

Cell-Free Productions of Anthocyanins:

I. Field of Invention:

5 The invention is related to materials and methods for production of anthocyanins. The invention provides methods and materials for cell-free production of one or more anthocyanins.

II. Reference to Sequence Listing

 The present application is being filed along with a Sequence Listing in electronic format. The Sequence Listing is provided as a file named DEBU-021-01WO.xml, created on June 28,
10 2024, which is 69 kilobytes in size. The information in the electronic format of the Sequence Listing is incorporated herein by reference in its entirety.

III. Background

 In the food coloring and pigment industry, there is a need for natural pigments and anthocyanins are highly sought after compounds. These pigments range in color from red to purple
15 to blue depending on molecular functional groups and pH, and are in demand for not only their color but also for their health promoting properties. These molecules are responsible for a variety of plant colors and also possess numerous beneficial attributes that range from anti-cancer therapies to helping advance solar energy technology.

 Common sources of anthocyanins used commercially as natural food colorants are grape
20 skin extract, red cabbage, purple carrot, and elderberry. They are natural pH indicators, being red in acidic conditions where they are most stable and becoming bluer and more unstable as the pH increases. Thus, a source of stable natural blue color that can be used in the food and beverage industry remains to be identified.

IV. Summary of the Invention

25 Anthocyanins are currently produced by chopping or crushing the fruit or vegetable and subsequent infusion of water acidified with a common food acid. This extract is then concentrated by non-chemical separation techniques. Pigment extracts from plant sources generally contain mixtures of different anthocyanin molecules, which vary by their level of hydroxylation, methylation and acylation. This increases the cost of production because of the purification
30 required from these mixtures. Moreover, these factors can vary in the source plant from year to

year, and are influenced by weather and environmental factors. Thus, another challenge for the commercial production of anthocyanin pigments from plants is that harvest is often limited to once a year. This means a large volume of extract has to be prepared and stored for an extended period of time to supply the needs of the food industry throughout the year. Special storage conditions
5 often have to be available due to the instability of anthocyanins.

Thus, new methods for production of anthocyanins are required. The invention provides methods for cell-free production of anthocyanins. The methods of the invention comprise: providing one or more enzymes in a cell-free medium, wherein the one or more enzymes result in transformation of a substrate to anthocyanins. In certain aspects of the invention, the methods of
10 the invention may provide for production of one or more intermediates during the transformation of one or more substrates to anthocyanin. In certain aspects of the invention, the anthocyanin is cyanidin-3-glucoside. In certain embodiments, the substrate is a catechin. In certain embodiments, the catechin is (+)-catechin. In certain embodiments, the one or more enzymes are selected from a group consisting of: anthocyanidin synthase enzyme (ANS) and anthocyanidin 3-O-
15 glucosyltransferase (3GT).

In certain embodiments of the invention, the anthocyanidin synthase (ANS) enzyme catalyzes the conversion of catechin to cyanidin. In certain embodiments, the anthocyanidin 3-O-glucosyltransferase (3GT) enzyme catalyzes the conversion of cyanidin to cyanidin-3-glucoside. In certain preferred embodiments, the cyanidin produced by transformation of catechin by
20 anthocyanidin synthase (ANS) enzyme is further transformed to cyanidin-3-glucoside by anthocyanidin 3-O-glucosyltransferase (3GT) enzyme. An overview of the conversion of catechin to cyanidin-3-glucoside is provided in **FIG. 1**.

In certain embodiments, the cell-free production of anthocyanins involves a cell-free medium. In certain embodiments, the cell-free medium comprises a cell lysate. In certain
25 embodiments, the cell-free medium comprises an activated sugar. In certain embodiments, the activated sugar is UDP-glucose. In certain embodiments, the UDP-glucose is added to the cell-free medium. In certain other embodiments, the UDP-glucose is synthesized in the cell-free medium by the one or more enzymes. In certain embodiments, UDP-glucose is synthesized from sucrose. In certain embodiments, sucrose synthase (SuSy) enzyme transforms sucrose to UDP-glucose. An

overview of the conversion of cyanidin and cyanidin-3-glucoside, wherein the UDP-glucose is synthesized from sucrose is provided in **FIG. 2**.

In certain beneficial aspects, the invention provides that the UDP-glucose used in the reaction is generated from other substrates in course of the reaction. In certain embodiments, the UDP moiety in UDP-glucose is recycled in the cell-free medium. The recycling of UDP provides economic efficiency of the processes of the invention. Accordingly, in certain aspects, the production of cyanidin-3-glucoside from cyanidin catalyzed by 3GT is conducted in conjunction with other methods for UDP-glucose production or recycling of UDP. In certain embodiments, the overview of the synthetic scheme involving the UDP-glucose production and/or recycling is provided in **FIG. 3**. In certain embodiments, UDP-glucose is generated by the reaction of uridine triphosphate (UTP) with glucose-1-phosphate. In certain preferred embodiments, the reaction of UTP and glucose-1-phosphate is catalyzed by UTP-glucose-1-phosphate uridylyltransferase (UGP). In certain preferred embodiments, the UGP may be galU.

In certain aspects, glucose-1-phosphate is the source for formation of UDP-glucose. Accordingly, in certain embodiments, glucose-1-phosphate may be added to the reaction for generation of cyanidin-3-glucoside. In certain embodiments, the glucose-1-phosphate may be supplied to the reaction for preparation of cyanidin-3-glucoside.

In certain embodiments, glucose-1-phosphate is generated during the course of the reaction. In certain embodiments, glucose-1-phosphate is generated enzymatically during the course of the reaction. Accordingly, in certain embodiments, glucose is converted to glucose-6-phosphate by a reaction of ATP (adenosine triphosphate) and glucose. In certain embodiments, reaction between glucose and ATP results in generation of glucose-6-phosphate and adenosine diphosphate (ADP). In certain embodiments, conversion of glucose to glucose-6-phosphate is catalyzed by glucokinase (GLK). In certain embodiments, conversion of glucose to glucose-6-phosphate is catalyzed by hexokinase (HK). In certain embodiments, glucose-6-phosphate is converted to glucose-1-phosphate. In certain embodiments, the conversion of glucose-6-phosphate to glucose-1-phosphate is catalyzed by phosphoglucomutase (PGM).

In certain aspects, the invention further provides that the nucleoside triphosphate species used in the reaction are recycled. In certain embodiments, the recycled nucleoside triphosphates

are ATP and UTP. In certain embodiments, UTP is generated by reaction of UDP and ATP. In certain embodiments, the generation of UTP from UDP and ATP is catalyzed by nucleoside diphosphate kinase (NDK). In certain embodiments, ATP may be generated from ADP and phosphate or polyphosphate. In certain embodiments, the conversion of ADP to ATP is catalyzed by polyphosphate kinase (PPK).

In certain beneficial aspects, the invention provides that the processes of the invention are cost effective because of the recycling of chemical intermediates involved in the reaction. In certain embodiments, the invention provides that chemical intermediates like UDP-glucose, UDP, and glucose-1-phosphate are recycled. Indeed, in certain embodiments, the only reagents required in non-catalytic quantities for the production of cyanidin-3-glucoside by conversion of catechin and/or cyanidin are polyphosphate/phosphate and glucose. Because the processes provided in the invention are conducted in a cell free medium and the ingredients (required in non-catalytic quantities) are economic, the processes of the invention are cost effective.

In certain embodiments, the one or more enzymes used in the transformation of a substrate to anthocyanins were expressed in a host organism. In certain embodiments, the cell lysate in the cell free medium is the cell lysate from the host organism expressing one or more enzymes for transformation of a substrate to an anthocyanin. In certain embodiments, the host organism is selected from a group consisting of: bacteria, yeast, and/or mammalian cells. In certain embodiments, the one or more enzymes are introduced in the host organism by integration into genome of the host organism or on a plasmid. In certain embodiments, wherein the host organisms expressing the one or more enzymes are cultured until a pre-determined biomass is achieved to produce the requisite quantity of the one or more enzymes for transformation of a substrate to anthocyanins. In certain embodiments, the method comprises lysing of cells followed by removal of cell debris to generate a cell lysate for use in the cell-free medium for cell-free production of anthocyanins.

In certain embodiments, anthocyanidin synthase enzyme (ANS), anthocyanidin 3-O-glucosyltransferase (3GT), and/or sucrose synthase (SuSy) are present in the cell-free medium for cell-free production of anthocyanins. In certain embodiments, the method does not include separating and/or purifying anthocyanidin synthase enzyme (ANS), anthocyanidin 3-O-glucosyltransferase (3GT), and sucrose synthase (SuSy) for cell-free production of anthocyanins.

It is beneficial to have a system where the method does not require separation of enzymes as it eliminates the excess steps and leads to reduced costs of production. In certain embodiments, the method comprises separating and/or purifying at least one of anthocyanidin synthase enzyme (ANS), anthocyanidin 3-O-glucosyltransferase (3GT), and sucrose synthase (SuSy) for cell-free
5 production of anthocyanins.

In certain embodiments, the one or more enzymes expressed in host cells are anthocyanidin synthase enzyme (ANS), anthocyanidin 3-O-glucosyltransferase (3GT), sucrose synthase (SuSy), glucokinase (GLK), hexokinase (HK), phosphoglucosyltransferase (PGM), polyphosphate kinase (PPK), UTP—glucose-1-phosphate uridylyltransferase (UGP), and/or nucleoside diphosphate kinase
10 (NDK). In certain embodiments, anthocyanidin synthase enzyme (ANS), anthocyanidin 3-O-glucosyltransferase (3GT), sucrose synthase (SuSy), glucokinase (GLK), hexokinase (HK), phosphoglucosyltransferase (PGM), polyphosphate kinase (PPK), UTP—glucose-1-phosphate uridylyltransferase (UGP), and/or nucleoside diphosphate kinase (NDK) are present in the cell-free medium for the cell-free production of cyanidin-3-glucoside.

In certain embodiments of the invention, the reaction mixture comprises purified enzymes for the cell-free production of cyanidin-3-glucoside. In certain embodiments, the purified enzymes are anthocyanidin synthase enzyme (ANS), anthocyanidin 3-O-glucosyltransferase (3GT), sucrose synthase (SuSy), glucokinase (GLK), hexokinase (HK), phosphoglucosyltransferase (PGM), polyphosphate kinase (PPK), UTP—glucose-1-phosphate uridylyltransferase (UGP), and/or
20 nucleoside diphosphate kinase (NDK). In certain embodiments, the purified enzymes are anthocyanidin synthase enzyme (ANS), anthocyanidin 3-O-glucosyltransferase (3GT), and/or sucrose synthase (SuSy). In certain embodiments, the purified enzymes are anthocyanidin synthase enzyme (ANS), and/or anthocyanidin 3-O-glucosyltransferase (3GT).

In certain embodiments, the CGT, SuSy, GLK, HK, PGM, PPK, UGP, and/or NDK
25 enzymes are present in the cell-free medium for cell-free production of carminic acid. In certain embodiments, the compositions of the invention provide CGT, SuSy, GLK, HK, PGM, PPK, UGP, and/or NDK that have not been purified or separated.

In certain embodiments, the cell-free medium may further comprise any other additional ingredients required for the cell-free production of cyanidin-3-glucoside. In certain embodiments,

the cell-free medium comprises buffer, catechin, cyanidin, an activated sugar, magnesium chloride, cell lysate, sucrose, glucose, glucose-1-phosphate, glucose-6-phosphate, UDP, UTP, ATP, polyphosphate, and/or water. In certain embodiments, the cell-free medium comprises buffer, catechin, cyanidin, an activated sugar, magnesium chloride, cell lysate, sucrose, and/or water. In certain embodiments, the buffer used in the cell-free reaction medium is any buffer suitable for enzymatic conversion of catechin and/or cyanidin to cyanidin-3-glucoside. In certain embodiments, the buffer maintains the pH of about 5 to about 9 in the reaction mixture. In certain embodiments, the buffer maintains the pH of about 6 to about 8 in the reaction mixture. In certain embodiments, the buffer maintains the pH in the range of 6 to 8 in the reaction mixture. In certain
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embodiments, the buffer is a phosphate buffer. In certain embodiments, the buffer is present at a concentration of about 1 mM to about 200 mM. In certain embodiments, the buffer is present at a concentration of about 5 mM to about 100 mM.

In certain embodiments, the cell-free medium further comprises buffer, catechin, an activated sugar, magnesium chloride, cell lysate with enzymes, sucrose, and/or water. In certain
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embodiments, the cell-free medium further comprises sodium ascorbate, ascorbic acid, ammonium iron sulphate, and α -ketoglutaric acid/2-oxoglutarate.

In certain embodiments, the buffer in the cell-free medium maintains the pH of the reaction mixture in the optimal range. In certain embodiments, the pH of the reaction medium is from about 5.5 to about 10. In certain embodiments, the pH of cell-free medium is from about 6 to about 9. In
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certain embodiments, the pH of cell-free medium is from about 6 to about 8. In certain embodiments, the concentration of the buffer in the cell-free medium is from about 1 mM to about 1000 mM. In certain embodiments, the concentration of the buffer is from about 2.5 mM to about 750 mM. In certain embodiments, the concentration of the buffer is from about 5 mM to about 100 mM. In certain embodiments, the buffer concentration is from about 20 mM to about 100 mM. In
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certain embodiments, the buffer is a phosphate buffer.

In certain embodiments, the activated sugar in the cell-free medium is UDP-glucose. In certain embodiments, the UDP-glucose is added to the cell free medium. In certain embodiments, the UDP-glucose in the cell-free medium is synthesized from sucrose. In certain embodiments, the cell-free medium comprises UDP. In certain embodiments, the concentration of the activated sugar
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in the cell-free medium is from about 0.01 mM to about 10 mM. In certain embodiments, the

concentration of the activated sugar in the cell-free medium is from about 0.05 mM to about 5 mM. In certain embodiments, the concentration of the activated sugar in the cell-free medium is from about 0.1 mM to about 2.5 mM.

5 In certain embodiments, the substrate is a catechin. In certain embodiments, the concentration of catechin in the cell-free medium is from about 0.1 mM to about 50 mM. In certain embodiments, the concentration of catechin in the cell-free medium is from about 0.5 mM to about 40 mM. In certain embodiments, the concentration of catechin in the cell-free medium is from about 1 mM to about 20 mM.

10 In certain embodiments, the cell-free medium comprises magnesium chloride. The magnesium chloride may be present in the range of from about 1 mM to about 50 mM. In certain embodiments, the cell-free medium comprises magnesium chloride in the range of from about 1 mM to about 25 mM. In certain embodiments, the cell-free medium comprises magnesium chloride in the range of from about 1 mM to about 20 mM.

15 In certain embodiments, the cell-free medium comprises the lysate from host microbe with the one or more enzymes for the cell-free reaction medium. In certain embodiments, the cell-free medium comprises lysate with the one or more enzymes in a concentration of from about 1% (v/v) to about 50% (v/v). In certain embodiments, the cell-free medium comprises lysate with the one or more enzymes in a concentration of from about 5% (v/v) to about 40% (v/v). In certain embodiments, the cell-free medium comprises lysate with the one or more enzymes in a concentration of from about 10% (v/v) to about 30% (v/v).

In certain embodiments, the cell-free medium comprises sucrose. In certain embodiments, sucrose is present in a concentration of from about 10 mM to about 1000 mM. In certain embodiments, sucrose is present in a concentration of from about 20 mM to about 800 mM. In certain embodiments, sucrose is present in a concentration of from about 50 mM to about 600 mM.

25 In certain embodiments, the cell-free medium further comprises sodium ascorbate/ascorbic acid, ammonium iron sulphate, and α -ketoglutaric acid.

In certain embodiments, the cell-free medium comprises sodium ascorbate/ascorbic acid. In certain embodiments, the cell-free medium comprises from about 1 mM to about 100 mM. In

certain embodiments, the cell-free medium comprises from about 2 mM to about 50 mM. In certain embodiments, the cell-free medium comprises from about 5 mM to about 30 mM.

In certain embodiments, the cell-free medium comprises ammonium iron sulphate. In certain embodiments, the cell-free medium comprises ammonium iron sulphate in a concentration of from about 0.1 mM to about 10 mM. In certain embodiments, the cell-free medium comprises ammonium iron sulphate in a concentration of from about 0.5 mM to about 5 mM. In certain embodiments, the cell-free medium comprises ammonium iron sulphate in a concentration of from about 1 mM to about 3 mM.

In certain embodiments, the cell-free medium comprises α -ketoglutaric acid. In certain embodiments, the cell-free medium comprises α -ketoglutaric acid in a concentration from about 1 mM to about 100 mM. In certain embodiments, the cell-free medium comprises α -ketoglutaric acid in a concentration from about 2.5 mM to about 50 mM. In certain embodiments, the cell-free medium comprises α -ketoglutaric acid in a concentration from about 10 mM to about 30 mM.

In certain embodiments, the cell-free reaction for production of anthocyanins is conducted for the requisite duration till the desired quantity of the anthocyanins are produced in the said reaction. In certain embodiments, the reaction for cell-free production of anthocyanins is carried out for a duration of from about 0.1 hours to about 20 hours. In certain embodiments, the reaction for cell-free production of anthocyanins is carried out for a duration of from about 0.5 hours to about 20 hours. In certain embodiments, reaction for cell-free production anthocyanins is carried out for a duration of from about 1 hours to about 15 hours.

In certain embodiments, the reaction of the cell-free medium is adjusted for an optimal yield of anthocyanins to be produced. In certain embodiments, the temperature of the cell-free medium is from about 20 °C to about 40 °C. In certain embodiments, the reaction is conducted in a bubble column reactor, wherein the one or more enzymes are in a solution. In certain embodiments, the reaction is conducted in a packed bed reactor, wherein the one or more enzymes are immobilized.

In certain embodiments, the cell-free medium comprises anthocyanidin synthase (ANS) enzyme. In certain embodiments, the anthocyanidin synthase (ANS) used in the methods of the

invention is an enzyme with an amino acid sequence at least 95% identical to any one of enzymes selected from the group consisting of:

Accession #	Organism
XP_022736758.1	<i>Durio zibethinus</i>
XP_030501512.1	<i>Cannabis sativa</i>
XP_028762755.1	<i>Prosopis alba</i>
XP_021901846.1	<i>Carica papaya</i>
XP_021735950.1	<i>Chenopodium quinoa</i>
QCI56004.1	<i>Iris sanguinea</i>
XP_010538778.1	<i>Tarenaya hassleriana</i>
XP_027183517.1	<i>Coffea eugenoides</i>
AHU88620.1	<i>Magnolia sprengeri</i>
XP_028787846.1	<i>Prosopis alba</i>
EOY24569.1	<i>Theobroma cacao</i>
AEF14411.1	<i>Onobrychis viciifolia</i>
ACM62747.1	<i>Garcinia mangostana</i>
XP_017236102.1	<i>Daucus carota subsp. sativus</i>
XP_010934075.1	<i>Elaeis guineensis</i>

In certain embodiments, the anthocyanidin synthase (ANS) used in the methods of the invention may comprise further modifications designed to optimize the yield of the products produced by the methods of the invention.

In certain embodiments, the cell-free medium comprises anthocyanidin 3-O-glucosyltransferase (3GT) enzyme. In certain embodiments, the anthocyanidin 3-O-glucosyltransferase (3GT) used in the methods of the invention is an enzyme with an amino acid sequence at least 95% identical to any one of enzymes selected from the group consisting of:

Accession #	Organism
AFJ52972.1	<i>Linum usitatissimum</i>
ABR24135.1	<i>Vitis labrusca</i>

AHJ80981.1	<i>Erythranthe lewisii</i>
QCP68996.1	<i>Salvia miltiorrhiza</i>
XP_004307876.1	<i>Fragaria vesca subsp. Vesca</i>
XP_010549820.1	<i>Tarenaya hassleriana</i>
XP_010087491.1	<i>Morus notabilis</i>
BBC62118.1	<i>Nemophila menziesii</i>
PIN20524.1	<i>Handroanthus impetiginosus</i>
QJB23733.1	<i>Centaurea cyanus</i>
QBL54224.1	<i>Scutellaria baicalensis</i>
XP_024179381.1	<i>Rosa chinensis</i>
BAA89008.1	<i>Petunia x hybrida</i>

In certain embodiments, the anthocyanidin 3-O-glucosyltransferase (3GT) enzyme used in the methods of the invention may comprise further modifications designed to optimize the yield of the products produced by the methods of the invention.

- 5 In certain embodiments, the cell-free medium comprises sucrose synthase (SuSy) enzyme. In certain embodiments, the sucrose synthase (SuSy) is an enzyme with an amino acid sequence at least 95% identical to any one of enzymes selected from the group consisting of:

Accession #	Organism
WP_004872341.1	<i>Acidithiobacillus caldus</i>
P30298.2	<i>Oryza sativa Japonica Group</i>
Q820M5.1	<i>Nitrosomonas europaea ATCC 19718</i>
P13708.2	<i>Glycine max</i>
CAA09297.1	<i>Nostoc sp. PCC 7119</i>
P49036.1	<i>Zea mays</i>
P49040.3	<i>Arabidopsis thaliana</i>
Q9M111.1	<i>Arabidopsis thaliana</i>
Q8W517	<i>Ipomoea batatas</i>
WP_011056890	<i>Thermosynechococcus elongatus</i>

In certain embodiments, the sucrose synthase (SuSy) enzyme used in the methods of the invention may comprise further modifications designed to optimize the yield of the products and/or intermediates produced by the methods of the invention.

In certain embodiments, the one or more enzymes involved in generation of UDP-glucose and/or recycling of one or more reaction intermediates are provided below. In certain embodiments, the enzymes used for generation of UDP-glucose (by reaction with UTP) and/or recycling of one or more intermediates are selected from the enzymes having at least 95% amino acid sequence identity from the enzymes provided in the Table below.

Enzyme	Accession No.
GLK	AAA27694.1
GLK	WP_331012003.1
PGM	AAC73782.1
PGM	A0A1I0EI47
PGM	A0A7M2S2U2
UGP	EEW2752841.1
UGP	WP_021648042.1
UGP	WP_028847555.1
NDK	WP_000963837.1
NDK	CAF21035.1
NDK	WP_066795798
PPK2	WP_010968631
PPK2	WP_160857702

In certain embodiments, the invention provides engineered ANS/3GT/SuSy enzymes for the cell-free production of cyanidin-3-glucoside. In certain embodiments, the engineered ANS/3GT/SuSy enzymes were optimized for cell-free production of cyanidin-3-glucoside. In certain embodiments, engineered ANS/3GT/SuSy enzymes may include genetic modifications. In certain embodiments, the genetic modifications may be selected from a group consisting of: point mutations, insertions, deletions, and/or any other modifications such that those enzymes result in

efficient and optimal cell-free production of cyanidin-3-glucoside. In certain embodiments, the ANS/3GT/SuSy enzymes used in the cell-free production of cyanidin-3-glucoside are any enzymes disclosed in this application.

In certain embodiments, the invention provides engineered and optimized
5 GLK/PGM/UGP/NDK/PPK enzymes for generation of UDP-glucose and/or recycling of reaction intermediates involved in cell-free production of cyanidin-3-glucoside. In certain embodiments, the engineered and/or modified GLK/PGM/UGP/NDK/PPK enzymes may include genetic modifications. In certain embodiments, the genetic modifications may be selected from a group consisting of: point mutations, insertions, deletions, and/or any other modifications such that those
10 enzymes result in efficient and optimal cell-free production of cyanidin-3-glucoside.

In certain aspects of the invention, the method of the cell-free production does not require the purification of the one or more enzymes from the lysed host organisms. In certain embodiments, the methods of the invention do not require the purification of one or more enzymes from the lysed biomass comprising host cell expressing the one or more enzymes for the cell-free
15 production of carminic acid. In certain embodiments, the methods of the invention do not require the purification of CGT/SuSy/GLK/PGM/UGP/NDK/PPK from the lysed cells for cell-free production of cyanidin-3-glucoside. This is advantageous because it increases the efficiency of the method and reduces the costs associated with the purification of these enzymes.

In certain embodiments, the cell-free medium may further comprise any other additional
20 ingredients required for the cell-free production of cyanidin-3-glucoside. For example, in certain embodiments, the cell-free medium comprises buffer, catechin, cyanidin, an activated sugar, magnesium chloride, cell lysate, sucrose, and/or water. In certain embodiments, the cell-free reaction mixture further comprises uridine diphosphate (UDP). In certain embodiments, the cell-free reaction mixture further comprises polyphosphate and/or glucose. In certain embodiments, the
25 cell-free reaction mixture comprises UDP-glucose and/or UDP in catalytic quantities. In certain embodiments, UDP-glucose is produced enzymatically in the reaction. In certain embodiments, the reaction mixture comprises glucose-1-phosphate. Glucose-1-phosphate may be generated in the cell-free reaction. Thus, in certain embodiments, glucose-1-phosphate is also present in catalytic quantity. In certain embodiments, the reaction medium comprises glucose and
30 polyphosphate.

In certain preferred embodiments, the processes of the invention utilize catechin, glucose, and polyphosphate as the starting materials for production of cyanidin-3-glucoside. In certain embodiments, the processes of the invention utilize cyanidin, glucose, and polyphosphate as the starting materials for production of cyanidin-3-glucoside. In these embodiments, the invention provides that the other intermediates in the process for production of cyanidin-3-glucoside may be recycled.

The conventional methods for production of anthocyanins either requires the extraction of the product from the plants, which is expensive and unpredictable, or certain cell-based approaches, for example, as described in U.S. Patent No. 8,962,327, which is incorporated by reference. However, the cell-based approaches have complications, such as the processes leading to low yields, a higher number of by-products, and expensive purification and separation protocols. The methods of the current invention beneficially provide cell-free approaches for bioproduction of anthocyanins. In particular, the product titers for anthocyanins produced by the methods of the invention are higher than the product titer produced by any other known methods for production of anthocyanins. In certain embodiments, the product titers produced by methods of the invention are at least two-fold higher than other known methods for production of anthocyanins. In certain embodiments, the product titers produced by methods of the invention are at least five-fold higher than other known methods for production of anthocyanins. In certain embodiments, the product titers produced by methods of the invention are at least ten-fold higher than other known methods for production of anthocyanins. In certain embodiments, the product titers produced by methods of the invention are at least hundred-fold higher than other known methods for production of anthocyanins. In certain embodiments, the product titers produced by methods of the invention are at least five-hundred-fold higher than other known methods for production of anthocyanins. In certain embodiments, the product titers produced by methods of the invention are at least thousand-fold higher than other known methods for production of anthocyanins. In certain embodiments, the product titers produced by methods of the invention are at least five-thousand-fold higher than other known methods for production of anthocyanins.

In other beneficial aspects of the method of the invention, the methods of the invention provide economically efficient methods of production of anthocyanins. Because the methods of the invention provide for cell-free production of anthocyanins, it leads to decreased costs of

purification of the reaction products. Moreover, in certain aspects of the invention, the one or more enzymes are included in the same reaction mixture, *i.e.*, the enzymes do not need to be isolated after being expressed in the host organisms. The one or more enzymes could be expressed in the host organisms at the same time and be utilized for the cell-free production without the need of
5 any additional purification and/or separation steps.

In certain other beneficial aspects of the invention, the use of the one or more enzymes for the cell-free production of anthocyanins leads to high yield of the product to be produced. The one more enzyme may be further modified to optimize the yield of the product being produced in the cell-free reaction.

10 V. Brief Description of Drawings

FIG. 1 provides a schematic pathway for conversion of catechin to cyanidin and cyanidin-3-glucoside mediated by ANS and 3GT respectively.

FIG. 2 provides a schematic pathway for conversion of catechin to cyanidin-3-glucoside mediated by ANS and 3GT, wherein the UDP-glucose is provided by SuSy mediated conversion
15 of sucrose to UDP-glucose.

FIG. 3 provides a schematic pathway for conversion of cyanidin to cyanidin-3-glucoside mediated by 3GT and medication of , wherein the UDP-glucose is generated by recycling and/or generation of UDP-glucose in course of reaction.

FIG. 4 provides HPLC plots demonstrating the conversion of cyanidin to cyanidin-3-glucoside. The top two panels provide HPLC plots for cyanidin-3-glucoside and cyanidin
20 respectively, and bottom two panels provide the HPLC plots to monitor the reaction in a system with and without SuSy mediated conversion of sucrose to UDP-glucose respectively.

VI. Detailed Description

25 The present application provides compositions and methods for production of anthocyanins in a cell-free medium, wherein one or more enzymes in a cell-free medium, wherein the one or more enzymes result in transformation of an organic material to anthocyanins. The one or more enzymes may be engineered. The engineered enzyme may be non-naturally occurring.

The term “non-naturally occurring”, when used in reference to an enzyme is intended to mean that nucleic acids or polypeptides include at least one genetic alteration not normally found in a naturally occurring polypeptide or nucleic acid sequence. Naturally occurring nucleic acids, and polypeptides can be referred to as “wild-type” or “original”. A host cell, organism, or
5 microorganism that includes at least one genetic modification generated by human intervention can also be referred to as “non-naturally occurring”, “engineered”, “genetically engineered,” or “recombinant”.

As used herein, the singular forms “a,” “an,” and “the” are intended to include the plural forms as well, unless the context clearly indicates otherwise. Furthermore, to the extent that the
10 terms “including,” “includes,” “having,” “has,” “with,” or variants thereof are used in either the detailed description and/or the claims, such terms are intended to be inclusive in a manner similar to the term “comprising.”

As used herein, “reaction solution” may refer to all components necessary for enzyme-based chemical transformation. This is typically, but not limited to, buffering agent, salts, cofactor,
15 and substrate (starting material).

As used herein, “reaction mixture” may refer to all components from the “reaction solution” plus the enzyme(s) and/or products from the reaction. In some embodiments, the “reaction mixture” may refer to just the reaction solution without any enzymes or reaction products. In some embodiments, “reaction solution” and “reaction mixture” may be used
20 interchangeably.

As used herein, “buffering agents” may refer to chemicals added to water-based solutions that resist changes in pH by the action of acid-base conjugate components.

As used herein, “cofactors” may refer to a non-protein chemical compound that may bind to a protein and assist with a biological chemical reaction. Non-limiting examples of cofactors may
25 include but are not limited to NADPH and NADH.

As used herein, the term used for enzyme anthocyanin synthase (ANS), may also refer to the enzyme known in the field as leucoanthocyanidin dioxygenase. The enzyme anthocyanin synthase (ANS) may also be known as leucoanthocyanidin dioxygenase.

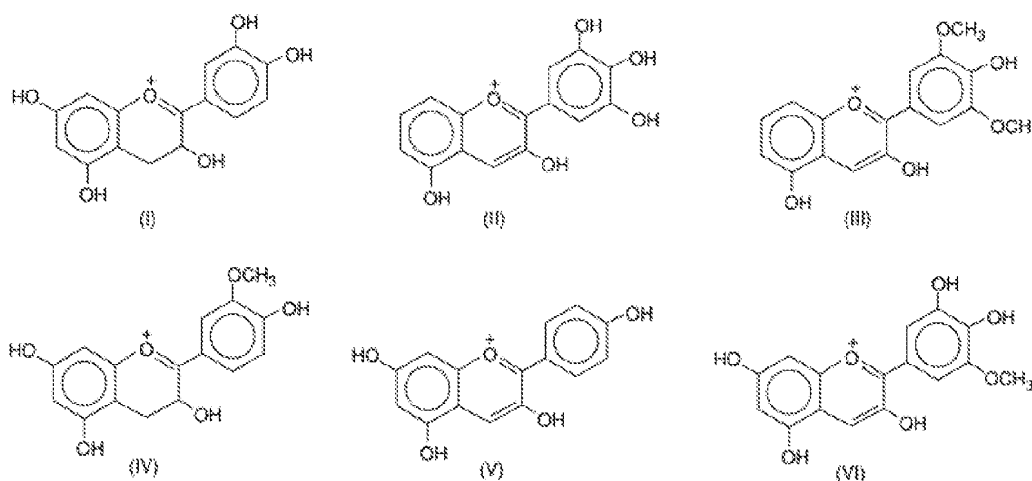
In the cell-free systems described herein, the critical components of the cell, namely
30 cofactors and enzymes, are used in a chemical reaction without cellular components that can directly or indirectly inhibit the desired biochemical reaction. The same enzymes found in plants

and other organisms may be created in vivo (typically through protein overexpression in hosts such as bacteria), isolated via chromatography and/or any other methods, and then added into a bioreactor with a substrate (starting material). The enzymes may also be used directly from plants without any isolation. The enzymes transform the substrate in the same way that occurs in the original organism without the organism's complexity. Additionally, the biochemical reaction may be enhanced by the addition of co-solvents, detergents, or both, which would not be tolerated by, or simply would not work in a whole cell-based manufacturing method. In this way, natural products can be created without the plant, cell, or chemical synthesis.

Anthocyanins:

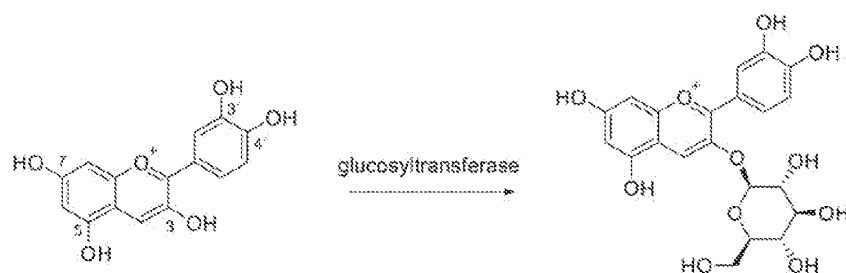
Anthocyanins are natural plant pigments that have beneficial effects for the plant as well as for humans and animals. Dietary sources of anthocyanins are generally easy to identify due to their red, blue, or purple color. Examples include berries and red-skinned grapes, apples, and pears and various vegetables such as radishes and red/purple cabbage. Anthocyanins may also be ingested through their use as a food additive and as dietary supplements, procured as anthocyanin-rich fruit extracts, powders, and purified compounds. Dietary consumption of anthocyanins is high compared with that of other flavonoids because of their wide distribution in plant foods. Numerous studies indicate the potential effect that this flavonoid family may have in reducing cardiovascular disease, cancer, hyperlipidemia, and other insulin resistance-related disease incidence through anthocyanin-rich food intake. Anthocyanins are also found in rich concentrations in teas, wines, fruits, vegetables, nuts, olive oil, cocoa, and cereals, and they appear vivid red to blue in color.

Anthocyanins are glycosides composed of the anthocyanidin aglycone plus one or more glycosidically bonded mono- or oligosaccharidic units. The structures of commonly known anthocyanidins are provided below:



As evident from the structures of anthocyanidins provided above, all anthocyanidins have hydroxyl groups in the 3-, 5- and 7-positions, although each structure may have its own characteristic hydroxyl or methoxyl groups on the so-called B ring: (I) cyanidin, (II) delphinidin, (III) malvidin, (IV) peonidin, (V) pelargonidin, and (VI) petunidin.

Glycosylation of anthocyanidins to form the respective anthocyanins can occur on different hydroxyl moieties of the molecule with 3-OH as the most abundant glycosylation site in nature to produce 3-O- β -glucosides. The reaction provided below demonstrates the conversion of cyanidin (anthocyanidin) to cyanidin-3-glucoside (anthocyanin).



10

Anthocyanins and anthocyanidins, as other polyphenols and flavonoids, possess the ability to act as free radical scavengers against harmful oxidants such as reactive oxygen and nitrogen species (ROS and RNS). A central role of the antioxidant activity is the oxidation of anthocyanins'

phenolic hydroxyl groups; in particular, para- and ortho- phenolic groups are important for the formation of semiquinones and for the stabilization of one-electron oxidation products.

Preparation of anthocyanins:

There are different strategies for anthocyanins and anthocyanidin extraction from biological matrices based on their complexity and selective search for specific molecules with particular chemical features. For example, the extraction of total anthocyanidins generally is performed by using aqueous/organic mixtures with a significant contribution of the organic part. Instead, the anthocyanins are often extracted with more hydrophilic solvents or with more prominent aqueous-based mixtures. In all this cases, it is very useful to maintain the ionization state of the compounds in the flavylium form, and this goal can be achieved by adding inorganic or organic acids to the aqueous phase. Plants, food and agricultural samples are usually extracted with ethanol/methanol: water mixtures (70-95:30-5) acidified with HCl, formic acid or other organic acids such as citric acid.

After the extraction, depending on the matrices (fruits, leaves or liquid samples), the first step for purification procedures is to distinguish between green and red pigment-rich extracts. The first case indicates a strong chlorophyll component that must be preliminary purified, and the second one indicates a matrix richer in anthocyanin (fruits, flowers or liquid biological samples). After chlorophyll elimination, the purification is generally carried out by different chromatographic steps involving differential stationary phases on the basis of the purposes.

The biosynthetic pathway of anthocyanins has been well characterized both in an *Arabidopsis thaliana* model plant and in various crops, and appears to be strongly conserved. The biosynthetic pathway of anthocyanins constitutes an important branch of the phenylpropanoid pathway and shares, in the initial stages, some biosynthetic enzymes for other flavonoids such as flavones and flavonones. It starts with phenylalanine which is converted into cinnamic acid by phenylalanine ammonia-lyase (PAL). The cinnamic acid is then converted into coumaric acid by the action of cinnamate-4-hydroxylase (C4H) and subsequently converted into 4-coumaroil CoA by the 4-coumaroil CoA ligase (4CL). Following condensation of 4-coumaroil CoA with malonyl CoA, naringenin chalcone is produced by chalcone synthase (CHS) and subsequently converted into naringenin by chalcone isomerase (CHI) and dihydroflavonols, such as dihydrokaempferol

and dihydroquercetin from flavanone 3-hydroxylase (F3H) and the flavonoid 3'-hydroxylase (F3'H), respectively. The last steps of the biosynthetic pathway lead to the production of leucocyanidins, cyanidins and anthocyanins by dihydroflavonol reductase (DFR), anthocyanidin synthase (ANS) and UDP-glucose:flavonoid-3-O-glycosyltransferase (UGT), respectively.

5 While the biosynthesis of anthocyanins occurs in the cytosol, they are stored in the vacuole by specific transporters. In Arabidopsis for example, the TT12 and AHA10 genes have been associated with the transport and vacuolar accumulation of anthocyanins in the seed. In particular TT12, which encodes for a membrane protein belonging to the "multidrug and toxic efflux antiporter" family, has been shown to be involved in the accumulation, at the vacuolar level, of

10 glycosylated flavan-3-ols and protoanthocyanidins. AHA10, which encodes for a plasma membrane H⁺-ATPase, is instead responsible for the acidification of the vacuole. A detailed description of the biosynthetic pathway is provided in Mattioli et al., Anthocyanins: A Comprehensive Review of Their Chemical Properties and Health Effects on Cardiovascular and Neurodegenerative Diseases, *Molecules*. 2020 Sep; 25(17): 3809, which is incorporated herein in

15 its entirety by reference.

Although biotechnological approaches to increase the level of anthocyanins in food have been explored, none of the previous approaches provide cell-free synthesis of anthocyanins. Anthocyanins are currently produced by chopping or crushing the fruit or vegetable and subsequent infusion of water acidified with a common food acid. This extract is then concentrated

20 by non-chemical separation techniques. Pigment extracts from plant sources generally contain mixtures of different anthocyanin molecules, which vary by their level of hydroxylation, methylation and acylation. This increases the cost of production because of the purification required from these mixtures. Moreover, these factors can vary in the source plant from year to year, and are influenced by weather and environmental factors. Thus, another challenge for the

25 commercial production of anthocyanin pigments from plants is that harvest is often limited to once a year. This means a large volume of extract has to be prepared and stored for an extended period of time to supply the needs of the food industry throughout the year. Special storage conditions often have to be available due to the instability of anthocyanins.

Thus, new methods for production of anthocyanins are required. The invention provides

30 methods for cell-free production of anthocyanins. The methods of the invention comprise:

providing one or more enzymes in a cell-free medium, wherein the one or more enzymes result in transformation of a substrate to anthocyanins. In certain aspects of the invention, the methods of the invention may provide for production of one or more intermediates during the transformation of a substrate to anthocyanin. In certain aspects of the invention, the substrate may be an anthocyanidin or a precursor thereof. In certain embodiments, the anthocyanidin is selected from a group consisting of: delphinidin, malvidin, peonidin, pelargonidin, and petunidin. In certain embodiments, the anthocyanins may be glucosylated delphinidin, malvidin, peonidin, pelargonidin, and petunidin.

In certain aspects of the invention, the anthocyanin is cyanidin-3-glucoside. In certain embodiments, the substrate is a catechin. In certain embodiments, the catechin is (+)-catechin. In certain embodiments, the one or more enzymes are selected from a group consisting of: anthocyanidin synthase enzyme (ANS) and anthocyanidin 3-O-glucosyltransferase (3GT).

In certain embodiments of the invention, the anthocyanidin synthase (ANS) enzyme catalyzes the conversion of catechin to cyanidin. In certain embodiments, the anthocyanidin 3-O-glucosyltransferase (3GT) enzyme catalyzes the conversion of cyanidin to cyanidin-3-glucoside. A schematic of the reaction pathway for conversion of catechin to cyanidin-3-glucoside is provided in **FIG. 1**. In certain preferred embodiments, the cyanidin produced by transformation of catechin by anthocyanidin synthase (ANS) enzyme is further transformed to cyanidin-3-glucoside by anthocyanidin 3-O-glucosyltransferase (3GT) enzyme.

In certain embodiments, the cell-free production of anthocyanins involves a cell-free medium. In certain embodiments, the cell-free medium comprises a cell lysate. In certain embodiments, the cell-free medium comprises an activated sugar. In certain embodiments, the activated sugar is UDP-glucose. In certain embodiments, the UDP-glucose is added to the cell-free medium. In certain other embodiments, the UDP-glucose is synthesized in the cell-free medium by the one or more enzymes. In certain embodiments, UDP-glucose is synthesized from sucrose. In certain embodiments, sucrose synthase (SuSy) enzyme transforms sucrose to UDP-glucose. **FIG. 2** provides the schematic reaction scheme for the production of UDP-glucose from sucrose mediated by SuSy, wherein the UDP-glucose is involved in the production of cyanidin-3-glucoside from catechin.

In certain beneficial aspects, the invention provides that the UDP-glucose used in the reaction is generated from other substrates in course of the reaction. In certain embodiments, the UDP moiety in UDP-glucose is recycled in the cell-free medium. The recycling of UDP provides economic efficiency of the processes of the invention. Accordingly, in certain aspects, the production of cyanidin-3-glucoside from cyanidin catalyzed by 3GT is conducted in conjunction with other methods for UDP-glucose production or recycling of UDP. In certain embodiments, the overview of the synthetic scheme involving the UDP-glucose production and/or recycling is provided in FIG. 3. In certain embodiments, UDP-glucose is generated by the reaction of uridine triphosphate (UTP) with glucose-1-phosphate. In certain preferred embodiments, the reaction of UTP and glucose-1-phosphate is catalyzed by UTP-glucose-1-phosphate uridylyltransferase (UGP). In certain preferred embodiments, the UGP may be galU.

In certain aspects, glucose-1-phosphate is the source for formation of UDP-glucose. Accordingly, in certain embodiments, glucose-1-phosphate may be added to the reaction for generation of cyanidin-3-glucoside. In certain embodiments, the glucose-1-phosphate may be supplied to the reaction for preparation of cyanidin-3-glucoside.

In certain embodiments, glucose-1-phosphate is generated during the course of the reaction. In certain embodiments, glucose-1-phosphate is generated enzymatically during the course of the reaction. Accordingly, in certain embodiments, glucose is converted to glucose-6-phosphate by a reaction of ATP (adenosine triphosphate) and glucose. In certain embodiments, reaction between glucose and ATP results in generation of glucose-6-phosphate and adenosine diphosphate (ADP). In certain embodiments, conversion of glucose to glucose-6-phosphate is catalyzed by glucokinase (GLK). In certain embodiments, conversion of glucose to glucose-6-phosphate is catalyzed by hexokinase (HK). In certain embodiments, glucose-6-phosphate is converted to glucose-1-phosphate. In certain embodiments, the conversion of glucose-6-phosphate to glucose-1-phosphate is catalyzed by phosphoglucomutase (PGM).

In certain aspects, the invention further provides that the nucleoside triphosphate species used in the reaction are recycled. In certain embodiments, the recycled nucleoside triphosphates are ATP and UTP. In certain embodiments, UTP is generated by reaction of UDP and ATP. In certain embodiments, the generation of UTP from UDP and ATP is catalyzed by nucleoside diphosphate kinase (NDK). In certain embodiments, ATP may be generated from ADP and

phosphate or polyphosphate. In certain embodiments, the conversion of ADP to ATP is catalyzed by polyphosphate kinase (PPK).

In certain beneficial aspects, the invention provides that the processes of the invention are cost effective because of the recycling of chemical intermediates involved in the reaction. In certain embodiments, the invention provides that chemical intermediates like UDP-glucose, UDP, and glucose-1-phosphate are recycled. Indeed, in certain embodiments, the only reagents required in non-catalytic quantities for the production of cyanidin-3-glucoside by conversion of catechin are polyphosphate/phosphate and glucose. Because the processes provided in the invention are conducted in a cell free medium and the ingredients (required in non-catalytic quantities) are economic, the processes of the invention are cost effective.

In certain embodiments, the one or more enzymes used in the transformation of a substrate to anthocyanins were expressed in a host organism. In certain embodiments, the cell lysate in the cell free medium is the cell lysate from the host organism expressing one or more enzymes for transformation of a substrate to an anthocyanin. In certain embodiments, the host organism is selected from a group consisting of: bacteria, yeast, and/or mammalian cells. In certain embodiments, the one or more enzymes are introduced in the host organism by integration into genome of the host organism or on a plasmid. In certain embodiments, wherein the host organisms expressing the one or more enzymes are cultured until a pre-determined biomass is achieved to produce the requisite quantity of the one or more enzymes for transformation of a substrate to anthocyanins. In certain embodiments, the method comprises lysing of cells followed by removal of cell debris to generate a cell lysate for use in the cell-free medium for cell-free production of anthocyanins.

In certain embodiments, anthocyanidin synthase enzyme (ANS), anthocyanidin 3-O-glucosyltransferase (3GT), and/or sucrose synthase (SuSy) are present in the cell-free medium for cell-free production of anthocyanins. In certain embodiments, the method does not include separating and/or purifying anthocyanidin synthase enzyme (ANS), anthocyanidin 3-O-glucosyltransferase (3GT), and sucrose synthase (SuSy) for cell-free production of anthocyanins. Similarly, in certain embodiments, the invention provides that the enzymes involved in UDP-glucose recycling pathways are also not engineered. It is beneficial to have a system where the method does not require separation of enzymes as it eliminates the excess steps and leads to

reduced costs of production. In certain embodiments, the method comprises separating and/or purifying at least one of anthocyanidin synthase enzyme (ANS), anthocyanidin 3-O-glucosyltransferase (3GT), and sucrose synthase (SuSy) for cell-free production of anthocyanins.

In certain embodiments of the invention, the reaction mixture comprises purified enzymes for the cell-free production of cyanidin-3-glucoside. In certain embodiments, the purified enzymes are anthocyanidin synthase enzyme (ANS), anthocyanidin 3-O-glucosyltransferase (3GT), and/or sucrose synthase (SuSy).

In certain embodiments, the one or more enzymes expressed in host cells are anthocyanidin synthase enzyme (ANS), anthocyanidin 3-O-glucosyltransferase (3GT), sucrose synthase (SuSy), glucokinase (GLK), hexokinase (HK), phosphoglucosyltransferase (PGM), polyphosphate kinase (PPK), UTP—glucose-1-phosphate uridylyltransferase (UGP), and/or nucleoside diphosphate kinase (NDK). In certain embodiments, C-glucosyltransferase (CGT), sucrose synthase (SuSy), glucokinase (GLK), hexokinase (HK), phosphoglucosyltransferase (PGM), polyphosphate kinase (PPK), UTP—glucose-1-phosphate uridylyltransferase (UGP), and/or nucleoside diphosphate kinase (NDK) are present in the cell-free medium for the cell-free production of cyanidin-3-glucoside.

In certain embodiments of the invention, the reaction mixture comprises purified enzymes for the cell-free production of cyanidin-3-glucoside. In certain embodiments, the purified enzymes are anthocyanidin synthase enzyme (ANS), anthocyanidin 3-O-glucosyltransferase (3GT), sucrose synthase (SuSy), glucokinase (GLK), hexokinase (HK), phosphoglucosyltransferase (PGM), polyphosphate kinase (PPK), UTP—glucose-1-phosphate uridylyltransferase (UGP), and/or nucleoside diphosphate kinase (NDK). In certain embodiments, C-glucosyltransferase (CGT), sucrose synthase (SuSy), glucokinase (GLK), hexokinase (HK), phosphoglucosyltransferase (PGM), polyphosphate kinase (PPK), UTP—glucose-1-phosphate uridylyltransferase (UGP), and/or nucleoside diphosphate kinase (NDK) are not purified. In certain embodiments, the purified enzymes are anthocyanidin synthase enzyme (ANS), anthocyanidin 3-O-glucosyltransferase (3GT), and/or sucrose synthase (SuSy).

In certain embodiments, the cell-free medium further comprises buffer, catechin, an activated sugar, magnesium chloride, cell lysate with enzymes, sucrose, and/or water. In certain

embodiments, the cell-free medium further comprises sodium ascorbate, ascorbic acid, ammonium iron sulphate, and α -ketoglutaric acid/2-oxoglutarate.

In certain embodiments, the buffer in the cell-free medium maintains the pH of the reaction mixture in the optimal range. In certain embodiments, the pH of the reaction medium is from about 5.5 to about 10. In certain embodiments, the pH of cell-free medium is from about 6 to about 9. In certain embodiments, the pH of cell-free medium is from about 6 to about 8.

In certain embodiments, the buffer is selected from a group consisting of: Tris, HEPES, phosphate, carbonate/bicarbonate, acetate, sodium hydroxide-glycine, and/or citrate. The concentration of the buffer for the reaction mixture would depend on the concentration of the starting materials and other ingredients in the reaction mixture. In certain embodiments, the buffer is a phosphate buffer. In certain embodiments, the concentration of the buffer in the cell-free medium is from about 1 mM to about 1000 mM. In certain embodiments, the concentration of the buffer is from about 2.5 mM to about 750 mM. In certain embodiments, the concentration of the buffer is from about 5 mM to about 100 mM. In certain embodiments, the buffer concentration is from about 20 mM to about 100 mM. In certain embodiments, the buffer is a phosphate buffer.

In certain embodiments, the activated sugar in the cell-free medium is UDP-glucose. In certain embodiments, the UDP-glucose is added to the cell free medium. In certain embodiments, the UDP-glucose in the cell-free medium is synthesized from sucrose. In certain embodiments, the cell-free medium comprises UDP. In certain embodiments, the concentration of the activated sugar in the cell-free medium is from about 0.01 mM to about 10 mM. In certain embodiments, the concentration of the activated sugar in the cell-free medium is from about 0.05 mM to about 5 mM. In certain embodiments, the concentration of the activated sugar in the cell-free medium is from about 0.1 mM to about 2.5 mM.

In certain embodiments, the substrate is a catechin. In certain embodiments, the concentration of catechin in the cell-free medium is from about 0.1 mM to about 50 mM. In certain embodiments, the concentration of catechin in the cell-free medium is from about 0.5 mM to about 40 mM. In certain embodiments, the concentration of catechin in the cell-free medium is from about 1 mM to about 20 mM.

In certain embodiments, the cell-free medium comprises magnesium chloride. The magnesium chloride may be present in the range of from about 1 mM to about 50 mM. In certain embodiments, the cell-free medium comprises magnesium chloride in the range of from about 1 mM to about 25 mM. In certain embodiments, the cell-free medium comprises magnesium chloride
5 in the range of from about 1 mM to about 20 mM.

In certain embodiments, the cell-free medium comprises the lysate from host microbe with the one or more enzymes for the cell-free reaction medium. In certain embodiments, the cell-free medium comprises lysate with the one or more enzymes in a concentration of from about 1% (v/v) to about 50% (v/v). In certain embodiments, the cell-free medium comprises lysate with the one
10 or more enzymes in a concentration of from about 5% (v/v) to about 40% (v/v). In certain embodiments, the cell-free medium comprises lysate with the one or more enzymes in a concentration of from about 10% (v/v) to about 30% (v/v).

In certain embodiments, the cell-free medium comprises sucrose. In certain embodiments, sucrose is present in a concentration of from about 10 mM to about 1000 mM. In certain
15 embodiments, sucrose is present in a concentration of from about 20 mM to about 800 mM. In certain embodiments, sucrose is present in a concentration of from about 50 mM to about 600 mM.

In certain embodiments, the cell-free reaction medium further comprises glucose. In certain embodiments, glucose is a starting material to provide UDP-glucose for the conversion of catechin/cyanidin to cyanidin-3-glucoside. Thus, the concentration of glucose in the reaction
20 mixture is determined by the quantity of UDP-glucose needed for the reaction for cell-free production of cyanidin-3-glucoside. In certain embodiments, glucose is present at a concentration of about 10 mM to about 1000 mM in the cell-free reaction medium. In certain embodiments, glucose is present at a concentration of about 20 mM to about 800 mM in the cell-free reaction medium. In certain embodiments, glucose is present at a concentration of about 50 mM to about
25 600 mM in the cell-free reaction medium.

In certain embodiments, the cell-free reaction medium further comprises polyphosphate. In certain embodiments, polyphosphate is also a starting material to provide UDP-glucose for the conversion of catechin/cyanidin to cyanidin-3-glucoside. Thus, the concentration of polyphosphate in the reaction mixture is determined by the quantity of UDP-glucose needed for

the reaction for cell-free production of cyanidin-3-glucoside. In certain embodiments, polyphosphate is present at a concentration of about 10 mM to about 1000 mM in the cell-free reaction medium. In certain embodiments, polyphosphate is present at a concentration of about 20 mM to about 800 mM in the cell-free reaction medium. In certain embodiments, polyphosphate is present at a concentration of about 50 mM to about 600 mM in the cell-free reaction medium.

In certain embodiments, the cell-free medium further comprises sodium ascorbate/ascorbic acid, ammonium iron sulphate, and α -ketoglutaric acid.

In certain embodiments, the cell-free medium comprises sodium ascorbate/ascorbic acid. In certain embodiments, the cell-free medium comprises from about 1 mM to about 100 mM. In certain embodiments, the cell-free medium comprises from about 2 mM to about 50 mM. In certain embodiments, the cell-free medium comprises from about 5 mM to about 30 mM.

In certain embodiments, the cell-free medium comprises ammonium iron sulphate. In certain embodiments, the cell-free medium comprises ammonium iron sulphate in a concentration of from about 0.1 mM to about 10 mM. In certain embodiments, the cell-free medium comprises ammonium iron sulphate in a concentration of from about 0.5 mM to about 5 mM. In certain embodiments, the cell-free medium comprises ammonium iron sulphate in a concentration of from about 1 mM to about 3 mM.

In certain embodiments, the cell-free medium comprises α -ketoglutaric acid. In certain embodiments, the cell-free medium comprises α -ketoglutaric acid in a concentration from about 1 mM to about 100 mM. In certain embodiments, the cell-free medium comprises α -ketoglutaric acid in a concentration from about 2.5 mM to about 50 mM. In certain embodiments, the cell-free medium comprises α -ketoglutaric acid in a concentration from about 10 mM to about 30 mM.

In certain embodiments, the cell-free reaction for production of anthocyanins is conducted for the requisite duration till the desired quantity of the anthocyanins are produced in the said reaction. In certain embodiments, the reaction for cell-free production of anthocyanins is carried out for a duration of from about 0.1 hours to about 20 hours. In certain embodiments, the reaction for cell-free production of anthocyanins is carried out for a duration of from about 0.5 hours to about 20 hours. In certain embodiments, reaction for cell-free production anthocyanins is carried out for a duration of from about 1 hours to about 15 hours.

In certain embodiments, the temperature of the reaction mixture for cell-free production of anthocyanins is varied to obtain optimal results for the production of anthocyanins. In certain embodiments, the temperature of the reaction mixture for cell-free production of anthocyanins is from about 15 °C to about 45 °C. In certain embodiments, the temperature of the reaction mixture for cell-free production of anthocyanins is from about 20 °C to about 40 °C. In certain
5 embodiments, the temperature of the reaction mixture for cell-free production of anthocyanins is from about 22.5 °C to about 37.5 °C. The temperature of the reaction mixture for cell-free production of anthocyanins may influence the rate of production of anthocyanins. Consequently, the duration of reaction may be adjusted according to the temperature of the reaction mixture to
10 obtain optimal yield of anthocyanins in cell-free production of anthocyanins.

In certain aspects, the methods provided in the invention may be carried out in any reactor suitable for carrying out the cell-free production of anthocyanins. In certain embodiments, the reaction for cell-free production of anthocyanins is conducted in a bubble column reactor/bioreactor. In certain embodiments, in the bubble column reactor/bioreactor, the one or
15 more enzymes involved in cell-free production of anthocyanins are in a solution. In certain embodiments, the reaction for cell-free production of anthocyanins is conducted in a bubble column reactor/bioreactor comprises the lysate from the host organism. In certain embodiments, it is advantageous to use the bubble column reactor/bioreactor for cell-free production of anthocyanins when the reaction mixture involves the lysate (or lysate with cellular debris removed)
20 from the host cell organisms in which the one or more enzymes responsible for cell-free production of anthocyanins were utilized. In certain embodiments, the reaction for cell-free production of anthocyanins is conducted in a packed bed reactor/bioreactor. In certain embodiments, the one or more enzymes are immobilized in the packed bed reactor/bioreactor. The packed bed reactors/bioreactors are preferred for the purified enzymes playing a role in cell-free production of
25 anthocyanins. In certain embodiments, the one or more enzymes may be immobilized in a single reactor/bioreactor. In certain other embodiments, the one or more enzymes may be immobilized in different reactors/bioreactors, wherein these reactors/bioreactors are linked sequentially. In certain embodiments, the bioreactor system provided in PCT/US2021/064049, incorporated by reference in its entirety.

The methods provided in the invention are advantageous over other conventional methods of production of anthocyanins. In certain embodiments, the methods of the invention provide cell-free production of anthocyanins. Because the methods of the invention are conducted in cell-free medium, they provide significant economic efficiency by reducing the cost of production of anthocyanins in other conventional methods. In certain embodiments, because the reaction for production of anthocyanins is conducted from the lysates from the host organisms expressing the one or more enzymes involved in the reaction, the methods of the invention are cost-efficient. In particular, in certain embodiments, the methods of the invention do not involve the purification of the one or more enzymes. Because purification of individual enzymes is not required in the methods of the invention, provides further economic efficiency by reducing the cost that would have been otherwise required in purifying individual enzymes.

Advantageously, the cell-free production of anthocyanins provided herein provide a significantly higher titer value for anthocyanins as compared to the conventional methods. The higher titer values of anthocyanins provide additional cost advantages for production of anthocyanins because the higher titer anthocyanins would provide efficiency in purifying and/or concentrating anthocyanins from the reaction mixture. In certain embodiments, the methods of the invention provide anthocyanins titer value of from at least 5-fold higher than the conventional methods. In certain embodiments, the methods of the invention provide anthocyanins titer value of from at least 10-fold higher than the conventional methods. In certain embodiments, the methods of the invention provide anthocyanins titer value of from at least 50-fold higher than the conventional methods. In certain embodiments, the methods of the invention provide anthocyanins titer value of from at least 100-fold higher than the conventional methods. In certain embodiments, the methods of the invention provide anthocyanins titer value of from at least 500-fold higher than the conventional methods. In certain embodiments, the methods of the invention provide anthocyanins titer value of from at least 1000-fold higher than the conventional methods. In certain embodiments, the methods of the invention provide anthocyanins titer value of from at least 5000-fold higher than the conventional methods.

In some embodiments, the isolated anthocyanin has a purity of about 10%, or about 20%, or about 30%, or about 40%, or about 50%, or about 60%, or about 70%, or about 80%, or about 90%, or about 95%, or about 99%, or about 100%.

In other embodiments, the isolated anthocyanin has a purity of from about 10% to 95%, or from about 10% to 90%, or from about 10% to 80% or from about 10% to 70%, or from about 10% to 60%, or from about 10% to 50%, or from about 10% to 40%, or from about 20% to 95%, or from about 20% to 90%, or from about 20% to 80% or from about 20% to 70%, or from about 20% to 60%, or from about 20% to 50%, or from about 20% to 40%, or from about 50% to 95%, or from about 50% to 90%, or from about 50% to 80% or from about 50% to 70%, or from about 50% to 60%.

In certain embodiments, the cell-free medium comprises anthocyanidin synthase (ANS) enzyme. In certain embodiments, the anthocyanidin synthase (ANS) used in the methods of the invention is an enzyme with an amino acid sequence at least 95% identical to any one of enzymes selected from the group consisting of:

Accession #	Organism
XP_022736758.1	<i>Durio zibethinus</i>
XP_030501512.1	<i>Cannabis sativa</i>
XP_028762755.1	<i>Prosopis alba</i>
XP_021901846.1	<i>Carica papaya</i>
XP_021735950.1	<i>Chenopodium quinoa</i>
QCI56004.1	<i>Iris sanguinea</i>
XP_010538778.1	<i>Tarenaya hassleriana</i>
XP_027183517.1	<i>Coffea eugenioides</i>
AHU88620.1	<i>Magnolia sprengeri</i>
XP_028787846.1	<i>Prosopis alba</i>
EOY24569.1	<i>Theobroma cacao</i>
AEF14411.1	<i>Onobrychis viciifolia</i>
ACM62747.1	<i>Garcinia mangostana</i>
XP_017236102.1	<i>Daucus carota subsp. sativus</i>
XP_010934075.1	<i>Elaeis guineensis</i>

In certain embodiments, the anthocyanidin synthase (ANS) used in the methods of the invention may comprise further modifications designed to optimize the yield of the products produced by the methods of the invention.

In certain embodiments, the cell-free medium comprises anthocyanidin 3-O-glucosyltransferase (3GT) enzyme. In certain embodiments, the anthocyanidin 3-O-glucosyltransferase (3GT) used in the methods of the invention is an enzyme with an amino acid sequence at least 95% identical to any one of enzymes selected from the group consisting of:

Accession #	Organism
AFJ52972.1	<i>Linum usitatissimum</i>
ABR24135.1	<i>Vitis labrusca</i>
AHJ80981.1	<i>Erythranthe lewisii</i>
QCP68996.1	<i>Salvia miltiorrhiza</i>
XP_004307876.1	<i>Fragaria vesca subsp. Vesca</i>
XP_010549820.1	<i>Tarenaya hassleriana</i>
XP_010087491.1	<i>Morus notabilis</i>
BBC62118.1	<i>Nemophila menziesii</i>
PIN20524.1	<i>Handroanthus impetiginosus</i>
QJB23733.1	<i>Centaurea cyanus</i>
QBL54224.1	<i>Scutellaria baicalensis</i>
XP_024179381.1	<i>Rosa chinensis</i>
BAA89008.1	<i>Petunia x hybrida</i>

In certain embodiments, the anthocyanidin 3-O-glucosyltransferase (3GT) enzyme used in the methods of the invention may comprise further modifications designed to optimize the yield of the products produced by the methods of the invention.

In certain embodiments, the cell-free medium comprises sucrose synthase (SuSy) enzyme. In certain embodiments, the sucrose synthase (SuSy) is an enzyme with an amino acid sequence at least 95% identical to any one of enzymes selected from the group consisting of:

Accession #	Organism
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WP_004872341.1	<i>Acidithiobacillus caldus</i>
P30298.2	<i>Oryza sativa Japonica Group</i>
Q820M5.1	<i>Nitrosomonas europaea ATCC 19718</i>
P13708.2	<i>Glycine max</i>
CAA09297.1	<i>Nostoc sp. PCC 7119</i>
P49036.1	<i>Zea mays</i>
P49040.3	<i>Arabidopsis thaliana</i>
Q9M111.1	<i>Arabidopsis thaliana</i>
Q8W517	<i>Ipomoea batatas</i>
WP_011056890	<i>Thermosynechococcus elongatus</i>

In certain embodiments, the sucrose synthase (SuSy) enzyme used in the methods of the invention may comprise further modifications designed to optimize the yield of the products and/or intermediates produced by the methods of the invention.

- 5 In certain embodiments, the one or more enzymes involved in generation of UDP-glucose and/or recycling of one or more reaction intermediates are provided below. In certain embodiments, the enzymes used for generation of UDP-glucose (by reaction with UTP) and/or recycling of one or more intermediates are selected from the enzymes having at least 95% amino acid sequence identity from the enzymes provided in the Table below.

Enzyme	Accession No.
GLK	AAA27694.1
GLK	WP_331012003.1
PGM	AAC73782.1
PGM	A0A1H0E147
PGM	A0A7M2S2U2
UGP	EEW2752841.1
UGP	WP_021648042.1
UGP	WP_028847555.1

NDK	WP_000963837.1
NDK	CAF21035.1
NDK	WP_066795798
PPK2	WP_010968631
PPK2	WP_160857702

In certain embodiments, the invention provides engineered ANS/3GT/SuSy enzymes for the cell-free production of cyanidin-3-glucoside. In certain embodiments, the engineered ANS/3GT/SuSy enzymes were optimized for cell-free production of cyanidin-3-glucoside. In certain embodiments, engineered ANS/3GT/SuSy enzymes may include genetic modifications. In certain embodiments, the genetic modifications may be selected from a group consisting of: point mutations, insertions, deletions, and/or any other modifications such that those enzymes result in efficient and optimal cell-free production of cyanidin-3-glucoside. In certain embodiments, the ANS/3GT/SuSy enzymes used in the cell-free production of cyanidin-3-glucoside are any enzymes disclosed in this application.

In certain embodiments, the invention provides engineered and optimized GLK/PGM/UGP/NDK/PPK enzymes for generation of UDP-glucose and/or recycling of reaction intermediates involved in cell-free production of cyanidin-3-glucoside. In certain embodiments, the engineered and/or modified GLK/PGM/UGP/NDK/PPK enzymes may include genetic modifications. In certain embodiments, the genetic modifications may be selected from a group consisting of: point mutations, insertions, deletions, and/or any other modifications such that those enzymes result in efficient and optimal cell-free production of cyanidin-3-glucoside.

In certain aspects of the invention, the method of the cell-free production does not require the purification of the one or more enzymes from the lysed host organisms. In certain embodiments, the methods of the invention do not require the purification of one or more enzymes from the lysed biomass comprising host cell expressing the one or more enzymes for the cell-free production of carminic acid. In certain embodiments, the methods of the invention do not require the purification of CGT/SuSy/GLK/PGM/UGP/NDK/PPK from the lysed cells for cell-free

production of cyanidin-3-glucoside. This is advantageous because it increases the efficiency of the method and reduces the costs associated with the purification of these enzymes.

In certain embodiments, the cell-free medium may further comprise any other additional ingredients required for the cell-free production of cyanidin-3-glucoside. For example, in certain
5 embodiments, the cell-free medium comprises buffer, catechin, cyanidin, an activated sugar, magnesium chloride, cell lysate, sucrose, and/or water. In certain embodiments, the cell-free reaction mixture further comprises uridine diphosphate (UDP). In certain embodiments, the cell-free reaction mixture further comprises polyphosphate and/or glucose. In certain embodiments, the cell-free reaction mixture comprises UDP-glucose and/or UDP in catalytic quantities. In certain
10 embodiments, UDP-glucose is produced enzymatically in the reaction. In certain embodiments, the reaction mixture comprises glucose-1-phosphate. Glucose-1-phosphate may be generated in the cell-free reaction. Thus, in certain embodiments, glucose-1-phosphate is also present in catalytic quantity. In certain embodiments, the reaction medium comprises glucose and polyphosphate.

15 In certain preferred embodiments, the processes of the invention utilize catechin, glucose, and polyphosphate as the starting materials for production of cyanidin-3-glucoside. In certain embodiments, the processes of the invention utilize cyanidin, glucose, and polyphosphate as the starting materials for production of cyanidin-3-glucoside. In these embodiments, the invention provides that the other intermediates in the process for production of cyanidin-3-glucoside may be
20 recycled.

The conventional methods for production of anthocyanins either requires the extraction of the product from the plants, which is expensive and unpredictable, or certain cell-based approaches, for example, as described in U.S. Patent No. 8,962,327, which is incorporated by reference. However, the cell-based approaches have complications, such as the processes leading
25 to low yields, a higher number of by-products, and expensive purification and separation protocols. The methods of the current invention beneficially provide cell-free approaches for bioproduction of anthocyanins. In particular, the product titers for anthocyanins produced by the methods of the invention are higher than the product titer produced by any other known methods for production of anthocyanins. In certain embodiments, the product titers produced by methods of the invention
30 are at least two-fold higher than other known methods for production of anthocyanins. In certain

embodiments, the product titers produced by methods of the invention are at least five-fold higher than other known methods for production of anthocyanins. In certain embodiments, the product titers produced by methods of the invention are at least ten-fold higher than other known methods for production of anthocyanins. In certain embodiments, the product titers produced by methods of the invention are at least hundred-fold higher than other known methods for production of anthocyanins. In certain embodiments, the product titers produced by methods of the invention are at least five-hundred-fold higher than other known methods for production of anthocyanins. In certain embodiments, the product titers produced by methods of the invention are at least thousand-fold higher than other known methods for production of anthocyanins. In certain embodiments, the product titers produced by methods of the invention are at least five-thousand-fold higher than other known methods for production of anthocyanins.

In other beneficial aspects of the method of the invention, the methods of the invention provide economically efficient methods of production of anthocyanins. Because the methods of the invention provide for cell-free production of anthocyanins, it leads to decreased costs of purification of the reaction products. Moreover, in certain aspects of the invention, the one or more enzymes are included in the same reaction mixture, *i.e.*, the enzymes do not need to be isolated after being expressed in the host organisms. The one or more enzymes could be expressed in the host organisms at the same time and be utilized for the cell-free production without the need of any additional purification and/or separation steps.

In certain other beneficial aspects of the invention, the use of the one or more enzymes for the cell-free production of anthocyanins leads to high yield of the product to be produced. The one more enzyme may be further modified to optimize the yield of the product being produced in the cell-free reaction.

Compositions for cell-free production of anthocyanins

In certain aspects, the invention provides compositions for cell-free production of anthocyanins. The compositions of the invention are utilized for cell-free production of anthocyanins in accordance with the methods described above. In certain embodiments, the compositions comprise ingredients for cell-free production of cyanidin-3-glucoside.

In certain aspects, the invention provides compositions for cell-free production of cyanidin-3-glucoside. The compositions of the invention are utilized for cell-free production of cyanidin-3-glucoside in accordance with the methods described above.

In certain embodiments, the compositions of the invention comprise: one or more enzymes
5 in a cell-free medium, wherein the one or more enzymes result in transformation of a substrate to cyanidin-3-glucoside. In certain embodiments, the substrate converted to cyanidin-3-glucoside is cyanidin. In certain embodiments, the substrate converted to cyanidin-3-glucoside is catechin (with cyanidin as the intermediate). In certain embodiments, the one or more enzyme catalyzing the conversion of catechin to cyanidin is ANS. In certain embodiments, the one or more enzyme is
10 3GT. In certain embodiments, the composition is a cell-free medium. In certain embodiments, the cell-free medium comprises an activated sugar. In certain embodiments, the activated sugar is UDP-glucose. In certain embodiments, the UDP-glucose is added to the cell-free medium.

In certain aspects of the invention, the UDP-glucose in the composition is synthesized in the cell-free medium by the one or more enzymes. In certain embodiments, the UDP-glucose is
15 synthesized from sucrose. In certain embodiments, the one or more enzymes is sucrose synthase (SuSy) that synthesizes UDP-glucose from sucrose. In certain embodiments, the cell-free medium is a cell lysate. In certain embodiments, the cell lysate is a cell lysate from cells from a host organism expressing the one or more enzymes. In certain embodiments, the host organism is selected from a group consisting of: bacteria, yeast, and/or mammalian cells. In certain
20 embodiments, the one or more enzymes are introduced in the host organism by integration into genome of the host organism or on a plasmid. In certain embodiments, the host organisms expressing the one or more enzymes are cultured until a pre-determined biomass is achieved to produce the requisite quantity of the one or more enzymes. In certain embodiments, the composition further comprises cell lysate generated by lysis of cells and subsequent removal of
25 cell debris, for use in the cell-free medium for cell-free production of cyanidin-3-glycoside. In certain embodiments, the ANS, 3GT, and SuSy enzymes are present in the cell-free medium for cell-free production of cyanidin-3-glycoside. In certain embodiments, the compositions of the invention provide ANS, 3GT, and SuSy that have not been purified or separated.

In certain embodiments, the composition further comprises glucose-1-phosphate. In certain
30 embodiments, glucose-1-phosphate is the source for formation of UDP-glucose. Accordingly, in

certain embodiments, glucose-1-phosphate may be added to the reaction for generation of carminic acid. In certain embodiments, glucose-1-phosphate may be supplied to the reaction for preparation of carminic acid.

In certain embodiments, in the compositions of the invention, glucose-1-phosphate is generated during the course of the reaction. In certain embodiments, glucose-1-phosphate is generated enzymatically during the course of the reaction. Accordingly, in certain embodiments, the compositions of the invention comprise glucose and/or ATP. In certain embodiments, glucose is converted to glucose-6-phosphate by a reaction of ATP and glucose. In certain embodiments, reaction between glucose and ATP results in generation of glucose-6-phosphate and adenosine diphosphate (ADP). In certain embodiments, conversion of glucose to glucose-6-phosphate is catalyzed by glucokinase (GLK). In certain embodiments, conversion of glucose to glucose-6-phosphate is catalyzed by hexokinase (HK). In certain embodiments, glucose-6-phosphate is converted to glucose-1-phosphate. In certain embodiments, the conversion of glucose-6-phosphate to glucose-1-phosphate is catalyzed by phosphoglucomutase (PGM). Accordingly, in certain embodiments, the composition comprises GLK, HK, and/or PGM.

In certain aspects, the invention further provides that the nucleoside triphosphate species used in the reaction are recycled. In certain embodiments, the recycled nucleoside triphosphates are ATP and UTP. In certain embodiments, UTP is generated by reaction of UDP and ATP. In certain embodiments, the generation of UTP from UDP and ATP is catalyzed by nucleoside diphosphate kinase (NDK). In certain embodiments, ATP may be generated from ADP and phosphate or polyphosphate. In certain embodiments, the conversion of ADP to ATP is catalyzed by polyphosphate kinase (PPK). In certain embodiments, the compositions of the invention further comprise ATP, UTP, and/or UDP. In certain embodiments, the compositions of the invention further comprise PPK.

In certain embodiments, the composition further comprises anthocyanidin synthase enzyme (ANS), anthocyanidin 3-O-glucosyltransferase (3GT), sucrose synthase (SuSy), glucokinase (GLK), hexokinase (HK), phosphoglucomutase (PGM), polyphosphate kinase (PPK), UTP—glucose-1-phosphate uridylyltransferase (UGP), and/or nucleoside diphosphate kinase (NDK). In certain embodiments, C-glucosyltransferase (CGT), sucrose synthase (SuSy), glucokinase (GLK), hexokinase (HK), phosphoglucomutase (PGM), polyphosphate kinase (PPK),

UTP—glucose-1-phosphate uridylyltransferase (UGP), and/or nucleoside diphosphate kinase (NDK) are present in the cell-free medium for the cell-free production of cyanidin-3-glycoside.

In certain embodiments, the cell-free medium may further comprise any other additional ingredients required for the cell-free production of cyanidin-3-glycoside. For example, in certain
5 embodiments, the cell-free medium comprises buffer, catechin, cyanidin, an activated sugar, magnesium chloride, cell lysate, sucrose, and/or water. In certain embodiments, the buffer used in the cell-free reaction medium is any buffer suitable for enzymatic conversion of catechin/cyanidin to cyanidin-3-glycoside. In certain embodiments, the buffer maintains the pH of about 5 to about 9 in the reaction mixture. In certain embodiments, the buffer maintains the pH of about 6 to about
10 8 in the reaction mixture. In certain embodiments, the buffer maintains the pH in the range of 6 to 8 in the reaction mixture. In certain embodiments, the buffer is a phosphate buffer. In certain embodiments, the buffer is present at a concentration of about 1 mM to about 200 mM. In certain embodiments, the buffer is present at a concentration of about 5 mM to about 100 mM.

In certain embodiments, the cell-free reaction medium comprises activated sugar at a
15 concentration of about 0.001 mM to about 50 mM. In certain embodiments, the cell-free reaction medium comprises activated sugar at a concentration of about 0.01 mM to about 5 mM. In certain embodiments, the activated sugar is UDP-glucose.

In certain embodiments, the cell-free reaction medium comprises magnesium chloride at a concentration of about 0.5 mM to about 40 mM. In certain embodiments, the cell-free reaction
20 medium comprises magnesium chloride at a concentration of about 1 mM to about 20 mM.

In certain embodiments, the cell-free reaction medium further comprises sucrose. In certain embodiments, the sucrose is converted to UDP-glucose. Thus, the concentration of sucrose in the reaction mixture is determined by the quantity of UDP-glucose needed for the reaction for cell-free production of cyanidin-3-glycoside. In certain embodiments, sucrose is present at a
25 concentration of about 10 mM to about 1000 mM in the cell-free reaction medium. In certain embodiments, sucrose is present at a concentration of about 20 mM to about 800 mM in the cell-free reaction medium. In certain embodiments, sucrose is present at a concentration of about 50 mM to about 600 mM in the cell-free reaction medium.

In certain embodiments, the cell-free reaction medium further comprises glucose. In certain embodiments, the concentration of glucose in the reaction mixture is determined by the quantity of UDP-glucose needed for the reaction for cell-free production of cyanidin-3-glycoside. In certain embodiments, glucose is present at a concentration of about 1 mM to about 1000 mM in the cell-free reaction medium. In certain embodiments, glucose is present at a concentration of about 10 mM to about 1000 mM in the cell-free reaction medium. In certain embodiments, glucose is present at a concentration of about 50 mM to about 600 mM in the cell-free reaction medium.

In certain embodiments, the cell-free reaction medium further comprises phosphate/polyphosphate. In certain embodiments, the cell-free reaction medium further comprises polyphosphate. In certain embodiments, the concentration of polyphosphate in the reaction mixture is determined by the quantity of UDP-glucose needed for the reaction for cell-free production of carminic acid. In certain embodiments, polyphosphate is present at a concentration of about 0.1 g/L to about 500 g/L in the cell-free reaction medium. In certain embodiments, polyphosphate is present at a concentration of about 1 g/L to about 100 g/L in the cell-free reaction medium. In certain embodiments, polyphosphate is present at a concentration of about 1 g/L to about 60 g/L in the cell-free reaction medium. In certain embodiments, polyphosphate is present at a concentration of about 5 g/L to about 40 g/L in the cell-free reaction medium. In certain embodiments, polyphosphate is present at a concentration of about 25 g/L.

In certain embodiments, the cell-free reaction medium further comprises UTP and/or UDP. In certain embodiments, the concentration of UTP and/or UDP in the reaction mixture is determined by the quantity of UDP-glucose needed for the reaction for cell-free production of carminic acid. In certain embodiments, UTP and/or UDP is in the concentration of about 0.01 μ M to about 100 μ M in the cell-free medium. In certain embodiments, UTP and/or UDP is in the concentration of about 0.1 μ M to about 50 μ M in the cell-free medium. In certain embodiments, UTP and/or UDP is in the concentration of about 0.1 μ M to about 10 μ M in the cell-free medium. In certain embodiments, UTP and/or UDP is in the concentration of about 1 μ M in the cell-free medium. In certain embodiments, UTP and/or UDP is in the concentration of about 5 μ M in the cell-free medium.

In certain embodiments, the cell-free reaction medium further comprises ATP. In certain embodiments, the concentration of ATP in the reaction mixture is determined by the quantity of

UDP-glucose needed for the reaction for cell-free production of carminic acid. In certain embodiments, ATP is in the concentration of about 0.01 μM to about 100 μM in the cell-free medium. In certain embodiments, ATP is in the concentration of about 0.1 μM to about 50 μM in the cell-free medium. In certain embodiments, ATP is in the concentration of about 0.1 μM to about 10 μM in the cell-free medium. In certain embodiments, ATP is in the concentration of about 1 μM in the cell-free medium. In certain embodiments, ATP is in the concentration of about 5 μM in the cell-free medium.

In certain embodiments, the cell-free reaction medium comprises glucose-6-phosphate and/or glucose-1-phosphate. In certain embodiments, glucose-6-phosphate and/or glucose-1-phosphate are generated and/or recycled in the reaction medium. Thus, in certain embodiments, glucose-6-phosphate and/or glucose-1-phosphate are present in catalytic quantities in the reaction medium. In certain embodiments, glucose-6-phosphate and/or glucose-1-phosphate may be added in the reaction medium. In certain embodiments, glucose-6-phosphate and/or glucose-1-phosphate are present at a concentration of from about 0.01 mM to about 500 mM. In certain embodiments, glucose-6-phosphate and/or glucose-1-phosphate are present at a concentration of from about 0.01 mM to about 1 mM. In certain embodiments, glucose-6-phosphate and/or glucose-1-phosphate are present at a concentration of from about 1 mM to about 500 mM. In certain embodiments, glucose-6-phosphate and/or glucose-1-phosphate are present at a concentration of from about 10 mM to about 500 mM. In certain embodiments, glucose-6-phosphate and/or glucose-1-phosphate are present at a concentration of from about 10 mM to about 100 mM.

In certain embodiments, the quantity of the one or more enzymes for the cell-free production of carminic acid is dependent on the target quantity of carminic acid to be produced and/or the concentration of other ingredients present in the reaction mixture. In certain embodiments, the one or more enzymes are present in a concentration of from about 1% to about 50% (v/v). In certain embodiments, the one or more enzymes are present in a concentration of from about 2.5% to about 45% (v/v). In certain embodiments, the one or more enzymes are present in a concentration of from about 5% to about 40% (v/v). In certain embodiments, the one or more enzymes are present in a concentration of from about 7.5% to about 30% (v/v).

In certain aspects of the invention, the compositions of the invention are in a bubble column reactor, wherein the one or more enzymes are in a solution. In certain embodiments, the

compositions of the invention are in a packed bed reactor, wherein the one or more enzymes are immobilized.

In some embodiments, the enzymes may be immobilized. In some embodiments, immobilized enzymes may be immobilized onto solid supports. Non-limiting examples of solid supports may include (but are not limited to) epoxy methacrylate, carboxymethyl-cellulose, starch, collagen, ion exchange resins, amino C₆ methacrylate, or microporous polymethacrylate. In further embodiments, various surface chemistries may be used for linking the immobilized enzyme to a solid surface, including but not limited to covalent, adsorption, ionic, affinity, encapsulation, or entrapment. In other embodiments, immobilized enzymes may be immobilized in crosslinked enzyme aggregates. In other embodiments, the enzymes are non-immobilized. Either immobilized or non-immobilized enzymes may be employed in batch or continuous synthesis. For example, an immobilized enzyme on a solid support may be used in a cartridge through which a reaction mixture passes, whereby an immobilized enzyme may catalyze modification of substrate to produce the product at a high titer. Alternatively, a continuous method may comprise micro mixing of enzyme solution and substrate to produce the product at a high titer, while continuously removing product, removing (e.g., recovering) substrate, or both. In some embodiments removed (e.g., recovered) substrate may be recycled to increase process efficiency and overall yield.

In some embodiments, anthocyanidin synthase enzyme (ANS), anthocyanidin 3-O-glucosyltransferase (3GT), sucrose synthase (SuSy), glucokinase (GLK), hexokinase (HK), phosphoglucomutase (PGM), polyphosphate kinase (PPK), UTP—glucose-1-phosphate uridylyltransferase (UGP), and/or nucleoside diphosphate kinase (NDK) are immobilized.

In some embodiments, anthocyanidin synthase enzyme (ANS), anthocyanidin 3-O-glucosyltransferase (3GT), sucrose synthase (SuSy), glucokinase (GLK), hexokinase (HK), phosphoglucomutase (PGM), polyphosphate kinase (PPK), UTP—glucose-1-phosphate uridylyltransferase (UGP), and/or nucleoside diphosphate kinase (NDK) are non-immobilized.

In some embodiments, enzymes are recycled by ultrafiltration. In some embodiments ion exchange resins may be used to capture carminic acid during production. For example, amine-functionalized solid support may be added to capture carminic acid for continuous purification

from reaction mixture. In certain embodiments, the bioreactor system provided in PCT/US2021/064049, incorporated by reference in its entirety.

Advantageously, the cell-free production of carminic acid provided herein provides a significantly higher titer value for cyanidin-3-glycoside as compared to the conventional methods.

5 The higher titer values of cyanidin-3-glycoside provide additional cost advantages for production of cyanidin-3-glycoside because the higher titer cyanidin-3-glycoside would provide efficiency in purifying and/or concentrating carminic acid from the reaction mixture. In certain embodiments, the methods of the invention provide carminic acid titer value of from at least 5-fold higher than the conventional methods. In certain embodiments, the methods of the invention provide carminic

10 acid titer value of from at least 10-fold higher than the conventional methods. In certain embodiments, the methods of the invention provide cyanidin-3-glycoside titer value of from at least 50-fold higher than the conventional methods. In certain embodiments, the methods of the invention provide carminic acid titer value of from at least 100-fold higher than the conventional methods. In certain embodiments, the methods of the invention provide cyanidin-3-glycoside titer

15 value of from at least 500-fold higher than the conventional methods. In certain embodiments, the methods of the invention provide carminic acid titer value of from at least 1000-fold higher than the conventional methods.

In some embodiments, the isolated cyanidin-3-glycoside has a purity of about 10%, or about 20%, or about 30%, or about 40%, or about 50%, or about 60%, or about 70%, or about

20 80%, or about 90%, or about 95%, or about 99%, or about 100%.

In other embodiments, the isolated cyanidin-3-glycoside has a purity of from about 10% to 95%, or from about 10% to 90%, or from about 10% to 80% or from about 10% to 70%, or from about 10% to 60%, or from about 10% to 50%, or from about 10% to 40%, or from about 20% to 95%, or from about 20% to 90%, or from about 20% to 80% or from about 20% to 70%, or from

25 about 20% to 60%, or from about 20% to 50%, or from about 20% to 40%, or from about 50% to 95%, or from about 50% to 90%, or from about 50% to 80% or from about 50% to 70%, or from about 50% to 60%.

VII. Examples

Example 1: Bioproduction of cyanidin-3-glucoside:

The reaction conditions for the cell-free production of cyanidin-3-glucoside are provided below:

Component	Working Range
Buffer	pH 6-8, 5-100 mM
Ascorbic acid	5-30 mM
Ammonium iron sulfate	1-3 mM
α -Ketoglutaric acid	20-30 mM
Catechin	1-20 mM
UDP/UDP-glucose	0.01-5mM
Magnesium chloride	1-20 mM
Sucrose	50-600 mM
Host microbe lysate with enzymes/Purified enzymes	5-40% (v/v)
Time	0.5h-20h
Temperature	20C-40C

FIG. 4 provides the HPLC chromatograms for the formation of cyanidin and cyanidin-3-glucoside.

Example 2: Reaction for generation of UDP-glucose and/or recycling of intermediates

The reaction conditions for the reactions for generation of UDP-glucose and/or recycling of intermediates are provided in the Table below.

Component	Working Range
Buffer	pH 6-8, 5-200 mM
Magnesium chloride	1-100 mM
Catechin and/or Cyanidin	0.1 – 50 g/L
Polyphosphate	1-60 g/L
UTP or UDP	0.1-10 μ M
ATP	0.1-10 μ M
Glucose, glucose-1-phosphate, glucose-6-phosphate, or UDP-glucose	0.01-500 mM
Host microbe lysate(s) with enzymes	0.5-40% (v/v)
Time	0.5h-48h

Temperature	20 °C-40 °C
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Table 1 provides exemplary sequences for anthocyanin synthase (ANS) enzymes in accordance with the methods of invention.

Table 1:

Anthocyanidin Synthase Enzymes			
SEQ ID NO:	Accession #	Organism	Sequence
1	XP_02273675 8.1	<i>Durio zibethinus</i>	MVTSVGSRVESLASSGIQSIPKEYIRPQEELTSI GNVFEEEEKKEEGPQVPTIDLKEIESEDKEVRER CHQQLKKAAMEWGVMLVNHGISDELTDREV KKAGQKFFELPIEEKEKYANDQASGKIQGYGS KLANNASGQLEWEDYFFHLVYPEEKRDLSIWP KTPSDYTEVTSEYARQLRGLASKILSALSCLG LEEGRLEKEVGGLEELLQMKINYYPKCPQPE LALGVEAHTDVSALTFFLHNMVPGQLQFYEGK WVTAKCVPNSIIMHIGDTIEILSNGKYKSILHRG LVNKEKVRISWAVFCEPPKEKILKPLPETVSET EPPLFPPTFAQHIOHKLFRKTQDGLSN
2	XP_03050151 2.1	<i>Camabis sativa</i>	MVSAAQRVESLSSSGIQSIPKEYVRPEEELKNIG NVFEEEEKKDEGPQVPTIDLKGINSEDEPGVREKC REELKRAAAEWGVMLVNHGISDELIDRVKK AGQTFEFLPVEEKEKYANDQASTNIQGYGSKL ANNASGQLEWQDYFFHLVFPEDKRDLSIWPKI PTDYIEATSEYARLLRGLTTILSVLSLGLGLEE DRLEREVGGLEELLQMKINYYPKCPQPELAL GVEAHTDVSALTFLHNMVPGQLQFYQGNWV TAKCVPNSIIMHIGDTVEILSNGKYKSILHRSLV NEEKVRVSWAVFCEPPKEKILKPLPEVVSEAE PPLFPPTFAQHIEHKLFRKTQELNGSK
3	XP_02876275 5.1	<i>Prosopis alba</i>	MVSSAAVVAPRVERLATSGIKSIPKEYVRPQEE LTNIGNVFEEEEKKEGPEVPTIDLTEIESEDEVVR ARCHETLKKAAQEWGMNLVNHGIPPELLNQ LRKAGETFFSLPIEEKEKYANDQASGKIQGYGS KLANNASGQLEWEDYFFHLVFPEDKCDLSIWP RTPSDYIEVTSEYARQLRGLATKILGALSGLG LEKGRLEEEVGGMEELLQMKINYYPICPQPEL ALGVEAHTDVSSLTFLHNMVPGQLQFYNGQ WITAKCVPNSIFMHIGDTVEILSNGRYKSILHR

			GLVNKEKVRISWAVFCEPPKEKILKPLPELVT DDEPARFPPTFAQHIQHKLFRKCQEGLSK
4	XP_02190184 6.1	<i>Carica papaya</i>	MFSSVAVPRVEILASSGIESIPKEYVRPQEELTTI GNIFDEEKKDEGPQVPTIDLRDIDSDDQQVRQR CRDELKKAADVWGVMLVNHGIPDHLIDRVK KAGQAFFELPVEVKEKYANDQASGNIQGYGS KLANNASGQLEWEDYYFHLIFPEEKRD LAIWP NNPADYIEVTSEYARQLRRLVSKILGVLSLGLG LEEGRLEKEVGGLEDLLQMKINYYPTCPQPE LALGVEAHTDISALTFILHNMPGLQLFYEGK WVTAKCVPNSIVMHVGD TIEILSNGKYKSILHR GLVNKEKVRISWAVFCEPPKEKILKPLPETVSE NEPPLFPPTFAQHIQHKLFRKNQENLEAK
5	XP_02173595 0.1	<i>Chenopodium quinoa</i>	MTQNKEPVNQKSEHDEQRVESLASSGIESIPK EYVRLNEELTSMGNVFEEKKKEEGSQVPTIDIK DIASEDPEVRGKAIQELKRAAMEWGVMHLVN HGISDELIDRVKVAGQTFELPVEEKEKYAND QASGNVQGYGSKLANSASGRLEWEDYYFHL YPEDKRDLSIWPETPADYIPAVSEYSKELRYLA TKILSALSALGLEEGRLEKEVGGLEELLQFKI NYYPKCPQPELALGVEAHTDVSALTFILHNMP PGLQLFYEGKWVTAKCVPNSIIMHIGDTIEILSN GKYKSILHRGLVNKEKVRISWAVFCEPPKEKII LKPLPDLVSDEEPARYPPPTFAQHVVQYKLFK TQGPQTITKN
6	QCI56004.1	<i>Iris sanguinea</i>	MASSKVMPAPARVESLASSGLASIPTEYVRPE WERDDSLGDALEEIKKTEEGPQIPIVDLRGFD GDEKERLHCMEEVKEAAVEWGVMHIVNHGIA PELIERVRAAGKGFFDLPVEAKERYANNQSEG KIQGYGSKLANNASGQLEWEDYFFHLIFPSDK VDLSIWPKEPADYTEVMMEFAKQLRVVVTM LSILSLGLGFEEKLEKKLGGMEELLMQMKIN YYPKCPQPELALGVEAHTDVSSLSFILHNGVPG LQVFHGGRWVNARLVPGSLVVHVGD TLEILS NGRYKSVLHRGLVNKEKVRISWAVFCEPPKEK IVLEPLAELVDKRSPAKYPPPTFAQHIQHKLFRK KAQEQLAGGVHIPEAIQN
7	XP_01053877 8.1	<i>Tarenaya hassleriana</i>	MVATTRVESLVNSGIESVPKEYIRPKEELMSIG DIFOEEKNEEGPQLPTIDLQAIDSEDET VRRKC MEELRNAAMEWGVMHLINHGIPEDLIERLKKS GEGFFRLPVEEKEKYANDQAAGKIQGYGSKLA NNASGQLEWEDYFFHLVFPEDKRDLSLWPKTP SDYTEVTS DYAKRLRALAGKIFSCLSVGLGLEP ERLEKEVGGMEELLQMKINYYPKCPQPELAL GVEAHTDVSALTFILHNMPGLQLFYEGKWV TAKCVPNSIIMHIGDTLEILSNGAYKSILHRGLV

			NKEKVRISWAVFCEPPKEKIVLKPLPETVTAES PAKFPPRTFAQHIIHKLFRKAQEDEDGKLATK EDAN
8	XP_02718351 7.1	<i>Coffea eugenoides</i>	MTTAAVTSRVERLASSGIQLIPKEYVRLQEELT NMGNVFEEEEKNNEGPQVPTIDLGDIEAEDEVV RERCNELKKAAMEWGVMHLVNHGISNELIN RVKVAGEAFFNLPVEEKEYANDQASGNLQG YGSKLANSASGQLEWEDYFFHCAFPEDKRDLS IWPKTPEDYIPAASEYAKQLRGLATKILAVLSL GLGLEEGRLEKEVGGIEELILQMKINYYPKCPQ PELALGVEAHTDVSSLTFILHNMVPGQLQFYE DKWITAKCVPNSIIMHIGDTIEILSNGKYKSILH RSLVNKDKVRISWAVFCEPPKEKIIILKPLPETVS ETEPPLYLPRTFAQHIDHKLFRKTEGAVEKNQS TEDNKSPEDNQPREDGKRS
9	AHU88620.1	<i>Magnolia sprengeri</i>	MATQVASIPRVEMLASAGIAIPTEYVRPEAER NSIGDVFEEEEKLEGQPQIPVVDLMGLEWENEE VFKKVEEDMKKAASEWGVMHIINHGISMELM DRVRIAGKAFFDLPIEEKEMYANDQASGKIAG YGSKLANNASGQLEWEDYFFHLIFPEDKRDMS IWPKQPSDYVEATEEFAKQLRGLVTKVLVLLS RGLGVEEDRLEKEFGGMEELLQMKINYYPKC PQPDALGVEAHTDVSALTFILHNMVPGQLQVF FDDKWVTAKCIPGALVVHIGDSLEILSNGKYR SILHRGLVNKEKVRISWAIFCEPPKEKVVLQPL PELVSEAEPARFTPRTFSQHVRQKLFKKQQDA LENLKSE
10	XP_02878784 6.1	<i>Prosopis alba</i>	MVSSAAVVATRVERLATSGIKSIPKEYVRPQEE LTNIGNVFEEEEKKEGPEVPTIDLTEIESEDEVVR ARCHETLKKAQEWGVMNLVNHGPIPELLNQ LRKAGETFFSLPIEKEKYANDQASGKIQGYGS KLANNASGQLEWEDYFFHLVFPEDKCDLSIWP RTPSDYIEVTSEYARQLRGLATKILGALSGLG LEKGRLEEEVGGMEELLQMKINYYPICPQPEL ALGVEAHTDVSSLTFLHNMVPGQLQFYNGQ WITAKCVPNSIFMHIGDTVEILSNGRYKSILHR GLVNKEKVRISWAVFCEPPKEKIIILKPLPELVT DDEPARFPPRTFAQHIIHKLFRKCQEGLSK
11	EOY24569.1	<i>Theobroma cacao</i>	MVTSMAPRVESLASSGIQSIPKEYIRPQEELTSI GNVFEEEEKKEEGPQVPTIDLKEIDSEDREVRER CRQELKKAATEWGVMHLVNHGISDELMERVK KAGQKFFELSVEEKEYANDQALGKIQGYGS KLANNASGQLEWEDYFFHLVYPEDKRDLSIWP QTPSDYTEVTSEYARQLRVLASKILSALSCLG LEEGRLEKEVGGLEELLQMKINYYPKCPQPE LALGVEAHTDVSALTFILHNMVPGQLQFYEGK

			WITAKCVPNSIIMHIGDTVEILSNGKYKSILHRG LVNKEKVRISWAVFCEPPKEKIILKPLPETVSET EPPLFPPRTFAQHIIHKLFRKTQDGLSN
12	AEF14411.1	<i>Onobrychis viciifolia</i>	MGTLAQRVESLSDSGIQSIPKEYVRPKEELANI GNIFEEEEKKEGPVPTIDLKEIESKDELVRGKCR EKLRKAAEEWGVMHLVNHGIPDELLNRLKEA GAFFSLPVEEKEYANDQATGNVQGYGSKL ANNASGQLEWEDYFFHLIFPEDKRDLSIWPKTP PHYTEVTSEYAKRLRVLASKILKALSLELGL GRLEKEVGGMEELLQMKINYYPICPQPELAL GVEAHTDISSLTFLHNMVPGQLQFYQGWVT AKCVPGSILMHIGDTIEILSNGKFKSILHRGLVN KEKVRISWAVFCEPPKEKIILKPLPELVTPTEPA RFPRTFAQHIIHKLFRKDQEEAAAAAANSK
13	ACM62747.1	<i>Garcinia mangostana</i>	MVTSVAPRVETLASSGIQIPKEYIRPQEELTNL GNIFEQEKKEGPVPTIDLEGIVSEDKEVRDKC WDELMKAAKEWGVMHLVNHGISNELTEKVKI AGEAFFQLPIEKEYANDQSGMIQGYGSKL ANNASGRLEWEDYFFHLVPPEEKRDLSIWPKT PSDYIEVTSEYARQLRALATKVLSSLSLCLGLE EGRLEKEVGGIEELALQMKINYYPKCPQPELA LGVEAHTDVSALTFILHNMVPGQLQFYEGEWV TAKCVPNSIIMHIGDTLEILSNGKLSILHRGVV NKEKVRISWAVFCEPPKDKIILKPLPELVSETEP PMFPPRTFSQHIQHKLFRKNQDDVGP
14	XP_01723610 2.1	<i>Daucus carota</i> <i>subsp. sativus</i>	MVATTLESVRVETLAGSGIQLIPKEYVRPKDEL VSITNIFEEEEKSDEGPQVPTVDIADILSDDRAVR EKCYERIKDAAVDWGVMHLVNHGISNELMDR VRVAGQAFFAEPIGEKEYANDPGTGMIOGYG SKLANNASGQLEWEDYFFHLVYPEEKADLSIW PKKPQDYIPATREYAKELRGLATKLLSALSIGL GLEEGRLEKEVGGMEELILQMKINYYPKCPQP ELALGVEAHTDVSALSFILHNMVPGQLQFYGG KWVTAKCVPDSIIVHIGDTLEILSNGKYKSILH RGLVNKEKVRISWAVFCEPPKEKIILKPLPETV TEAEPPLFPPRTFAQHMAHKLFRKSQEAIDDSK KVQPQEQNNAETDIPQPEEQKTVESKPQKIEI LKPGEESTSP
15	XP_01093407 5.1	<i>Elaeis guineensis</i>	MASKVASAVPRVESLAKRGLEAIPTEYVRPES ERRDLGDAFEEEEKAAEGPQIPVVDLKGFDSE DEVVRRACAEVRRVASEWGVMHIVNHGIPL ELIEKVRRVGKEFFDLPIEKEYANDQSSGMI AGYGSKLANNASGQLEWEDYFFHLIFPHDKA DMSIWPKNPANYVEVTKEFAERLRGVVSKML AILSLGLGLDEAKLEKELGGMEDLLLQMKINY YPRCPQPDALGVEAHTDVSSLSFILHNMVPG

			LQVYKDNKWVTAKCVPDSMIVHIGDTLEILSN GNYKSILHRGLVNKEKVRISWAVFCEPPKEKIV LKPLPELVTDETPAKFPPTFAQHIQHKLFKKT QEDFISPK
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Table 2 provides exemplary sequences for anthocyanidin 3-O-glucosyltransferase (3GT) enzymes in accordance with the methods of invention.

Table 2:

Anthocyanidin 3-O-glucosyltransferase (3GT) Enzymes			
SEQ ID NO:	Accession #	Organism	Sequence
16	AFJ52972.1	<i>Linum usitatissimum</i>	MPPPATTPDGPVVVLSFPFGTHAAPLLAVVKRL AALSPATHFSFFGTEQSNASAFKNQAAVSESPN LRAHMOVWNGIPEGYELTGRPQEAIEFMKAAPG EFRKGIEAAVEESGRKVS WLVTDAFYWFASEMG IPWIAFWTAGPNSLSAHLHTDPLREALGSPDAV VGREEETLK GIVAGMSKLRFRDLPEGVVKG NLQ SIFSTMLHNMATHLPKAAAVFINSFHALDPTITDD LSSKLNFLNIGPFHLLYPSASKEQQQPSDCIS WLNDQRHLPASVAYLSFGSVVTPPPHELA AVE ALEASKVPFIWSLKEHKAHL PDGFLDWSKGNG VVVPWAPQMEVLGHQAVGVFITHCGWNSVLESI AGGVPMICRPFFGDQIINSQMVEKVWEIGVNLEG GAFTKSGLV SCLDRVLRQEEGKKVRVNTRHLRE KAEATQSKGSSSADFFKLVELVSKST
17	ABR24135.1	<i>Vitis labrusca</i>	MSQTTTNP HVAVLAFPFSTHAAPLLAVVRR LAV AAPHA VFSFFSTSESNASIFHDSMIHTMQCNISYD VSDGVPEGYVFTGRPQEGIDLFMRAAPESFRQGM VMAVAETGRPVSCLVADAFIWFAADMAAEMGV AWLPFWTAGPNSLSTHVYIDEIREKIGVSGIQGRE DELLNFIPGMSKVRFRDLQEGIVFGNLNLSLFSRLL HRMGQVLPKATAVFINSFEELDDSLTNDLKSCLK TYLNIGPFNLITPPPVPNTTGCLQWLKERKPTSV VYISFGTVTTPPPAELVALAEALASRVVPFIWSLR DKARMHLPEGFLEKTRGHGMVVPWAPQAEVLA HEAVGAFVTHCGWNSLWESVAGGVPLICRPFFG DQRLNGRMVEDVLEIGVRIEGGVFTKSGLMSCFD QILSQEKGKKLRENLRALRETADRAVGPKGSSTE NFKTLVDLVSKPKDV
18	AHJ80981.1	<i>Erythranthe lewisii</i>	MSFHLHIGVLAFPFPGTHAAPLLALVQRLATASAP AGEAGARFSFFNSAVSNSSMSFSNSCENIKAYDV

			WDGTPEGEVFGGTHSDAVGLFLKASPRNFEKAIE EAEEDAGMKISCLISDAFLWFACDLAERRGVPW VPFWTAASCSLSAHFYTEEILTAFVGFKETAEEQ QTLTFIPGLSKVHFSDLPPEIFVEQNPSPLAITINN MVGKLPKATAVVLNSFEEIDPIITKDLKSKFQDFL NVGPSILASPDTPPSPDHDKTGCLSWLETQNKPKS VVYISFGTVVIPSEKELLALAETLETRKLPFLWSL GGNAKKYLPEGFIERTSGFGKIVPWAPQAGVLAH NSVGVFVTHCGWNSVLESVSRGVPLICRPFFGDQ KINSRMVEVSWKIGVRVEGGVFTKASTMKALDR IMSGEAGKVIRENVRRLKDKAVSAVELKGSSSKN FEKLEHSVPN
19	QCP68996.1	<i>Salvia miltiorrhiza</i>	MGFKLHIGVLAFFPGTHAPPLLALVQRLAASAPD ALFSFFNSATSNAALFNNTSYDNIRPCDVWDGTPH GEAFTGSHFEAVGLFLKASPGNFDKVIQEAHQ GLKICCLITDAFLWFACDMAEKRGVPWLPFWTA ASCSLSSHLYTDQIVKAIQSTETAKQDKALSFIPG LEMVTTSDLPPEVFLDNPSPLAITINKMVQQLPK STAVVLNSFEEIDPIITKDLKSKFRHFLNVGPSILA SPARAAPDDKTGCLSWLGSQNRPNVSVYISFGTV ITPPENELAALAEALETCGFPFLWSLKEHAKKSLP TGFLERTNSLGKIVAWAPQQQVLAHENVGVFVT HCGWNSILESISCVPLICRPFFGDQMLNSRMVQD SWKVGVRVKGGVFTKSETVESLRELMTEKGEK MRENVGVLKDKAMAAVEPQGTSSKNFIKLEIIR APESR
20	XP__004307876.1	<i>Fragaria vesca subsp. Vesca</i>	MAPVSNQVGGHVAVLAFFPFSTHAAPLLNIVCRL ATAAPSTLFSFFNTKQSNSSILAGNTSVLRYSNVS VCEVADGVPEGYVFGKPPQEDIELFMKAAPDNF RRCLEASVAESGREVSCLVTDAFFWFGAHMADD MGGVPWVPFWTAGPASLSAHVHTDLIRNTTGGG GHDEKETITVIAGMSKVRPQDLPEGHIFGNLESLS RMLHQMGMQMLPLATAVFINSFEELDPVITNDLKS KFKRFLNVGPLDLLEPPASAATTPQTAEAVAGD GCLSWLDKQKAASVVYVSFGSVTRPSPEELMAL AEALEASRVPLWLSRLDNLKNPQLDEFLSKGKLN GMVVPWAPQPQVLAHGSVGAFVTHCGWNSVLE SVAGGVPLICRPFFGDQKLNARMVEDVWKIGLR LEGGVFTKNGMLKSLDMLLSQDEGAKMKNKINT LKQLAQQAPEPKGSSTRNFESLLEMTTIN
21	XP__010549820.1	<i>Tarenaya hassleriana</i>	MAEPSHAARDTHVAVLAFFPGTHAPLLSVTRCL AAAAPAARFSFFGTAQSNSASVGGDGLPANLRT YDVSDGVPEGYVFTGKPQEAIELFLTAAPESFR GMGTAEAEVGRRVTCLVTDAFYWFAAEMAAEM EAVVWAFWTAGPCSLSAHLCTDLIRRTIGLQEV GRKEEKLGFITGMEKMRVKDLPEGVVFGNLESIF

			SRMLHQMGGLALPKASALFLNSFEELDPLLSTLSS NLNRFLNVGPLPLSTPPKVSTLNDTYNCLAWLDN QALGSVAYISFGTVMTPPPSELQAMAEGLEASRV PFIWSLKDKDRAHLPKGFQDRTREQGLVVPWAP QVKVLEHRATGVFVTHCGWNSVLESVAGGIPMI CRPFFGDQRLNGRAVEAVWEIGVTIDGGAFTRD GFVKCLDQILGRDSGRKMRGNACKKLKGLAHEA VSANGSSTQNFKDLSEIIVM
22	XP_010087491.1	<i>Morus notabilis</i>	MAPSPCHVAVAAFPFGTHAAPLLAIVRRLAAAS PGTTFFENTRQSNNSIFNKDTATQFPNITAYDIW DGVPEGHVFSGKPKQERVELFVKAAPESLRFNIEM AVVETGREVSCLLTDAFFWFGGELAQDMRVAS WVQFWTAGPCSLSAHFYTDLRQRISIAAKGSEN EPLSFIPGMSKVLVQDLPEGVLFGKLESVFSNML HKMGLLLPRAKAVFINSFEELDKTITHDLKSKFN KFLNVGPLNVVSPSLPPTPDESGCLRWLDEQKAD ASVAYISFGSVMVPPREELEEIAAALEGGGIPFIW SLRENAKENLPIGFLEKTRKTGMVVPWAPQIDIL AHRAVGVFVTHCGWNSVLESIVGGVPMICRPFFG DQRLNGRMVQDVWEIGVKVEGGAFTAGLERC LDLVLCQERRKKMRDKIKSLKEAANEAVGPRGS STENFKLLLDVVTRPQGVVRLI
23	BBC62118.1	<i>Nemophila menziesii</i>	MSKQIHVAVLAFPGTHGAPLLNLVQRLAKALP NAIFSFSTHKSNSLSFSSSLTLDNVKAYDVDDGV PEGYVLGKSQEDIELFLKVAREGFKNEIAVAEEK VGRKISCVIADPFLWFTCEMAEEMGVDWIAFWT SGVTPLSAHFHTDLVRQTIALDGANERKEELVTFI PGFSELSLGDLPDGILFGNLHSPFSQMLHKMGQT LPRASAIILNSFEDLNPHIVKDLRTNFKKFLNIGPF GLISPTPISKVSDVHGCLSWLDQQKEGSVAYIGFG TVVAPPPHELKALAEALEERKTPFLWSLKDHKLK NLPEGFLERTKEIGKIVPWPQVQVLGHKATGVFI FHGGWNSMLESVVGGVVPVIGRPFFGDQHVNVSV VERVWKTGIKIEGGVFSGDGVVLQGLELVLSSERG KEMKERVRELRELAFKSVEPNGSSSKNFETLLSM LL
24	PIN20524.1	<i>Handroanthus impetiginosus</i>	MVSPLHIGVLAFPGTHAAPLLTLVQRLAASAPW IQSFENTASNDKIFNAHVSGSYDNIKVYNVWD GTSEGGFSGHFEAVGLFLKASPGNFEEKVIGEAEEE TGLEICCFISDAFLWFACDLADRKGVPWVAFWT AASCSLSAHVYTQEIIVKAGGLSSTETAEETTTSF VPGLEMVRFSIDLPEIFLDKNPSPFALTINIMVQK LPQSTAVVLNSFEEIDPVITNDLKSKFQHFLNIGPS ILSSPTPTSPDDKTGCLSWLEKQSRPKSVIYISFGS VVTTPPENELVALAEALCTCKFPFLWSLKDHAKKS LPEGFLNRTIAFGKIVPWPQVQVLGHKSVGLFV

			THCGWNSILESICGSVPMICRPFFGDQKLNGRMV EDSWKIGVRIKGGVFTKNETIEVLNNMMSGEEG KKVRENIYQLKEKAVNAVDFEGSSTKNFKKLEI IGGPKCDKKLV
25	QJB23733.1	<i>Centaurea cyanus</i>	MEDLVLTSGNHNNGRSRPERHVAVFAFPSSSHP SLLTLIRRLSSASPTTVFSFFNTAKFNRPVFSDFS SPDIRRYDISDGVPEGYVFRGKPQEDTNLFLAVA EEELRKGVKVAEADIGLKVSCLVVDAFYWFSGD MAEEMNVPWVSFWTAGACSLAAHVYTDLIRSKS SQIHKSSAVPDEDEIVDFIPGFSKLRLSDLP SGVLF GNLESPFSTMLHKMGKNMAKATAVPINSFQQLD PNLTKNLSSELNNFLNIGPFNLITSTQNPPSEVDEF SCISWLESQEPRSVAYISFGTVCKPAPHELVAIAE ALEEAKTPFLWSISNDSKKHFPEGFLERTTANGSG KVVWPAPQVQVLEHIAIGVFVTHGGWNSVLESV GAGVPMICRPLRGDQQINSWMVEKVWGIGVRIE GGSFTRRGTRCALEQVLSRDGSRKGFKERIEALK ELAHKAVEPNGSSNENFKILVDVVTRVTL
26	QBL54224.1	<i>Scutellaria baicalensis</i>	MVFQSHIGVLAFFPGTHAAPLLTVVQRLATSSPH TLFSFFNSAVSNSTLFNNGVLDSDYDNIRVYHVWD GTPQQGAFTGSHFEAVGLFLKASPGNFDKVIDEA EVETGLKISCLITDAFLWFGYDLAEKRGVPWLAF WTSAQCALSAHMYTHEILKAVGSNGVGETAEIII LIQSLIPGLEMAHLSLDPPEIFFDKNPNPPLAITINK MVLKLPKSTAVILNSFEEIDPIITDLKSKFHHFLN IGPSILSSPTPPPPDDKTGCLAWLDSQTRPKSVVYI SFGTVITPPENELAALSEALET CNYPFLWSLNDRA KKSLPTGFLDRTKELGMIVPWAPQPRVLAHRSVG VFVTHCGWNSILESICSGVPLICRPFFGDQKLNSR MVEDSWKIGVRLEGGVLSKTATVEALGRVMMS EEGEIIRENVNEMNEKAKIAVEPKGSSFKNFNKL EIIINAPQSS
27	XP_024179381.1	<i>Rosa chinensis</i>	MAPASNQVGGHVAVLAFFPFSTHAAPLLNIVCRL AAATPNTLFSFFNTKQSNSSILASNTSSILRNSNR VCEVADGVPEGYVFGKPQEDIELFMKAAPDNF RRCLEASEAETGRKVSCLVTD AFFWFGAHMADD LGGLPWVPFWTAGPASLSAHVHTDLIRNTTSMG GHDGNETVTVIAGMSKVRPQDLPEGHIFGKLDSLF SRMLHQMGMLPLATAVFINSFEELDPVITNDLK SKFKRYLNVGPFDLLES PAPAATTTLQTGDVVVG DGCLSWLDKQKAASVVYVSFGSVTRPSPEELMA LAEALEASRVFPLWSLRNNLKT PKLDEFISKAEL NGMVVPWVPQPQVLAHGSVGA FVTHCGWNSVL ESVAGGVPMICRPFFGDQKL NARMVEDEWKIGL KLEGGVFTKNGMLKSLDILLSQKKGNIMRDKINT FKQLAQQA VEPKGSSSTRNFESLLEVISTAN

28	BAA89008.1	<i>Petunia x hybrida</i>	MTTSQLHIALLAFFPGSHAAPLLTLVQKLSPLPS DTIFSFNTSQSNTSIFSEGSKPDNIK VYNVWDGV TETNGNKPVGLEAIKLFIQATPTNFEKVMKEAEE ETGVKPFSCIFSDAFLWFSYKLAEKINVPWIAFWT AASGSLSVHLYTDFIRSNDETSLNPGFSSTLKISD MPPEVMAENLDLPMPSMLYNMALNLHKA AAVV LNSFEELDPTINKDLKVKLQKVLNIGPLVLQPTSP KKVLDACDERGCHWLEKQKEESVVYLSFGTVTT LPPNEIVAVAEALEAKKFFIWSLKDNGIKNLPTG FLERTGQFGKIVSWAPQLEILNHS AVGVFVTHCG WNSILEGISC GVP MICRPFFGDQKLNSRMVESVW QIGLQIEGGSFTKIGTISALDTFFSEEKGKVLRENV KGLKERALEAVKPDGSSSKNFKDLVELVKCHKL T
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Table 3 provides exemplary sequences for sucrose synthase (SuSy) enzymes in accordance with the methods of invention.

Table 3:

Sucrose Synthase Enzymes			
SEQ ID NO:	Accession #	Organism	Sequence
29	WP_004872341.1	<i>Acidithiobacillus caldus</i>	MIEALRQQLDDPRSWYAFRLHLVASQRDS WLYTDLQRACADFREQLP EGYAEGIGPLEDF VAHTQEVI FRDPWMVF AWRPRPGRWIYVRI HREQLALEELSTDAYLQAKEGIVGLGAEGEA VLTVD FRDFRPVSRRLRDESTIGDGLTHLNR RLAGRIFSDLAAGRSQILEFLSLHRLDGQNL MLSNGNTDFDSL RQTVQYLGTLPRETPWAEI REDMRRRGFAPGWGNTAGRVRETMRLLMD LLDSPSPA ALESFLDRIPMISRILIVSIHG WFA QDKVLGRPDTGGQVVYILDQARALEREMRN RLRQQGV DVEPRILIATRLIPESDGTTC DQRL EPVVGAENVQILRV PFRYPDGRIPHWHISRFK IWPWLERYAQDLEREVL AELGSRPDLIIGNY SDGNLVATLLSERLGVTQC NIAHALEKSKYL YSDLHWRDHEQDHHFACQFTADLIAMNAA DIIVTSTYQEIAGNDREIGQYEGHQDYTL PGL YRVENGIDVFDSKF NIVSPGADPRFYFSYART EERPSFLEPEIESLLFGREPGADRRGVLEDRO KPLLLSMARMDRIKNLSGLAELYGRSSRLRG LANLVIIGGHVDVGNSRDAEERE EIRRMHEI MDHYQLD GQLRWVGALLDKTVAGEL YRVV

			ADGRGVFVQPALFEAFGLTVIEAMSSGLPVF ATRFGGPLEIHEDGVSGFHIDPNDHEATAERL ADFLEAARERPKEYWLEISDAALARVAERYT WERYAERLMTIARIFGFWRFLDRESQVME RYLOMFRHLOWRPLAHAVPME
30	P30298.2	<i>Oryza sativa</i> <i>Japonica Group</i>	MAAKLARLHSLRERLGATFSSHPNELIALFS RYVNQGGKMLQRHQLLAEFDAIEADKEY APFEDILRAAQEAIVLPPWVALAIRPRPGVW DYIRVNVSELAVEELSVSEYLAKEQLVDGH TNSNFVLELDFEPFNASFPRPSMSKSGNGVQ FLNRHLSKLFQDKESLYPLNFKAHNHKG TTMMLNDRIQSLRGLQSSLRKAEYLMGIPQ DTPYSEFNHRFQELGLEKGWGDCAKRVLDT IHLLLDLLEAPDPANLEKFLGTIPMMFNVVIL SPHGYFAQSNVLGYPDTGGQVVYILDQVRA LENEMLLRIKQQGLDITPKILIVTRLPLDAVG TTCGQRVKVGTEHTDILRVPRSENGILRK WISRFDVWPFLETYTEDVANEIMREMQAKP DLIIGNYSDGNLVATLLAHKLGVTQCTIAHA LEKTKYPNSDIYLDKFDSQYHFSCQFTADLIA MNHTDFIITSTFQEIAGSKDTVGGQYESHIAFT LPGLYRVVHGIDVDFPKFNIVSPGADMVSYF PYTEADKRLTAFHPEIEELLYSEVENDEHKF VLKDKNKPIIFSMARLDRVKNMTGLVEMYG KNAHLRDLANLVIVCGDHGNOSKDREEQAE FKKMYGLIDQYKLGHIRWISAQMNRVRNG ELRYRICDTKGVFVQPAFYEAFLTVIEAMT CGLPTIATCHGGPAEIIVDGVSGLHIDPYHSD KAADILVNFFEKCKQDSTYWDNISQGGQRI YEKYTWKLYSERLMTLTGVYGFVKYVSNL ERRETRRYIEMFYALKYRSLASAVPLAVDGE STSK
31	Q820M5.1	<i>Nitrosomonas</i> <i>europaea ATCC</i> <i>19718</i>	MTTIDTLATCTQQNRDAVYTLLRRYFTANR TLLQLQSDLREGLLQTEQDCGQSDMLRAFVFR LQEGIFSSPWAYLALRPEIAKWEFMRIHQEH LIPEKLTISEFLKFKETVVKGEATESVLEVDF GPFNRGFPRLKESRSIGQGVIFLNRKLSSEMF SRIEAGHTSLLHFLGVHAIEGQQLMFSNNSH DIHAVRNQLRQALEMLETLDGTTTPWIELAPK MNQLGFAPGWGHANRVAETMNMMLMDILE APSPSALEEFACIPMISRLILSPHGYFGQDN VLGLPDTGGQVVYILDQVRALEKEMHDRLQ LQGVQVEPKILIVTRLIPDAGDTCNQRLEKV SGCTNTWILRVPRKHNGEIIPHWISRFEIWP HLEIFAGDVEREALAEELGGHPDLIIGNYSDG NLVATLLSRRLGVTQCNIAHALEKTKYLHSD

			IYWQENEDKYHFSCQYTADLLAMNSADFIV TSTYQEIAGTREAEQYESYQAFSMPDL YRV IHGIDLFDPKFNIVSPGANADIYFPYSDPNRRL HSLIPEIESLIFDDATNLPARGYLQDPDKPLIF TMARLDRIKNITGLVELYAASPRLRSLANLVI VGGKIDPQHSSDHEEQEQIHRMHQLMDEHE LDQQVRWLGMRLDKNLAGELYRYIADKRGI FVQPALFEAFGLTIIEMASGLPTFATRYGGP LEIIQNNRSGFHIDPNQGAATADLIADFFKKN LENPQEWERISQGALDRVASRYTWKLYAER MMTSLRIYGFWKFVSGLEREETDRYLNMFY HLQFRPLANRLAHEI
32	P13708.2	<i>Glycine max</i>	MATDRLTRVHSLRERLDETLTANRNEILALL SRIEAKGKGILQHHQVIAEFEEIPEENRQKLT DGAFGEVLRSTQEAIPLPPWVALAVRPRPGV WEYLRVNVHALVVEELQPAEYLHFKEELVD GSSNGNFVLELDFEFPNAAFPRPTLNKSIGNG VQFLNRHLSAKLFHDKESLHPLLEFLRLHSV KGKTLMLNDRIQNPDALQHVLKAAEYLG VPPETPYSEFEHKFQEIGLERGWGDNAERV ESIQLLDLLEAPDPCTLETFLGRIPMVFNVI LSPHGYFAQDNVLGYPDITGGQVVYILDQVR ALENEMLHRIKQQGLDIVPRILITRLLPDAV GTTCGORLEKVFGEHSHILRVPRTEKGIVR KWISRFVWPYLETYTEDVAHELAKELQGK PDLIVGNYS DGNIVASLLAHKLGVTQCTIAH ALEKTKYPESDIYWKLEERYHFSCQFTADL FAMNHTDFIITSTFQEIAGSKDTVGGYESHTA FTLPGLYRVVHGIDVDFDPKFNIVSPGADQTIY FPHTETSRRLTSFHIPEIEELLYSSVENEHICV LKDRSKPIIFTMARLDRVKNITGLVEWYGKN AKLREL VNLVVVAGDRRKESKDLEEKAE KKMYGLIETYKLNGQFRWISSQMNRVRNGE LYRVICDTRGAFVQPAVYEAFFGLTVVEAMT CGLPTFATCNGGPAEIIHVGKSGFHIDPYHGD RAADLLVDFFEKCKLDPTHWDKISKAGLQRI EEKYTWQIYSQRLLTLTGYYGFVKHVSNDL RRESRRYLEMFYALKYRKLAEVPLAAE
33	CAA09297.1	<i>Nostoc sp. PCC 7119</i>	MSELMQAILDSEEKHDLRGFISELRQQDKNY LLRNDILNVYAEYCSKCQKPETSYSKFSNLSK LIYYTQEIIPEDSNFCFIIRPKIAAQEVYRLTAD LDVEPMTVQELDLRLVNFHHPYEGDILE LDFGPFYDYTPITRDPKNIGKGVQYLNRYLS SKLFQDSQQWLESFLNFLRLHNYNGIQLLIN HQIQSQQLSQQVKNALNFVSDRPNDPEYE QFRLQLQTMGFEPGWGNTASRV RDTLNLID

			ELIDSPDPQTLEAFISRIPMIFRIVLVSAHGWF GQEGVLGRPDTGGQVVYVLDQAKNLEKQL QEDAILAGLEVLNVQPKVIILTRLIPNSDGT CNQRLEKVYGTENAWILRVPLREFNPKMTQ NWISRFEFWPYLETFAIDSERELLAEFQGRPD LIVGNYTDGNLVAFLLTRMKVTQCNIAHA LEKSKYLFSNLYWQDLEEKYHFSLQFTADLI AMNAANFVISSTYQEIVGTPDSIGQYESYKCF TMPELYHVVNIGIELFSPKFNVVPPGVNENSY FPYTQTQNRIESDRDRLEEMLFTLEDSSQIFG KLDDPNKRPIFSMARLDRIKNLTGLAECFGQ SQELQERCNLILVAGKLRIEESDNEEKDEIV KLYRIIDEYNLHGKIRWLGVRLSKNDSGEIY RVICDRQGIFVQPALFEAFGLTILESMISGLPT FATQFGGPLEIIQDKINGFYINPTHLEETATKI LDFVTKCEQNPNYWNIISEKAIDRVYSTYTW KIHTTKLLTLARIYGFWNFTSKEKREDLLRY LESLFYLIYKPRAQQLLEQHKYR
34	P49036.1	<i>Zea mays</i>	MGEGAGDRVLSRLHSVRERIGDSL SAHPNEL VAVFTRLK NLGKGMLQPHQIIAEYNN AIP EA EREK LKDGAFEDVLRAAQEAIVIPPWVALAI RPRPGVWEYVRVNVSELAVEELRVPEYLQF KEQLVEEGPNNNFVLELDFEPFNASFP RPSLS K SIGNGVQFLNRHLSSKLFHDKESMYPLLNF LRAHNYKGMTMMLNDRIRSLSALQGALRK AEEHLSTLQADTPYSEFHHRFQELGLEKGW GDCAKRAQETIHL LLDLLEAPDPSTLEKFLG TIPMVFN VVILSPHGYFAQANVLGYPDTGGQ VVYILDQVRAMENEMLLRIKQCGLDITPKILI VTRLLPDATGTTCGQRLEKVLGTEHCHILRV PFRTE NGIVRKWISRFEVWPYLETYTDDVAH EIAGELQANPDLIIGNYSDGNL VACLLAHKM GVTHCTIAHALEKTKYPNSDLYWKKFEDHY HFSCQFTTDLIAMNHADFIITSTFQEIAGNKD TVGQYESHMAFTMPGLYRVVHGIDVFDPKF NIVSPGADLSIYFPYTESHKRLTSLHPEIEELL YSQTENTEHKFVLNDRNKPIIFSMARLDRVK NLTGLVELYGRNKR LQELVNLVVVCGDHG NPSKDKEEQAEFKKMFDLIEQYNLNGHIRWI SAQMNRVRNGELYRYICDTKGAFVQPAFYE AFGLTVVEAMTCGLPTFATAYGGPAEIIVHG VSGYHIDPYQGDKASALLVDFFDKCCQAEP SH WSKISQGG LQRIEEKYTWKLYSERLMTLTG VYGFWKYVSNLERRETRRYLEMLYALKYRT MASTVPLAVEGEPSSK

35	P49040.3	<i>Arabidopsis thaliana</i>	MANAERMITRVHSQRERLNETLVSERNEVL ALLSRVEAKGKGILQQNQIIAEFEALPEQTRK KLEGGPFFDLLKSTQEAIVLPPWVALAVRPR PGVWEYLRVNLHALVVEELQPAEFLHFKEE LVDGVKNGNFTLELDFEPFNASIPRPTLHKYI GNGVDFLNRHLSAKLFHDKESLLPLLKFLRL HSHQGKNLMLSEKIQNLNTLQHTLRKAEYY LAELKSETLYEEFEAKFEEIGLERGWGDNAE RVLDMIRLLLDLLEAPDPCTLETFLGRVPMV FNVVILSPHGYFAQDNVLGYPDGTGGQVVYIL DQVRALEIEMLQRIKQQGLNIKPRILILTRLLP DAVGTTTCGERLERVYDSEYCDILRVPRTEK GIVRKWISRFEVWPYLETYTEDAAVELSKEL NGKPDLIIGNYSDGNLVASLLAHKLGVTQCT IAHALEKTKYPDSDIYWKKLDDKYHFSCQFT ADIFAMNHTDFIITSTFQEIAGSKETVGQYES HTAFTLPGLYRVVHGIDVFDPKFNIVSPGAD MSIYFPYTEEKRRLTKFHSEIEELLYSDVENK EHLCVLKDKKKPILFTMARLDRVKNLSGLV EWYGKNTRLRELANLVVVGDDRRESKDN EEKAEMKKMYDLIEEYKLNGQFRWISSQMD RVRNGEL YRYICDTKGAFVQPALYEAFLT VVEAMTCGLPTFATCKGGPAEIIHVGKSGFH IDPYHGDQAADTLADFFTKCKEDPSHWDEIS KGGLORIEEKYTWQIYSQRLTTLTGVIYGFV KHVSNLDRLEARRYLEMFYALKYRPLAQAV PLAQDD
36	Q9M111.1	<i>Arabidopsis thaliana</i>	MANPKLTRVLSTRDRVQDTLSAHRNELVAL LSRYVDQGGKILQPHNLIDELESVIGDDETK KSLSDGPFGEILKSAMEAIVVPPFVALAVRPR PGVWEYVRVNVFELSVEQLTVSEYLRFKEEL VDGPNSDPFCLELDFEPFNANVPRPSRSSSIG NGVQFLNRHLSSVMFRNKDCLEPLLDLFLRV HKYKGHPLMLNDRIQISIRLQIQLSKAEDHIS KLSQETPFSEFEYALQGMGFKEKGWGDAGR VLEMMHLLSDILQAPDPSSLEKFLGMVPMVF NVVILSPHGYFGQANVLGLPDTGGQVVYILD QVRALETEMLLRIRKQGLDISPSILIVTRLIPD AKGTTCNQRLEVRSGTEHTHILRVPRSEKGI LRKWISRFDVWPYLENYAQDAASEIVGELQ GVPDFIIGNYSDGNLVASLMAHRMGVTQCTI AHALEKTKYPDSDIYWKDFDNKYHFSCQFT ADLIAMNNADFIITSTYQEIAGTKNTVGQYES HGAFTLPGLYRVVHGIDVFDPKFNIVSPGAD MTIYFPYSEETRRLTALHGSIEEMLYSPDQTD EHVGTLSDRSKPILFSMARLDKVNISGLVE

			MYSKNTKLREL VNLVVIAGNIDVNKSKDRE EIVEIEKMHNLMKNYKLDGQFRWITAQTNR ARNGELYRYIADTRGAFAQPAFYEAFLTV VEAMTCGLPTFATCHGGPAEIEHGLSGFHID PYHPEQAGNIMADFFERCKEDPNHWKKVSD AGLQRIYERYTWKIYSERLMTLAGVYGFWK YVSKLERRETRRYLEM FYILKFRDLVKT VPS TADD
37	Q8W517	<i>Ipomoea batatas</i>	MAGNDWINSYLEAILDVGPGIDDAKSSLLLR ERGRFSPTRYFVEEVITGFDETDLHRSWVRA QATRSPQERNTRLENMCWRIWNLARQKKQL EGEQAQRLAKRRQERERGRREAVADMSEDL SEGEKGDAISDISAHGESIKGRLPRISSVETME SWANQQKGKKLYIVLISLHGLIRGENMELGR DSDTGGQVKYVVELARALGSM PGVYRVDL LTRQVSSPEVDWSYGEPTMLTPINSEGLMT EMGESSGAYIIRIPFGPRDKYIPKEDLWPIPE FVDGALNHILHVSKVLGGQIGSGRDVWPVAI HGHYADAGDSAALLSGALNVPMLFTGHSLG RDKLEQLLRQGRLSKDEINSTYKIMRRIEAEE LSLDASEIVITSTRQEIDEQWRLYDGFDPILER KLRARIKRVSCYGRFMPRMVVIPPGMEFH HIVPHEGDMDFETEGSEDGKAPDPHIWTEIM RFFSNPRKPMILALARPD PKKNLTTLVKA FG ECRPLRELANLTLIMGNRDNIDEMSSTNASV LLSILKMIDKYDLYGQVAYPKHHKQSEVPDI YRLAAKTKGVFINPAFIEPFGTLIEAAAHGL PIVATKNGGPVDIHRGSDNGLLVDPHDQHAI ADALLKLVADKHLWAKCRANGLKNIHLFS WPEHCKTYLSRIAGCKPRQPCWLRNADDDE NSESESPSDSLRDIQDISLNLKFSLDGDKNED SDNLFDPPDRKNKLENAVLAWSKGVKGTH KTSIDKIDQSSSAGKFPALRRRKQIFVIAVDC DSSTGLFENVRKIFA AVEAEGMEGSIGFHIGH FIQYIRSAFFSDFRGHESTDFDAFICNSGGDL YYSSSHSEDNPFVVDLYYHSHIEYRWGGEGL RKTLVRWAASISDKKGEKEEHIVVEDEKNSA DYCYTFKVQKSGGDPSVKELRKSMRIQALR CHVVYCQNGSRINVIPVLSSRSQALRYLYLR WGMDLSKL VV FVGESGDTDYEGLLGGLRK AVILKGVC SVSSQLLSNRNYPLTDVVPYNS PNVIQTTEECSSSELHASLEKLAVLKG
38	WP_011056890	<i>Thermosynechococcus elongatus</i>	MTCVLLKAVVESDERADLRQFSRILQLGEKR YLLRNDILDAFADYCRDQERPVP PPSESRLSK LVFYTQEIIVDNESLCWIVRPRIAQQEVCRL VEDLTIVPMTIPELLDLRDLVNHYHPNEGD

			VFEIDVQPFYDYSPHIRDAKNIGKGVFLNRY LSSKLFQDPRQWQQNLNLFRLHRYNGYQLL INERIRSPQHLSEQVKQALVVLSDRPPTAAYS EFRFELQNLGFEPGWGNTVARVRDTLEILDQ LLDSPDHQVLEAFVSRIPMLFRIALISPHGWF GQEGVLGRPDTGGQVVYILDQVKSLEKQMR EDLELAGLVLEAQPKIIVLTRLIPNAEGTLC NQRLEKIYGTNDAWILRVPFREFNPKVTQN WISRFEIWPYLETFAIDAERELRAEFVGHVVDL HIGNYSDGNLVAFLARRLKVTQCNIAHALE KSKYLFSNLYWQDLEDKYHFSLQFTADLIA MNAANFIISSTYQEIVGTPDSIGQYESYQSFT MPDL YHVVNGIELFSPKFNVVPPGVNEQVYF PYYHYTERLEGDRQRLEELLFTLEDPPQIYG YLEAPEKRPLFSMARLDRIKNLTGLAEAFGR SKALQERCNLILVAGKLRTADSSDREEIAEIE KLYQIIHQYNLHGKIRWLGIRLPKADSGEIYR IADRQGIFVQPALFEAFGLTILEAMISGLPTF GTRFGGPLEIIQDGVNGFYINPHTLEEMAETI VRFLEACDRDPQEWQRISKAGIERVYSTYTW KIHCTRLLSLAKIYGFWNFSSQENREDMMRY MEALFHLLYKPRQAQALLAEHLQR
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Table 4 provides exemplary sequences for enzymes involved in generation and/or recycling of UDP-glucose the reaction intermediates in accordance with the methods of invention.

5 Table 4:

SEQ ID NO	Gene	Accession	Sequence
39	GLK	AAA27694.1	MEIVAIIDIGGTHARFSIAEVSNGRVLSLGEETTFKTAEHASLQ LAWERFGEKLGRPLPRAAAIAWAGPVHGEVLKLTNNPWVL RPATLNEKLDIDTHVLINDFGAVAHAVAHMDSSYLDHICGP DEALPSDGVITILGPGTGLGVAHLRTEGRYFVIETEGGHIDF APLDRLEDKILARLRERFRVSIERIISGPGLGNIYEALAAIEG VPFSLDDIKLWQMALEGKDNLAEAALDRFCLSLGAIAGDL ALAQGRTSVVIGGGVGLRIASHLPESGFRQRFVSKGRFERV MSKIPVKLITYPQPGLLGAQLPMPTNILKLNNIF
40	GLK	WP_331012003.1	MEIVAVDIGGTHARFAIAEVDGHHVLSLGEPTLKTAEHGS LQLAWEAAGEALGRPIPRAGIAIATPIGGEVLKLTNNPWVI RPALIQSKLGVDSYVLVNDFEAVGHAVAQVGAEYLOHLGC

			PDDPLPDTGIITIVGPGTGLGVAHLLKTPAGYHVLPCEGGHI DFAPLDVLEDGILKTLRKQYRRVSVERIVSGPGLVNIYESLPP STSAALRPIDDKELWTAALAGTDSRAAAALDRFCLSLGAVA GDLALAHGAKAVVIAGGLGLRIADHLPRSGFSERFVAKGRF ERIMSAMPVKFITHPQPGLFGAAAFAGAHRS
41	PGM	AAC737 82.1	MAIHN RAGQPAQQSDLINVAQLTAQYYVLKPEAGNAEHAV KFGTSGHRGSAARHSFNPHILAIAQAIAEERAKNGITGPCY VGKDTHALSEPAFISVLEVLAAANGVDVIVQENNGFTPTPAVS NAILVHNKKGGPLADGIVITPSHNPPEDGGIKYNPPNGGPAD TNVTKVVEDRANALLADGLKGVKRISLDEAMASGHVKEQD LVQPFVEGLADIVDMAAIQKAGLTLGVDPLGGSGIEYWKRI GEYYNLNL TIVNDQVDQTFRFMHLDKDGAIRMDCSSECAM AGLLALRDKFDLAFANDPDYDRHGIVTPAGLMNPNHYLAV AINYLFQHRPQWGKDVAVGKTLVSSAMIDRVVNDLGRKLV EVPVGFKWFDGLFDGSFGFGGEESAGASFLRFDGTPWSTD KDGIMCLLAAEITAVTGKNPQEHYNELAKRFGAPSYNRLQ AAATSAQKAALSKLSPMVSASTLAGDPITARLTAAPGNGA SIGGLKVMTDNGWFAARPSGTEDAYKIYCESFLGEEHRKQI EKEAVEIVSEVLKNA
42	PGM	A0A1I0E I47	MAKHPRAGQQPTSCDLINVAKLTSQYYVLQPALHESAHAV KFGTSGHRGSSTKHTFNEAHILAIAQAIAEIRHAKGITGPCYV GKDTHALSEPAFISVLEVLAAANQVTVIIQPENGFTPTPAISHAI LTHNKSNIHLADGIVITPSHNPPEDGGIKYNPPNGGPADTDF TGLIEKRANQLLDERSSENAVLAGVKRISLSQAISSQFIIQQD MMMPYVEDLVNVVDLSVIQQAGLKLGVDPPLGGSGIAYWQ QIAEYYKLNLSLVNDQVDQTFRFMPLDHDGVIRMDCSSVD AMGGLRLKDKFDLAFANDPDYDRHGIVTPAGLMNPNHYL SVAINYLYQNRPGWPVDLGVGKTLVSSAMIDRVVSSLNRKL VEVPVGFKWFDGLYTGKLGFGGEESAGASFLRFDAPKWS TDKDGIMCLLSAEITARTGKNPQVHYDELSEKFGAPIYNRI QANATHEQKAILSKLSPDQVQAKTLAGDLITARITHAPSNN ASIGGLKVVTDNGWFAARPSGTEEAYKIYCESFISHEHLDLI SKEAVEIVDAAAFASNK
43	PGM	A0A7M2 S2U2	MAMHPRAGQKAQQEDLHNIPALVANYFLLQPDAANAHEHK VQFGTSGHRGTADKHTFNENHILAIAQAVAEVRSEQGTTGP LFVGKDTHALSEPAFSSVIEVLIANGVKVIVQQDNGYTPTPG ISHAILTYNIKHDEKADGIVITPSHNPPQDGGIKYNPTHGGPA EAELTQAIEDRANELIAEGLQGVRRLPLAEAKASDLFVEMD LVKPYIDDLVNVIDMEAIQKANLKGVDPLGGSGIDYWRQI GQAYNLDLTLVSEAIDPSFQFMSLDKDGVVVRMDCSSPYAM AGLLALKDEYDLAFGNPDYDRHGIVTPKGLMNPNHFLAV CIDYLYRHRDAWGKDVAVGKTLVSSALIDRVVADLGRALC EVPVGFKWFDGLYTGKFGFGGEESAGASFLRKDGTIPWST DKDGILLCLLAAEITAVTGKNPQDYEEELAAKHGESKYNRI

			QAVANGPQKDVLLKLSPEMVAAETLAGDAITARLTHAPGN GAAIGGLKVTTANGWFAARPSGTEDIYKIYCESFKGEEHLK QIEAEAQEIVNQVFAAAGL
44	UGP	EEW275 2841.1	MAAINTKVKKAVIPVAGLGTRMLPATKAIPKEMLPLVDKPL IQYVVNECIAAGITEIVLVTHSSKNSIENHFDTSFEEAMLEK RVKRQLLDEVQSICPPHVTIMQVRQGLAKGLGHAVLCAHP VVGDEPVAVILPDVILDEYESDLSQDNLAEMIRRFDETGHSSQ IMVEPVADVTAYGVVDCKGVELAPGESVPMVGVVEKPKA DVAPSNLAIVGRYVLSADIWPLLAKTPPGAGDEIQLTDAIDM LIEKETVEAYHMKGKSHDCGNKLGVMQAFVEYGIRHNTLG TEFKAWLEEEMGIKK
45	UGP	WP_021 648042.1	MFAEDLKRTEKMTVDDVFEQSAQKMREQGMSEIAISQFRH AYHVWASEKESAWIREDTVEPLHGVRSFHDVYKTIDHDKA VHAFAKTAFLKLNGLGTSMGLQCAKSLLPVRRHKARQMR FLDIILGQVLTARTRLNVPPLVTFMNSFRTSDDTMKALRHQR KFKQTDIPLEIIHQHEPKIDAATGAPASWPANPDLEWCPPGH GDLFSTLRESGLDITLLEHGFEYLFISNSDNLGARPSRTLAQ YFEDTGAPFMVEVANRTYADRKGGHIVRDTATGRLILREMS QVHPDDKDAQAQDIKHPYFNTNNIWVRIDVLRDMLAEHDG VLPLPVIINNKTVDPIDPQSPAVVQLETAMGAAIGLFEGAICV QVDRMRFLPVKTTNDLFIMRSDRFHLTDSYEMEDGNYIFPN VDLDPYYKNIEDFNERFPYNVPSLAAANSVSIKGDWTFGR DVIMFADARLEDRNEPSYVPNGEYVVGPMGIEPGDWV
46	UGP	WP_028 847555.1	MTASAEHQTFITTAIVPAAGLGTRFLPTTKSVPKELLPVVDTP AIELVADEARQAGAERLVIVTSPAKQSIAAYFRPAPELERSL EEKGKTGQLAKIRRAPELLEVEVAIQEQALGLGHAVACAEP NLGPEDDVVAVLLPDDLVLPHGILERMAKVRAEHGGSVLC AFDIPKEEISAYGVFVDVSDTDDADVVRVHGMVEKPPAEQAP STFAAAGRYLLDRAIFDALRRIEPGAGGELQLTDAVALLIQE GHPVHVVVHRGDRHDLGNPGGFLRAAVDFALQDPDYGPPEL RAWLTDRIARP
47	NDK	WP_000 963837.1	MAIERTFSIIKPNVAVAKNVIGNIFARFEAAAGFKIVGTKMLHLT VEQARGFYAEHDGKPFDFGLVEFMTSGPIVVSVLEGENAVQ RHRDLLGATNPANALAGTLRADYADSLTENGTHGSDSVES AAREIAYFFGEGEVCPTR
48	NDK	CAF2103 5.1	MTERTLILIKPDGVTNGHVGEIARIERKGLKLAALDLRVAD RETAEKHYEEHADKPFEGELVEFITSAPLIAGIVEGERAIDA WRQLAGGTDPAKATPGTIRGDFALTVGENVVHGSDSPES AEREISIWFPNL
49	NDK	WP_066 795798	MTERTLILIKPDGVANGHVGEIARIERKGLKLVELDLRTAD RETAEKHYEEHSDKPFEGELVEFITSAPLVAGIVEGERAIDA

			WRQLAGGTDVPVKATPGTIRGDFALTVGENVVHGSDSPESA EREIAIWFPNK
50	PPK2	WP_010 968631	MALDEAPAEARPGSRAVELEIDGRSRIFDIDDPDLPKWIDEE AFRSDDYPPYKKKLDREEYEETLTKLQIELVKVQFWMQATG KRVMAVFEGRDAAGKGGAIHATTANMNPVSARVVALTKP TETERGQWYFQRYVATFPTAGEFVLFDERSWYNRAGVEPVM GFCTPDQYEQFLKEAPRFEEMIANEGIHFLKFWINIGREMQ KRFHDDRHDPLKIWKLSMDIAALSKWDDYTGKRDRMLKE THTEHGPWAVIRGNDKRRSRINVIRHMLTKLDYDGKDEAAI GEVDEKILGSGPGFLR
51	PPK2	WP_160 857702	MVEKAESRAVELDIDGKKRVFDIDDPKLPDWIDKEAFQSD FPYDKKLDEDEYEEALEALQVELVKVQFWQOKTGARIMAI FEGRDAAGKGGAIHATMSNMNPVSARIVALTKPTETEQQG WYFQRYVATFPTSGEFVLFDERSWYNRAGVEPVMGFCTPEQ YEKFLKATPRIEKMIASEGIHFFKFWLNIGREMQKRFHDDR HDPLKVWKLSPMDIAALNKWDDYTEKRDRMLKATHTDRA PWTVIRANDKRRARINLIRHILTTLDYEGKDEKAIGKIDDKIV GSGPGFVK

Incorporation by Reference

References and citations to other documents, such as patents, patent applications, patent publications, journals, books, papers, web contents, publicly accessible databases, have been made throughout this disclosure. All such documents are hereby incorporated herein by reference in their entirety for all purposes.

Equivalents

Various modifications of the invention and many further embodiments thereof, in addition to those shown and described herein, will become apparent to those skilled in the art from the full contents of this document, including references to the scientific and patent literature cited herein. The subject matter herein contains important information, exemplification and guidance that can be adapted to the practice of this invention in its various embodiments and equivalents thereof.

Claims:

1. A method for cell-free production of anthocyanins, wherein the method comprises:
providing one or more enzymes in a cell-free medium, wherein the one or more enzymes
result in transformation of one or more substrates to anthocyanins.
2. The method of claim 1, wherein the substrate is a catechin.
3. The method of claim 2, wherein catechin is (+)-catechin.
4. The method of claim 3, wherein the anthocyanin is cyanidin-3-glucoside.
5. The method of claim 1, wherein the one or more enzymes are selected from a group
consisting of: anthocyanidin synthase enzyme (ANS) and anthocyanidin 3-O-
glucosyltransferase (3GT).
6. The method of claim 5, wherein the anthocyanidin synthase enzyme (ANS) catalyzes the
conversion of catechin to cyanidin.
7. The method of claim 5, wherein the anthocyanidin 3-O-glucosyltransferase (3GT)
catalyzes the conversion of cyanidin to cyanidin-3-glucoside.
8. The method of claim 1, wherein the cell-free medium comprises an activated sugar.
9. The method of claim 8, wherein the activated sugar is UDP-glucose.
10. The method of claim 9, wherein the UDP-glucose is added to the cell-free medium.
11. The method of claim 9, wherein the UDP-glucose is synthesized from one or more
ingredients selected from the group consisting of: sucrose, glucose, UTP, UDP, ATP,
glucose-6-phosphate, glucose-1-phosphate, and/or polyphosphate.
12. The method of claim 11, wherein the one or more enzymes is selected from the group
consisting of sucrose synthase (SuSy), glucokinase (GLK), hexokinase (HK),

phosphoglucosyltransferase (PGT), polyphosphate kinase (PPK), UTP—glucose-1-phosphate uridylyltransferase (UGP), and nucleoside diphosphate kinase (NDK).

13. The method of claim 11, wherein the one or more enzymes is sucrose synthase (SuSy).
14. The method of claim 1, wherein the cell-free medium is a cell lysate.
15. The method of claim 14, wherein the cell lysate is a cell lysate from cells from a host organism expressing the one or more enzymes.
16. The method of claim 15, wherein the host organism is selected from a group consisting of: bacteria, yeast, and/or mammalian cells.
17. The method of claim 16, wherein the one or more enzymes are introduced in the host organism by integration into genome of the host organism or on a plasmid.
18. The method of claim 16, wherein the host organisms expressing the one or more enzymes are cultured until a pre-determined biomass is achieved to produce the requisite quantity of the one or more enzymes.
19. The method of claim 18, further comprising lysing of cells followed by removal of cell debris to generate a cell lysate for use in the cell-free medium for cell-free production of anthocyanins.
20. The method of any of claims 1-19, wherein ANS, 3GT, SuSy, GLK, HK, PGM, PPK, UGP, and/or NDK are present in the cell-free medium for cell-free production of anthocyanins.
21. The method of claim 20, wherein the method does not include separating and/or purifying ANS, 3GT, SuSy, GLK, HK, PGM, PPK, UGP, and/or NDK, for cell-free production of anthocyanins.

22. The method of claim 20, wherein the method comprises separating and/or purifying at least one of a ANS, 3GT, SuSy, GLK, HK, PGM, PPK, UGP, and/or NDK, for cell-free production of anthocyanins.
23. The method of any of claims 1-22, wherein the cell-free medium further comprises: buffer, catechin, an activated sugar, magnesium chloride, cell lysate, sucrose, glucose, glucose-1-phosphate, glucose-6-phosphate, UDP, UTP, ATP, polyphosphate, and/or water.
24. The method of claim 23, wherein the buffer is a phosphate buffer.
25. The method of claim 23, wherein the activated sugar is UDP-glucose.
26. The method of claim 23, wherein the cell-free medium further comprises sodium ascorbate, ascorbic acid, ammonium iron sulphate, and 2-oxoglutarate.
27. The method of claim 23, wherein the catechin is (+)-catechin.
28. The methods of any of claims 1 – 27, wherein the method results in titer value of produced anthocyanins is from about 2 times to about 5000 times higher than methods for cell-based production of anthocyanins.
29. The methods of any of claims 1 – 27, wherein the method results in titer value of produced anthocyanins is from about 5 times to about 1000 times higher than methods for cell-based production of anthocyanins.
30. The method of claim 23, wherein pH of cell-free medium is from about 5 to about 10.
31. The method of claim 23, wherein pH of cell-free medium is from about 6 to about 8.
32. The method of claim 26, wherein the buffer is in the concentration of from about 1 mM to about 200 mM.

33. The method of claim 26, wherein ammonium iron sulfate concentration is from about 0.25 mM to about 10 mM.
34. The method of claim 26, wherein the cell-free medium further comprises α -ketoglutaric acid in a concentration of from about 5 mM to about 50 mM.
35. The method of claim 26, wherein the ascorbic acid is in the concentration of from about 1 mM to about 100 mM.
36. The method of claim 26, wherein catechin is the concentration of from about 0.5 mM to about 50 mM.
37. The method of claim of 26, wherein magnesium chloride is present in the range of about 1 mM to about 20 mM.
38. The method of claim of 26, wherein sucrose is present in the range of about 10 mM to about 600 mM.
39. The method of claim 26, wherein the cell lysate including the one or more enzymes is present at a concentration of about 5% (v/v) to about 40% (v/v).
40. The method of claim 26, wherein reaction for cell-free production is carried out for a duration of about 0.5 hours to about 48 hours.
41. The method of claim 26, wherein reaction for cell-free production is carried out for a duration of about 0.5 hours to about 20 hours.
42. The method of claim 26, wherein temperature of the cell-free medium is from about 20 °C to about 40 °C.
43. The method of claim 1, wherein reaction is conducted in a bubble column reactor, wherein the one or more enzymes are in a solution.

44. The method of claim 1, wherein reaction is conducted in a packed bed reactor, wherein the one or more enzymes are immobilized.
45. The method of claim 5, wherein the anthocyanidin synthase (ANS) is an enzyme with an amino acid sequence at least 95% identical to any one of enzymes selected from the group consisting of:

Accession #	Organism
XP_022736758.1	<i>Durio zibethinus</i>
XP_030501512.1	<i>Cannabis sativa</i>
XP_028762755.1	<i>Prosopis alba</i>
XP_021901846.1	<i>Carica papaya</i>
XP_021735950.1	<i>Chenopodium quinoa</i>
QCI56004.1	<i>Iris sanguinea</i>
XP_010538778.1	<i>Tarenaya hassleriana</i>
XP_027183517.1	<i>Coffea eugenioides</i>
AHU88620.1	<i>Magnolia sprengeri</i>
XP_028787846.1	<i>Prosopis alba</i>
EOY24569.1	<i>Theobroma cacao</i>
AEF14411.1	<i>Onobrychis viciifolia</i>
ACM62747.1	<i>Garcinia mangostana</i>
XP_017236102.1	<i>Daucus carota subsp. sativus</i>
XP_010934075.1	<i>Elaeis guineensis</i>

46. The method of claim 5, wherein the anthocyanidin 3-O-glucosyltransferase (3GT) is an enzyme with an amino acid sequence at least 95% identical to any one of enzymes selected from the group consisting of:

Accession #	Organism
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AFJ52972.1	<i>Linum usitatissimum</i>
ABR24135.1	<i>Vitis labrusca</i>
AHJ80981.1	<i>Erythranthe lewisii</i>
QCP68996.1	<i>Salvia miltiorrhiza</i>
XP_004307876.1	<i>Fragaria vesca subsp. Vesca</i>
XP_010549820.1	<i>Tarenaya hassleriana</i>
XP_010087491.1	<i>Morus notabilis</i>
BBC62118.1	<i>Nemophila menziesii</i>
PIN20524.1	<i>Handroanthus impetiginosus</i>
QJB23733.1	<i>Centaurea cyanus</i>
QBL54224.1	<i>Scutellaria baicalensis</i>
XP_024179381.1	<i>Rosa chinensis</i>
BAA89008.1	<i>Petunia x hybrida</i>

47. The method of claim 13, wherein the sucrose synthase (SuSy) is an enzyme with an amino acid sequence at least 95% identical to any one of enzymes selected from the group consisting of:

Accession #	Organism
WP_004872341.1	<i>Acidithiobacillus caldus</i>
P30298.2	<i>Oryza sativa Japonica Group</i>
Q820M5.1	<i>Nitrosomonas europaea ATCC 19718</i>
P13708.2	<i>Glycine max</i>
CAA09297.1	<i>Nostoc sp. PCC 7119</i>
P49036.1	<i>Zea mays</i>
P49040.3	<i>Arabidopsis thaliana</i>
Q9M111.1	<i>Arabidopsis thaliana</i>
Q8W517	<i>Ipomoea batatas</i>
WP_011056890	<i>Thermosynechococcus elongatus</i>

48. The methods of any of claims 5 or 13, wherein the ANS, 3GT, and/or SuSy enzymes are modified and optimized for the cell-free production of anthocyanins.
49. A composition for cell-free production of anthocyanins, wherein the composition comprises: one or more enzymes in a cell-free medium, wherein the one or more enzymes result in transformation of one or more substrates to an anthocyanin.
50. The composition of claim 49, wherein the substrate is a catechin.
51. The composition of claim 50, wherein catechin is (+)-catechin.
52. The composition of claim 51, wherein the anthocyanin is cyanidin-3-glucoside.
53. The composition of claim 49, wherein the one or more enzymes are selected from a group consisting of: anthocyanidin synthase enzyme (ANS) and anthocyanidin 3-O-glucosyltransferase (3GT).
54. The composition of claim 53, wherein the anthocyanidin synthase enzyme (ANS) catalyzes the conversion of catechin to cyanidin.
55. The composition of claim 53, wherein the anthocyanidin 3-O-glucosyltransferase (3GT) catalyzes the conversion of cyanidin to cyanidin-3-glucoside.
56. The composition of claim 49, wherein the cell-free medium comprises an activated sugar.
57. The composition of claim 56, wherein the activated sugar is UDP-glucose.
58. The composition of claim 57, wherein the UDP-glucose is added to the cell-free medium.
59. The composition of claim 58, wherein the UDP-glucose is synthesized in the cell-free medium by the one or more enzymes.

60. The composition of claim 57, wherein the UDP-glucose is synthesized from one or more ingredients selected from the group consisting of: sucrose, glucose, UTP, UDP, ATP, glucose-6-phosphate, glucose-1-phosphate, and/or polyphosphate.
61. The composition of claim 60, wherein the one or more enzymes is sucrose synthase (SuSy), glucokinase (GLK), hexokinase (HK), phosphoglucomutase (PGM), polyphosphate kinase (PPK), UTP—glucose-1-phosphate uridylyltransferase (UGP), and nucleoside diphosphate kinase (NDK).
62. The composition of claim 49, wherein the cell-free medium is a cell lysate.
63. The composition of claim 50, wherein the cell lysate is a cell lysate from cells from a host organism expressing the one or more enzymes.
64. The composition of claim 63, wherein the host organism is selected from a group consisting of: bacteria, yeast, and/or mammalian cells.
65. The composition of claim 64, wherein the one or more enzymes are introduced in the host organism by integration into genome of the host organism or on a plasmid.
66. The composition of claim 65, wherein the host organisms expressing the one or more enzymes are cultured until a pre-determined biomass is achieved to produce the requisite quantity of the one or more enzymes.
67. The composition of claim 66, further comprising lysing of cells followed by removal of cell debris to generate a cell lysate for use in the cell-free medium for cell-free production of anthocyanins.
68. The composition of any of claims 49-67, wherein ANS, 3GT, SuSy, GLK, HK, PGM, PPK, UGP, and/or NDK are present in the cell-free medium for cell-free production of anthocyanins.

69. The composition of claim 68, wherein the ANS, 3GT, SuSy, GLK, HK, PGM, PPK, UGP, and/or NDK for cell-free production of anthocyanins are not separated and/or purified.
70. The composition of claim 68, wherein the ANS, 3GT, SuSy, GLK, HK, PGM, PPK, UGP, and/or NDK for cell-free production of anthocyanins are separated and/or purified.
71. The composition of any of claims 49-70, wherein the cell-free medium further comprises: buffer, catechin, an activated sugar, magnesium chloride, cell lysate, sucrose, glucose, glucose-1-phosphate, glucose-6-phosphate, UDP, UTP, ATP, polyphosphate, and/or water.
72. The composition of claim 71, wherein the buffer is a phosphate buffer.
73. The composition of claim 71, wherein the activated sugar is UDP-glucose.
74. The composition of claim 71, wherein the cell-free medium further comprises sodium ascorbate, ascorbic acid, ammonium iron sulphate, and 2-oxoglutarate.
75. The composition of claim 71, wherein the catechin is (+)-catechin.
76. The compositions of any of claims 49 – 75, wherein the composition is used for producing anthocyanins with titer value of produced anthocyanins is from about 2 times to about 100 times higher than methods for cell-based production of anthocyanins.
77. The compositions of any of claims 49 – 75, wherein the composition is used for producing anthocyanins with titer value of produced anthocyanins is from about 5 times to about 10 times higher than methods for cell-based production of anthocyanins.
78. The composition of claim 71, wherein pH of cell-free medium is from about 5 to about 10.
79. The composition of claim 71, wherein pH of cell-free medium is from about 6 to about 8.

80. The composition of claim 72, wherein the buffer is in the concentration of from about 1 mM to about 200 mM.
81. The composition of claim 74, wherein ammonium iron sulfate is in the range of from about 0.25 mM to about 10 mM.
82. The composition of claim 74, wherein the cell-free medium further comprises α -ketoglutaric acid in a concentration of from about 5 mM to about 50 mM.
83. The composition of claim 74, wherein the ascorbic acid is in the concentration of from about 1 mM to about 100 mM.
84. The composition of claim 74, wherein catechin is the concentration of from about 0.5 mM to about 50 mM.
85. The composition of claim 74, wherein magnesium chloride is present in the range of from about 1 mM to about 20 mM.
86. The composition of claim 74, wherein sucrose is present in the range of from about 10 mM to about 600 mM.
87. The composition of claim 74, wherein the cell lysate including the one or more enzymes is present at a concentration of about 5% (v/v) to about 40% (v/v).
88. The composition of claim 74, wherein reaction for cell-free production is carried out for a duration of about 0.5 hours to about 48 hours.
89. The composition of claim 74, wherein reaction for cell-free production is carried out for a duration of about 0.5 hours to about 20 hours.
90. The composition of claim 74, wherein temperature of the cell-free medium is from about 20 °C to about 40 °C.

91. The composition of claim 49, wherein the composition is in a bubble column reactor, wherein the one or more enzymes are in a solution.
92. The composition of claim 90, wherein the composition is in a packed bed reactor, wherein the one or more enzymes are immobilized.
93. The composition of claim 53, wherein the anthocyanidin synthase (ANS) is an enzyme with an amino acid sequence at least 95% identical to any one of enzymes selected from the group consisting of:

Accession #	Organism
XP_022736758.1	<i>Durio zibethinus</i>
XP_030501512.1	<i>Cannabis sativa</i>
XP_028762755.1	<i>Prosopis alba</i>
XP_021901846.1	<i>Carica papaya</i>
XP_021735950.1	<i>Chenopodium quinoa</i>
QCI56004.1	<i>Iris sanguinea</i>
XP_010538778.1	<i>Tarenaya hassleriana</i>
XP_027183517.1	<i>Coffea eugenioides</i>
AHU88620.1	<i>Magnolia sprengeri</i>
XP_028787846.1	<i>Prosopis alba</i>
EOY24569.1	<i>Theobroma cacao</i>
AEF14411.1	<i>Onobrychis viciifolia</i>
ACM62747.1	<i>Garcinia mangostana</i>
XP_017236102.1	<i>Daucus carota subsp. sativus</i>
XP_010934075.1	<i>Elaeis guineensis</i>

94. The composition of claim 53, wherein the anthocyanidin 3-O-glucosyltransferase (3GT) is an enzyme with an amino acid sequence at least 95% identical to any one of enzymes selected from the group consisting of:

Accession #	Organism
AFJ52972.1	<i>Linum usitatissimum</i>
ABR24135.1	<i>Vitis labrusca</i>
AHJ80981.1	<i>Erythranthe lewisii</i>
QCP68996.1	<i>Salvia miltiorrhiza</i>
XP_004307876.1	<i>Fragaria vesca subsp. Vesca</i>
XP_010549820.1	<i>Tarenaya hassleriana</i>
XP_010087491.1	<i>Morus notabilis</i>
BBC62118.1	<i>Nemophila menziesii</i>
PIN20524.1	<i>Handroanthus impetiginosus</i>
QJB23733.1	<i>Centaurea cyanus</i>
QBL54224.1	<i>Scutellaria baicalensis</i>
XP_024179381.1	<i>Rosa chinensis</i>
BAA89008.1	<i>Petunia x hybrida</i>

95. The composition of claim 61, wherein the sucrose synthase (SuSy) is an enzyme with an amino acid sequence at least 95% identical to any one of enzymes selected from the group consisting of:

Accession #	Organism
WP_004872341.1	<i>Acidithiobacillus caldus</i>
P30298.2	<i>Oryza sativa Japonica Group</i>
Q820M5.1	<i>Nitrosomonas europaea ATCC 19718</i>
P13708.2	<i>Glycine max</i>
CAA09297.1	<i>Nostoc sp. PCC 7119</i>
P49036.1	<i>Zea mays</i>
P49040.3	<i>Arabidopsis thaliana</i>
Q9M111.1	<i>Arabidopsis thaliana</i>
Q8W517	<i>Ipomoea batatas</i>
WP_011056890	<i>Thermosynechococcus elongatus</i>

96. The composition of any of claims 53 or 61, wherein the ANS, 3GT, and/or SuSy enzymes are modified and optimized for the cell-free production of anthocyanins.