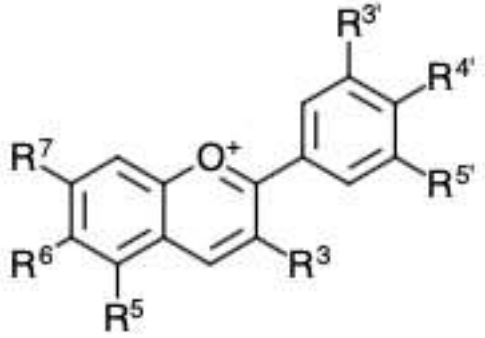
in vivo (typically through protein overexpression in hosts such as bacteria), isolated via chromatography, and then added into a bioreactor with a substrate (starting material). The enzymes transform the substrate in the same way that occurs in plants but without the complexity of the organism. In this way, natural products can begin to be created without the plant, cell, or chemical synthesis.

BRIEF SUMMARY

It is an objective of the present disclosure to provide a cell-free method that allows for the production of anthocyanidins (cyanidin glycosides, such as cyanidin-3-glycosides, cyanidin-5-glycosides, etc.) starting from anthocyanidins via a cell-free biosynthesis platform. Examples of the present disclosure can be freely combined with each other if they are not mutually exclusive. In one aspect, the present disclosure provides a method of producing an anthocyanin from an anthocyanidin, the method comprising:

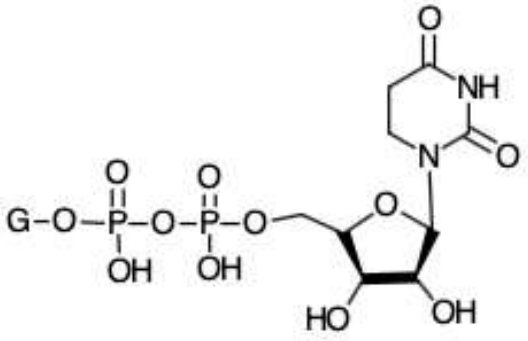
a) adding to a mixture comprising a suitable solvent (i) an anthocyanidin of Formula I:



Formula I

wherein each of R^3, R^5, R^6, R^7, $R^{3'}$, $R^{4'}$, and $R^{5'}$ are independently selected from the group consisting of H, OH, and OCH_3, wherein at least one of R^3, R^5, R^6, R^7, $R^{3'}$, $R^{4'}$, and $R^{5'}$ is OH or CH_3 and at least one of R^3, R^5, R^6, R^7, $R^{3'}$, $R^{4'}$, and $R^{5'}$ is OH;

(ii) a glycosylated uridine-5'-diphosphate of Formula II:



Formula II

wherein G is a glycosyl residue selected from the group consisting of 5- and 6-

membered monosaccharides; and

(iii) a glycosyltransferase enzyme to form a reaction mixture;

b) removing the supernatant from the reaction mixture from (a); and

c) isolating the anthocyanin.

In another aspect, the present disclosure provides a method of producing an anthocyanin from an anthocyanidin, the method comprising:

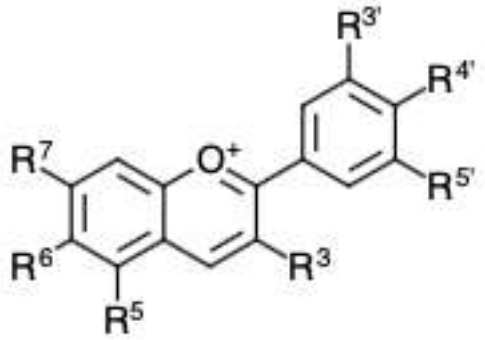
c) in a cell-free vessel, providing a glycosyltransferase enzyme;

d) adding an anthocyanidin and a glycosylated uridine diphosphate to the cell-free vessel to form the anthocyanin; and

c) removing the anthocyanin from the cell-free vessel.

In yet another aspect, the present disclosure provides a method of producing an anthocyanin from an anthocyanidin, the method comprising:

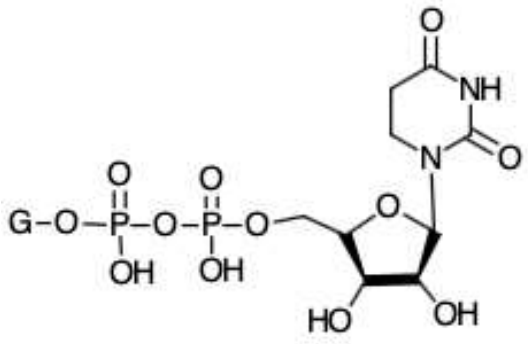
e) adding an anthocyanidin or anthocyanin of Formula I:



Formula I

wherein each of R^3, R^5, R^6, R^7, $R^{3'}$, $R^{4'}$, and $R^{5'}$ are independently selected from the group consisting of H, OH, OCH_3, or a sugar, wherein at least one of R^3, R^5, R^6, R^7, $R^{3'}$, $R^{4'}$, and $R^{5'}$ is OH;

a glycosylated uridine-5'-diphosphate of Formula II:



Formula II

wherein G is a glycosyl residue selected from the group consisting of 5- and 6-membered monosaccharides; and

a glycosyltransferase enzyme to form a reaction mixture;

f) removing the supernatant from the reaction mixture from (a); and

c) isolating the anthocyanin.

The present disclosure uses glycotransferase enzymes to convert anthocyanidins to several possible anthocyanidin glycosides, such as cyanidin-3-glycosides, depending on the glycosyl donor.

The presently claimed process uses a controlled enzymatic step to increase product titer in short reaction times. In some examples, the controlled enzymatic step may increase product titer by a factor of at least five. Additionally, enzyme immobilization may be used to enhance anthocyanidin conversion to corresponding anthocyanins. For example, enzyme immobilization was used to improve cyanidin conversion to cyanidin-3-glucoside (C3G) by avoiding enzyme precipitation, inactivation, and the unreliability of the non-immobilized system.

A unique and inventive technical feature of the present disclosure is the use of a cell-free system for the production of anthocyanins (anthocyanidin glycosides), such as cyanidin-3-glycoside. Without wishing to limit the disclosure to any theory or mechanism, it is believed that the technical feature of the present disclosure advantageously provides for higher reaction concentrations of starting materials, reagents, and enzymes, resulting in higher concentrations of final products. Additionally, the present disclosure eliminates the complications of cell walls, thereby eliminating a barrier to product and substrate diffusion. Furthermore, the present disclosure eliminates competition for carbon flux, which limits the efficiency of cell-based synthesis methods, and thus greatly reduces byproduct formation. Also, because there is no cell, the present disclosure is not vulnerable to degradation by cell death due to the formation of toxic compounds. The adaptability of the system to accommodate different starting anthocyanidins and glycosylated UDPs (UDP-sugars) lends the system flexibility to produce a multitude of products and the ability to use various solvents, such as organic solvents, to permit higher concentrations of solutes without

worrying about killing the cell.

The present disclosure is adaptable to various products. In this approach, one simply changes the anthocyanidin, UDP-sugar, and UGT enzyme in the pathway to synthesize the desired anthocyanin product.

Furthermore, prior references teach away from the present disclosure. The maximum titers achievable for current cell-based production of anthocyanins are limited. Previously published work involving extensive cellular reprogramming and metabolic engineering produce anthocyanins such as cyanidin-3-glucoside on the scale of 0-200 mg/L. Although there have been reports of microbial hosts producing titers approaching 300-400 mg/L, these experiments are either not technically reproducible (see, e.g., Shrestha, 2019), or contain data that is not calculated to a standard set forth in the field (Yan, 2008). The present disclosure affords a C3G production titer of at least 1.5 g/L, representing up to a 50-fold increase from reported values.

Furthermore, the inventive technical features of the present disclosure contributed to a surprising result that would not have been predicted from the cell-based synthesis literature. For example, the stability of the starting material cyanidin is dependent on the pH of the solution it is contained in, with the highest stability at pH < 5.0. These low pH values are not compatible with cell-based production, as the optimum pH for cells is 7. The present disclosure is completely functional at a range of pH values (4.0 – 9.0). In some examples, it is preferred to carry out the reaction at a pH at or near the anthocyanidin's pH of maximum stability, for example, at or near pH 5 (e.g., 4.0-6.0, 4.2-5.8, 4.3-5.7, 4.4-5.6, 4.5-5.5, 4.6-5.4, 4.7-5.3, 4.8-5.2, or 4.9-5.1), in order to maximize the stability of the anthocyanidin.

Additionally, the solubility of the starting material anthocyanidin may be enhanced by using a solvent, such as an organic solvent, which the present disclosure permits. For example, the solubility of cyanidin is only 49 mg/L in water, and it will degrade within minutes at this concentration. Addition of a co-solvents such as dimethylformamide (DMF), dimethylsulfoxide (DMSO), methanol (MeOH), or ethanol (EtOH) (or mixtures of two or more thereof) greatly improves the solubility and stability of cyanidin for the reactions. Some methods according to the present disclosure can operate at co-solvent levels >60% -- at least 12 times higher than cell-based anthocyanin production, as living cells are sensitive to most co-solvent concentrations above 5%.

The inventive technical features of the present disclosure contributed to a further unexpected result of permitting high concentrations of UDP-sugar in the reaction mixture. For example, the conversion of cyanidin to C3G requires a UDP-conjugated sugar donor molecule such as UDP-Glucose. The UDP-glucotransferase enzyme has low affinity for UDP-Glucose and the reactions

are dependent on large molar excess of this molecule. The level of UDP-Glucose in the present disclosure is controllable and can vary from 1 mM - 30 mM (0.5-17 g/L), compared to cell-based production which requires substantial cell engineering to reach UDP-Glucose levels approaching 2 g/L (Feng et al 2020).

Any feature or combination of features described herein are included within the scope of the present disclosure provided that the features included in any such combination are not mutually inconsistent as will be apparent from the context, this specification, and the knowledge of one of ordinary skill in the art. For example, while certain anthocyanins are presented as products of certain anthocyanidins, one skilled in the art will recognize that the methods described herein can be generalized to produce a wide range of anthocyanins from a range of anthocyanidins. Additional advantages and examples of the present disclosure are apparent in the following detailed description and claims.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

The features and advantages of the present invention will become apparent from a consideration of the following detailed description presented in connection with the accompanying drawings in which:

FIG. 1A shows the structures of common anthocyanidins. The structure on the left is the anthocyanidin core structure and the table on the right shows the “Rn” substituents at the indicated positions of the anthocyanidin core structure.

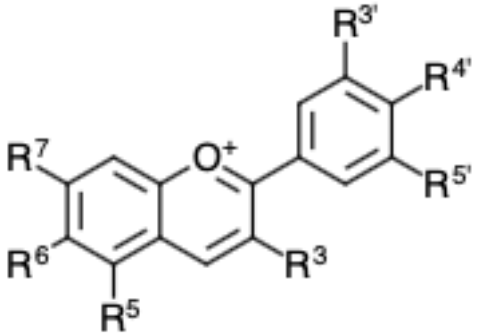
FIG. 1B shows the chemical transformation pathway for the production of an anthocyanin (e.g., cyanidin-3-O-glucoside, a.k.a. C3G) from an anthocyanidin (e.g., cyanidin) and a UDP-sugar (e.g., UDP-glucose).

FIGs. 2A-2C Shows HPLC traces for cyanidin, cyanidin-3-glycoside standards (top, FIG. 2A), non-immobilized enzyme reactions (middle, FIG. 2B), and immobilized enzyme reactions (bottom, FIG. 2C). The retention times of 6.95 minutes (C3G) and 8.9 minutes (cyanidin) at 530nm are noted.

FIGs. 3A-3B show the schematic diagram of the continuous reactor (top, FIG. 3A), and HPLC traces for molecule standards as well as cyanidin-3-glycoside produced in the continuous reactor (bottom, FIG. 3B). The retention times of 6.95 minutes (C3G) and 8.9 minutes (cyanidin) at 530 nm are noted.

CLAIMS

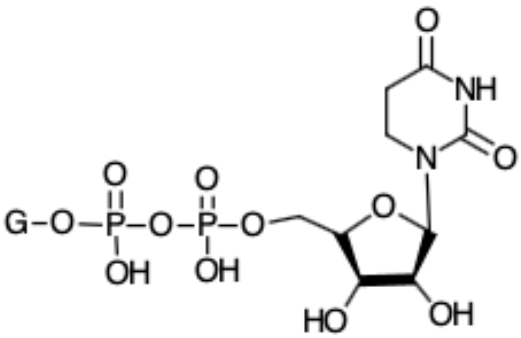
1. A method of producing an anthocyanin from an anthocyanidin, the method comprising:
    a) adding to a mixture comprising a suitable solvent (i) an anthocyanidin of Formula I:



Formula I

wherein each of R^3, R^5, R^6, R^7, $R^{3'}$, $R^{4'}$, and $R^{5'}$ are independently selected from the group consisting of H, OH, and OCH_3, wherein at least one of R^3, R^5, R^6, R^7, $R^{3'}$, $R^{4'}$, and $R^{5'}$ is OH or CH_3 and at least one of R^3, R^5, R^6, R^7, $R^{3'}$, $R^{4'}$, and $R^{5'}$ is OH;

(ii) a glycosylated uridine-5'-diphosphate of Formula II:



Formula II

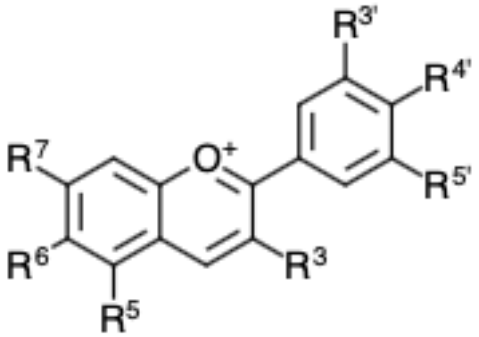
wherein G is a glycosyl residue selected from the group consisting of 5- and 6-membered monosaccharides; and

(iii) a glycosyltransferase enzyme to form a reaction mixture;

    b) removing the supernatant from the reaction mixture from (a); and
    c) isolating the anthocyanin.

2. A method of producing an anthocyanin from an anthocyanidin, the method comprising:
    a) in a cell-free vessel, providing a glycosyltransferase enzyme;
    b) adding an anthocyanidin and a glycosylated uridine diphosphate to the cell-free vessel to form the anthocyanin; and
    c) removing the anthocyanin from the cell-free vessel.

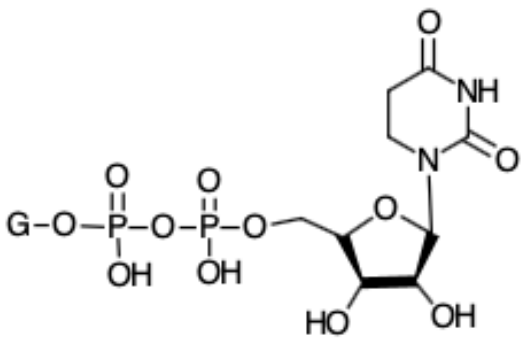
3. A method of producing an anthocyanin from an anthocyanidin, the method comprising:
    a) adding an anthocyanidin or anthocyanin of Formula I:



Formula I

wherein each of R^3, R^5, R^6, R^7, $R^{3'}$, $R^{4'}$, and $R^{5'}$ are independently selected from the group consisting of H, OH, OCH_3, or a sugar, wherein at least one of R^3, R^5, R^6, R^7, $R^{3'}$, $R^{4'}$, and $R^{5'}$ is OH;

a glycosylated uridine-5'-diphosphate of Formula II:



Formula II

wherein G is a glycosyl residue selected from the group consisting of 5- and 6-membered monosaccharides; and

a glycosyltransferase enzyme to form a reaction mixture;

    b) removing the supernatant from the reaction mixture from (a); and
    c) isolating the anthocyanin.

4. The method of any one of claims 1 to 3, wherein the anthocyanidin is selected from the group consisting of cyanidin, delphinidin, malvidin, pelargonidin, peonidin, petunidin, a glycosylated cyanidin, a glycosylated delphinidin, a glycosylated malvidin, a glycosylated pelargonidin, and a glycosylated peonidin.
5. The method of any one of claims 1 to 4, wherein the anthocyanidin is selected from the group consisting of cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin.
6. The method of any one of claims 1, 3, or 4, wherein G is selected from the group

consisting of glucose, galactose, xylose, arabinose, and rhamnose.

7. The method of any one of claims 1 to 6, wherein the anthocyanidin is selected from the group consisting of cyanidin, delphinidin, malvidin, pelargonidin, peonidin, petunidin, a glycosylated cyanidin, a glycosylated delphinidin, a glycosylated malvidin, a glycosylated pelargonidin, and a glycosylated peonidin.

8. The method of claim 7, wherein the anthocyanidin is selected from a mono-, di-, tri-, tetra- or penta-glycosylated cyanidin, a mono-, di-, tri-, tetra- or penta-glycosylated delphinidin, a mono-, di-, tri-, tetra- or penta-glycosylated malvidin, a mono-, di-, tri-, tetra- or penta-glycosylated pelargonidin, and a mono-, di-, tri-, tetra- or penta-glycosylated peonidin.

9. The method of any one of claims 1 to 8, wherein the glycosylated uridine diphosphate has an initial concentration of 1 mM to 30 mM in the reaction mixture.

10. The method of any one of claims 1 to 9, wherein the glycosyltransferase enzyme is a 3-O-glycosyltransferase or 5-O-glycosyltransferase.

11. The method of any one of claims 1 to 9, wherein the glycosyltransferase enzyme is a member of the group consisting of 3-O-glucoside-6"-O-rhamnosyltransferase (3RT), 3-O-glucoside-2"-O-glucotransferase (3GGT), 3'-O-glycosyltransferase, or 7-O-glycosyltransferase.

12. The method of any one of claims 1 to 11, wherein the sequence for the glycosyltransferase enzymes is one of SEQ ID NO 1-5, or an amino acid sequence with at least 90%, at least 92.5%, at least 95%, at least 97.5%, at least 99%, at least 99.5%, or at least 99.9% similarity to SEQ ID NO 1-5.

13. The method of any one of claims 1 to 12, wherein the reaction mixture has a starting pH, wherein the starting pH is 4 to 6.

14. The method of any one of claims 1 to 12, wherein the glycosyltransferase enzyme is non-immobilized.

15. The method of any one of claims 1 to 12, wherein the glycosyltransferase enzyme is immobilized.

16. The method of claims 14 and 15, wherein the glycosyltransferase enzyme is housed in a continuous reactor system.

17. The method of any one of claims 1 to 16, wherein R^3 is OH, and the glycosyltransferase enzyme is 3-O-glycotransferase (3GT).

18. The method any one of claims 1 to 16, wherein R^5 is OH, and the glycosyltransferase enzyme is 5-O-glycotransferase (5GT).

19. The method of any one of claims 1 to 18, wherein the glycosylated uridine-5'-diphosphate is selected from the group consisting of UDP-arabinose, UDP-galactose, UDP-glucose, UDP-rhamnose, and UDP-xylose.