria, a food-keeping box, called "Flavorator". *E. coli*, engineered to overproduce volatile flavors, geraniol and/or farnesol, to suppress unwanted microbial growth was used for the preservative for "Flavorator". Our strategy includes three steps. (1) Geraniol/farnesol precursors are overproduced by non-mevalonate (MEP) pathway, following superimposing four rate-limiting enzyme genes, *ispD*, *ispF*, *idi*, and *dxs*, in the *E. coli*. (2) Farnesyl diphosphate synthase gene (*ispA*) or its mutant (*m-ispA*, S80F) in combination with geraniol synthase gene from *Ocimum basilicum* (*ObGES*) were additionally introduced in the same *E. coli*. These gene-combinations are possible to convert IPP and DMAPP into to geraniol/farnesol, respectively. (3) An activator gene of AcrAB-TolC efflux pump (*marA*) was further additionally introduced in the same *E. coli* with increased tolerance to geraniol/farnesol by efficiently exporting them in the outer media. The results are as follows: (1) Ubiquinone 8, an end product of MEP pathway, was detected more in superimposed strain than the counterpart *E. coli*. (2) Farnesol was produced by *E. coli* engineered with farnesol production device (BBa_K1653025). (3) *E. coli* engineered with geraniol production device (BBa_K1653021), *ObGES*, showed different smell as compared with counterpart control (pSB1C3). (4) *E. coli* engineered with *marA* device (Bba_K1653020) showed exported intracellular geraniol. (5) *E. coli* engineered with *marA* device (BBa_K1653020) showed increased tolerance to the it as compared with the counterpart non-engineered strain.

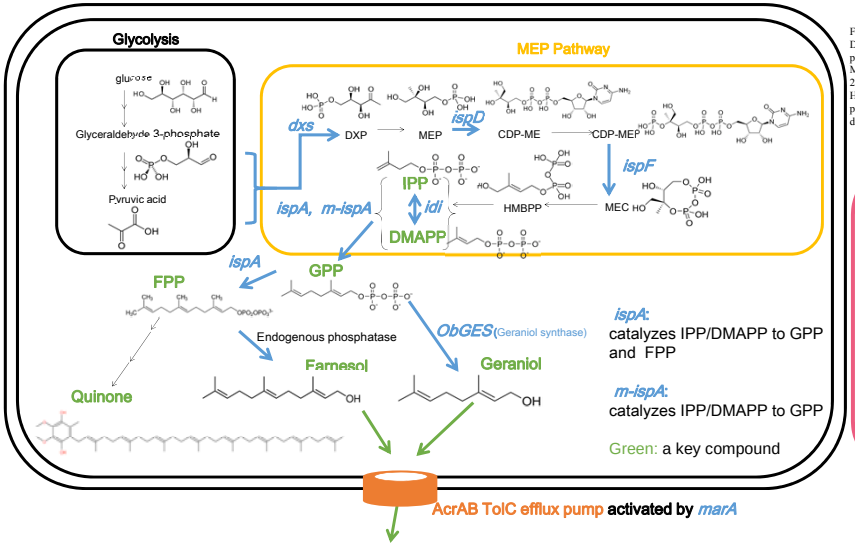

Fig. 1: Pathway of our design
DXP: 1-Deoxy-D-xylulose 5-phosphate. MEP: 2-C-methylerythritol 4-phosphate. CDP-ME: 4-diphosphocytidyl-2-C-methylerythritol. CDP-MEP: 4-diphosphocytidyl-2-C-methyl-D-erythritol 2-phosphate. MEC: 2-C-methyl-D-erythritol 2, 4-cyclodiphosphate. HMBPP: (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate. IPP: Isopentenyl pyrophosphate. DMAPP: Dimethylallyl pyrophosphate. GPP: geranyl diphosphate. FPP: farnesyl diphosphate.

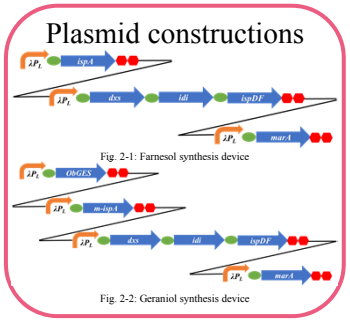

Fig. 2-1: Farnesol synthesis device

Fig. 2-2: Geraniol synthesis device

(2) Farnesol production

E. coli strain engineered with MEP pathway enzymes, *ispD*, *ispF*, *idi*, and *dxs* , in combination with the enzyme genes, *ispA*, produced farnesol (Fig. 10B), which was detected by the GC/MS (Fig. 10A-G), having the same retention time as the farnesol chemical sample (Fig. 10A), while the counterpart control *E. coli* did not produce farnesol under the same conditions (Fig. 10C). Neither *E. coli* engineered with MEP pathway enzymes only nor the one engineered *ispA* only showed any farnesol by the GC/MS (Figs. 10D and E). Farnesol is generated through hydrolysis of farnesyl diphosphate (FPP) by the endogenous phosphatases. Increase in farnesol should be associated with an increased intracellular FPP level. FPP is, in turn, converted from geranyl diphosphate (GPP), whose precursors are IPP and DMAPP. IPP and DMPP are end products of MEP pathway that exists in *E. coli*. Conversion to FPP from IPP or DMPP requires *ispA* (or *m-ispA*). Following this context, we speculate that *E. coli* could produce farnesol better than the counterpart control cells under the up-regulated cellular conditions of an increased intracellular MEP pathway enzymes by metabolic engineering in combination with the special enzyme that converts IPP or DMAPP into FPP.

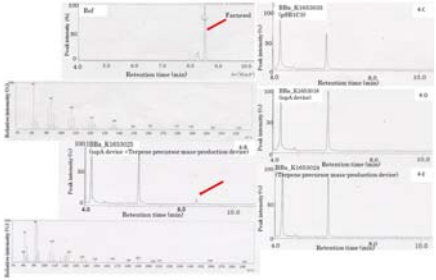

Fig. 10: The farnesol standard solution (Ref) was used as a control. The peak corresponding to the farnesol standard at 8.5 min is indicated by an arrow. The peak at 8.5 min was applied to GC/MS. The farnesol standard solution (Ref) was used as a control. *E. coli* JM109(BBa_K1653025) were compared with respect to farnesol formation using GC-MS.

(3) Geraniol production

Geraniol is generated through GPP hydrolysis by geraniol synthase. A MEP pathway has been shown to synthesize IPP and DMAPP efficiently in *E. coli*. *E. coli* engineered with Geraniol production device (BBa_K1653027) showed different smell as compared with counterpart control (pSB1C3) and WT. This result derived from questionnaire survey. And then we tried to detect that the geraniol generated by engineered E. coli by GC and GC-MS. However, the GOH were not detected. These result may indicate that *E. coli* with geraniol production device produce smaller amounts of than can be detected by GC and GC-MS.

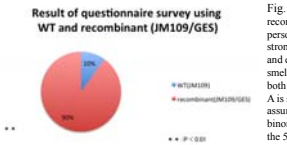

Fig. 11: Result of questionnaire survey using WT and recombinant (JM109/*GES*). Out of 20 persons, two persons (10%) answered the medium A (WT) smelled stronger than the medium B (recombinant (JM109/*GES*)) and eighteen persons (90%) answered the medium B smelled stronger than the medium A. If we assume that both media smell equally, the probability that the medium A is selected in the questionnaire must be 0.5. From this assumption, p-value of this result was calculated using binomial test. Because the p-value was much smaller than the 5% significance level (0.0004025), the smell of recombinant (JM109/*GES*) is stronger than that of WT significantly. This result indicate that the recombinant (JM109/*GES*) synthesize geraniol.

F. 12: Questionnaire survey of fragrance of geraniol A: WT or recombinant (JM109/pSB1C3) B: recombinant (JM109/BBa_K1653027) experimental cooperation persons: 20 persons Experiment smelling the smell of A and B Experiment collaborators chose a stronger smell by comparing the A and B.

Confirmation antibacterial activity of each volatile substances derived from plant

First, we examined our working hypothesis to "Flavorator" that the volatile gaseous substances from plants' origin can show either the antibacterial or bacteriostatic activity in a box like "KOZOKO". The results clearly showed that all the volatile substances of Wasabi (Japanese horse radish), rose, garlic and onion had antibacterial properties.
In the literatures, wasabi, rose, garlic and onion have antibacterial volatiles, such as allyl isothiocyanate (Wasabi), geraniol (rose), allicin (garlic), and lachrymatory-factor (onion)

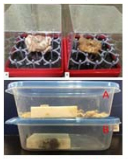

Fig. 3:Left figure (A): the grated garlic paste was put in a small box and it was put in a plastic cage with the separation from the pork meat without direct contact. Right figure (B): the above treatment was not done. These pork meats in a plastic cage were left in the box at 18℃ for 2 months

Fig. 4: The grated Wasabi root paste was put aside of rice cake in a plastic box without direct contact between rice cake and Wasabi root paste. These boxes were kept at 18℃ for 2 month.

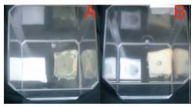

Fig. 5 : Storage of English bread by geraniol. A: ddH2O (300 µl), B: geraniol (300 µl of geraniol solution was spotted on the cotton). Chopsticks dipped in suspension of mold was put in the center of the bread. These boxes were kept for 35 days at room temperature.

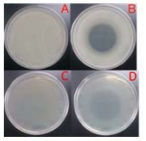

Fig. 6 : Effect of geraniol for growth of Bacillus subtilis ver. Natto (Chassie) (A and B) and *E. coli* (C and D). A, ddH2O (300 µl) ; B, geraniol (300 µl) . Geraniol was put in the center of the paper of the plate cover without direct contact with the bacteria. The dishes were incubated for 21 hours at 37 ℃.

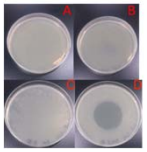

Fig. 7 : Effect of farnesol for growth of *E. coli* (A and B). A, ddH2O (300 µl) ; B, farnesol (300 µl) and Bacillus subtilis ver. Natto (Chassie) (C and D). Farnesol was put in the center of the paper of the plate cover without direct contact with the bacteria. The dishes were incubated 21 hours at 37 ℃.

(1) Increasing in the amount of terpene's precursors

We want to make the *E. coli* produces farnesol and geraniol which are one of the terpenes. To produce great quantity of terpenes they need many terpene precursor. *E. coli* produces a small amount of the terpene precursor in MEP pathway. In MEP pathway, there are four enzymes (*ispD*, *ispF*, *idi*, *dxs*) which are speed limiting enzyme for terpenes precursors produce in *E. coli*. In order to create a high-yield strains producing IPP and DMAPP, we exogenously engineer to superimpose these genes into *E. coli* to create strains overproducing IPP and DMAPP in a MEP pathway. To confirm increased production of terpene precursors by Terpene precursor mass-production device. We put attention on ubiquinone. Ubiquinone 8 is made from Farnesyl diphosphate (FPP) which is one of the terpene precursors. quinone is one of the electron carrier present in the cell membrane of prokaryotes. And also they glow when exposed to UV rays. In the measurement of production of quinone it was measured by thin-layer chromatography. (TLC silica gel)

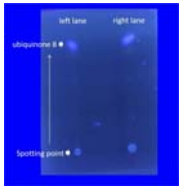

Fig. 8: Analysis of Ubiquinone-8 synthesized by *E. coli* JM109/BBa_K1653025 (Terpene precursor production device) by TLC. Right lane: IPTG plus, Left lane: IPTG minus

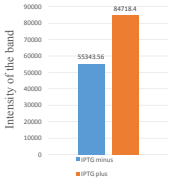

Fig. 9: Ubiquinone-8 content in spots
Each intensity of spot was measured indicating the content of Ubiquinone-8

(4) Export of gernaiol

We introduced an activator gene of AcrAB-TolC efflux pump (*marA*) to release the geraniol from the cells and to increase the content in the media that shows increase these flavors in the "Flavolator". In our study, we confirmed that overexpressing of *marA* gives host *E. coli* strain high resistance against geraniol and reduced intracellular geraniol concentration.

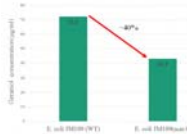

Fig. 13: Intracellular geraniol concentration of *E. coli* JM109 and its overexpressing of *marA* strain
The intracellular geraniol concentration of *E. coli* JM109(*marA*) was observed at 42.9 µg/ml, which was 40% lower than 72.2 µg/ml of *E. coli* JM109 (WT).

(5) Enhancement of geraniol resistance

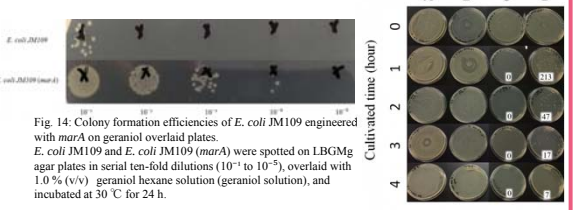

Fig. 14: Colony formation efficiencies of *E. coli* JM109 engineered with *marA* on geraniol overlaid plates.
E. coli JM109 and *E. coli* JM109 (*marA*) were spotted on LBGMg agar plates in serial ten-fold dilutions (10^{-1} to 10^{-5}), overlaid with 1.0 % (v/v) geraniol hexane solution (geraniol solution), and incubated at 30 ℃ for 24 h.

Fig. 15: Comparison of colony numbers after addition of 0.5 % (v/v) geraniol hexane solution (geraniol solution).
Time interval for treatment was set every 1 hour from 1 hour to 4 hours. A: *E. coli* JM109 (WT) + hexane; B: *E. coli* JM109 (*marA*) + hexane; C: *E. coli* JM109 (WT) + 0.5 % geraniol solution; D: *E. coli* JM109 (*marA*) + 0.5 % geraniol solution. As shown in Figs. 14 A and B, treatment with hexane of *E. coli* JM109 (WT) and of *E. coli* JM109 (*marA*) showed similar colony numbers during these treatment intervals to those of time zero.

Innovation HP: Cloud founding

In general, to the public audience or expert sectors, we explain our project, iGEM activities, and the topics of synthetic biology. After explanation, we discussed with all participants and often did a survey by questionnaire. Other team often uses the questionnaire, too. Our innovation is quite unique. The idea was to set "Cloud funding". Our milestone for our budget from funding was set up to 200,000 yen. Fortunately, we got the amount summed up to 310, 200 yen. This achievement clearly shows that we had fund-raisers more than we had expected. We had public consensus that our project is worth supporting. This type of activity seems unique in terms of not only the amount of raised fund, but also realization that so many public audiences support us. In Japan, only Nagahama team was successful in setting up " Cloud funding" and achieving the milestone

Fig. 16: Our team's page of academist site

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