Steviol Synthesis in Yeast

a student project

Stevio myces iGEM 12 / 13 it's gonna be sweet

An iGEM project on alternative sweeteners

Several diseases are thought to be connected with an increased sugar consumption. The use of non-sugar sweeteners could be a possible approach for both reducing the risk of sickening and a proper ingestion without high sugar intakes.
Several of the known problems with common sweeteners could be overcome by diterpene glycosides which are produced by the paraguayan herb *Stevia rebaudiana*.
One possibility to improved sweetening products based on steviol glycosides is the microbial production of the compound Rebaudioside A which could also lower the enviromental costs of sweetener production. We want to investigate the possibillty of such a microbial sweetener production in the baker yeast *Saccharomyces cerevisiae*.

Important advantages of steviol glycosides are that there is no effect on blood sugar level, no delay of satiety, almost no calories, pH and heat stability.

Need an ER? Go Yeast!

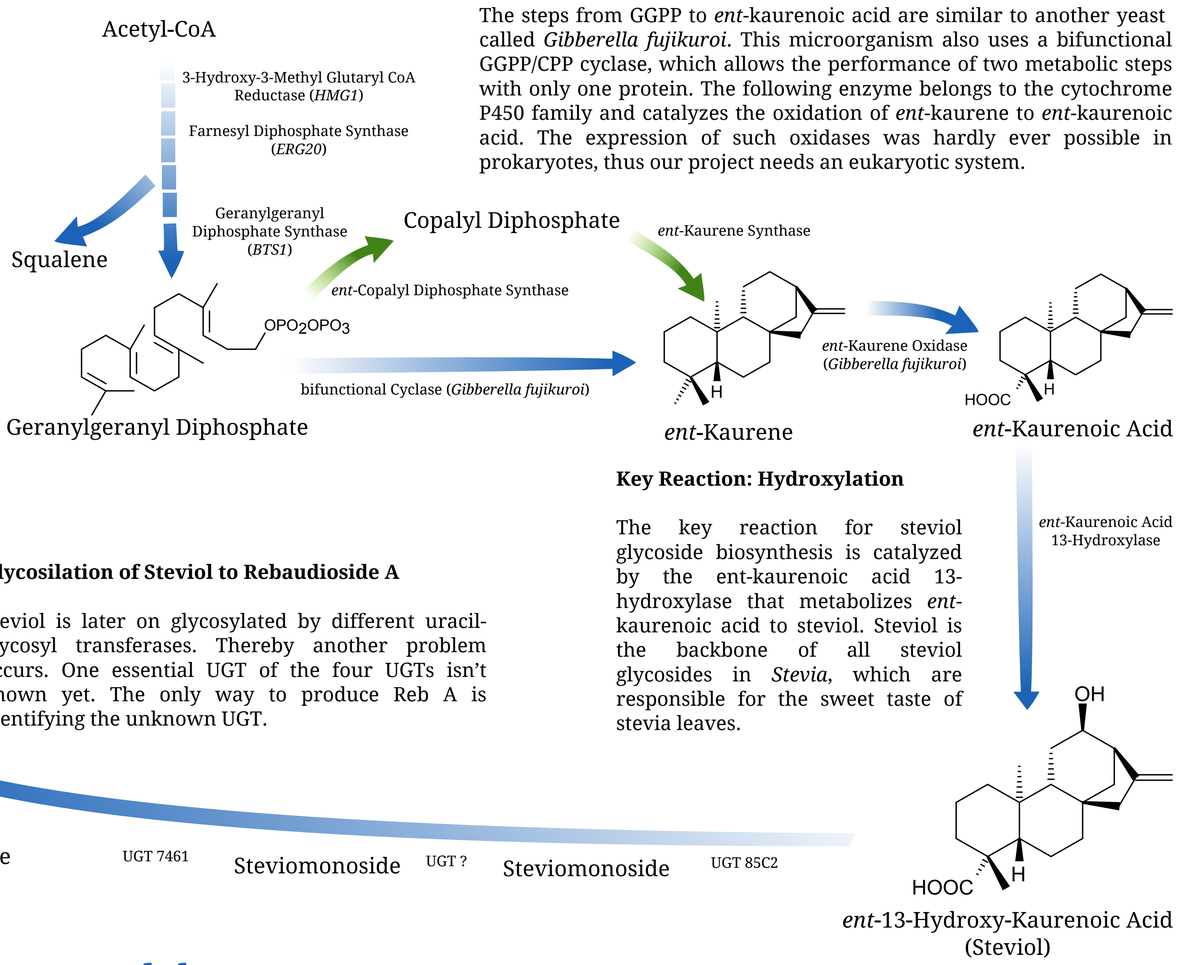
Yeast is an eukaryotic unicellular organism. In addition to this it is a popular model organism and in our case it is the yeast *Saccharomyces cerevisiae*. But E.coli is also a popular model organism and even grows faster than yeast. Moreover there are more biobricks available for *E.coli*. So why do we use yeast? For the simple reason that we need an endoplasmic reticulum to produce steviol, because the ent-kaurene oxidase and the ent-kaurenoic acid hydroxylase are localized in the ER. In addition yeast has got further advantages. It is an established food additive producer and it is closer related to Stevia.

Furthermore we utilize the yeast's ability for homologous recombination. Yeast only needs about 40 bp of a homologous area for recombination. That means that DNA sequences can be integrated into another DNA molecule if the DNA sequence has got homologous areas to the target DNA. Our aim was to assemble a mevalonate overexpression plasmid and a steviol synthesis plasmid via homologous recombination, which is called gap repair, in *S.cerevisiae*.

Acetyl-CoA to Rebaudioside A

Mevalonate Pathway

The mevalonate pathway of *Saccharomyces cerevisiae* uses acetyl-CoA for production of isoprenyl diphosphate, which serves as a basis for biosynthesis of steroids and other isoprenoids. The first important metabolite of the mevalonate pathway for steviol glycoside synthesis in *Stevia* is geranylgeranyl diphosphate (GGPP). GGPP is immediately abstracted by the squalene synthase. For a higher concentration of GGPP in *S. cerevisiae* the squalene synthase can either be inhibited or an overexpression of the key enzymes, GGPP synthase, farnesyl diphosphate synthase and 3-hydroxy-3-methyl glutaryl CoA reductase can be used.



Gibberellin Pathway

The steps from GGPP to *ent*-kaurenoic acid are similar to another yeast called *Gibberella fujikuroi*. This microorganism also uses a bifunctional GGPP/CPP cyclase, which allows the performance of two metabolic steps with only one protein. The following enzyme belongs to the cytochrome P450 family and catalyzes the oxidation of *ent*-kaurene to *ent*-kaurenoic acid. The expression of such oxidases was hardly ever possible in prokaryotes, thus our project needs an eukaryotic system.

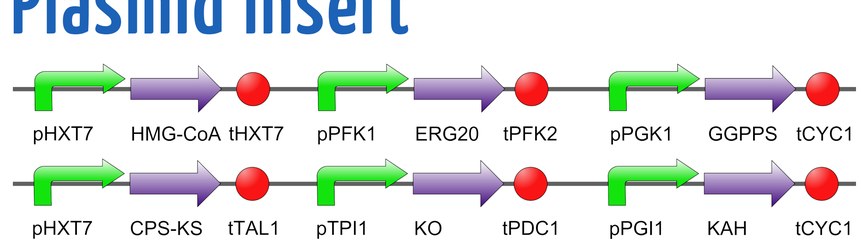
Key Reaction: Hydroxylation

The key reaction for steviol glycoside biosynthesis is catalyzed by the ent-kaurenoic acid 13-hydroxylase that metabolizes *ent*-kaurenoic acid to steviol. Steviol is the backbone of all steviol glycosides in *Stevia*, which are responsible for the sweet taste of stevia leaves.

Glycosilation of Steviol to Rebaudioside A

Steviol is later on glycosylated by different uracil-glycosyl transferases. Thereby another problem occurs. One essential UGT of the four UGTs isn't known yet. The only way to produce Reb A is identifying the unknown UGT.

Plasmid Insert

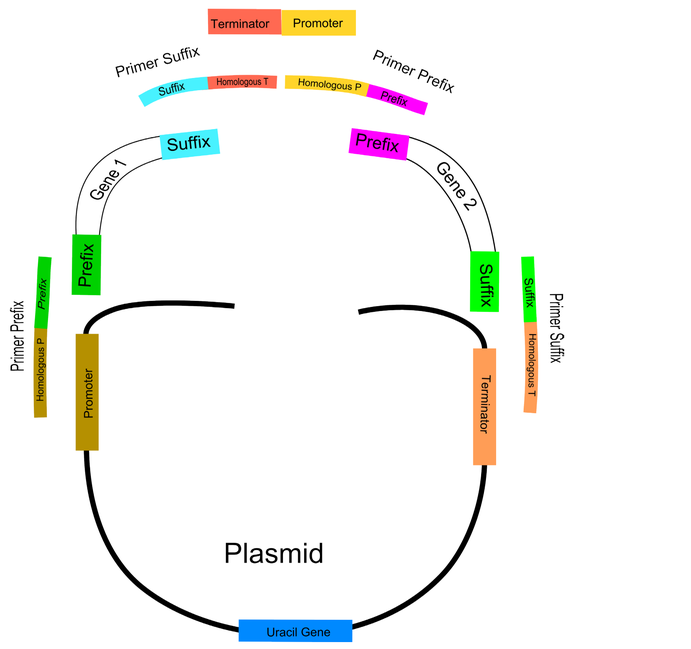


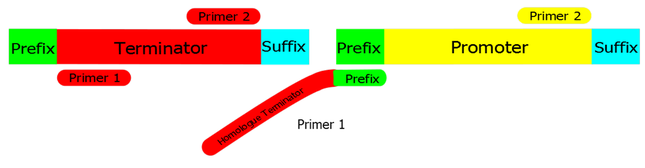
Results

- ✔ sponsoring, labspace, wiki, poster
- ✔ transform yeast with mevalonat plasmid
- ✔ new yeast biobrick assembly (RFC proposal)
- ✔ human practice: night of science, bioethics
- ✔ BioBricks: HMG-CoA, ERG20, GGPPS, CPS-KS, KO
- ✖ proof of GGPP increase via GC-MS
- ✖ transform yeast with steviol plasmid

Yeast BioBrick Assembly

The idea of YBA (new BioBrick RFC) is to design a new standard for the assembly of cloning vectors based on standardized PCR primers. The primers are used to create BioBricks which are suitable for gap repair cloning. We want to make yeast terminator/promoter-fragments available that can be used to assemble a plasmid with several genes via gap repair.
For this we constructed two standard primers for every BioBrick gene, which anneal at the prefix or the suffix and have homologue overlaps to the respective promoter or terminator. So you can amplify every coding sequence BioBrick with homologous overlaps to the terminator/promoter-fragment. Therefore gap repair cloning can be realized very simple.
(Figure right upper)
To allow more possibilities of combining terminators and promoters we constructed primers that bind at the prefix of a BioBrick promoter and have got a homologous overlap to a terminator. Thereby every promoter and terminator can be combined.
(Figure right lower)
Furthermore it is also possible to assemble genes that should be expressed in E.coli via gap repair cloning in yeast. Certainly you need a shuttle plasmid and an E.coli promoter and terminator. Because of this method you are able to amplify the pure gene without any BioBrick endings which might be important for some protein expressions.





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