[DESCRIPTION]

[Invention Title]

NOVEL VARIANT GLYCOSYLTRANSFERASE POLYPEPTIDE AND METHOD FOR PRODUCING STEVIOL GLYCOSIDES USING THE SAME

[Technical Field]

The present disclosure relates to a novel variant polypeptide; a polynucleotide encoding the variant polypeptide; a vector comprising the polynucleotide; a microorganism comprising any one or more of the variant polypeptide, the polynucleotide encoding the variant polypeptide, and the vector comprising the polynucleotide; a method of producing a steviol glycoside, the method comprising the step of transferring glucose to rebaudioside A by reacting rebaudioside A with the nucleotide diphosphate-linked glucose in the presence of the variant polypeptide or the microorganism; a composition for producing any one or more of rebaudioside D and rebaudioside M using the same; and use of the microorganism in producing any one or more of rebaudioside D and rebaudioside M.

[Background Art]

As the World Health Organization (WHO) recommends lowering the amount of daily sugar intake due to concerns about diseases (obesity) caused by sugar consumption, various policies aimed at reducing the amount of sugar intake are actively being discussed under the leadership of governments, mainly in developed countries. Therefore, the demand for various alternative sweeteners to replace sugar and high fructose is increasing in the market, and alternative sweeteners are continuously being developed and commercialized.

From this perspective, customers' need for natural sweeteners has been

serine, but is not limited thereto.

In one embodiment of the above-described embodiments, the variant polypeptide may consist of an amino acid sequence of SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 23, SEQ ID NO: 25, SEQ ID NO: 27, SEQ ID NO: 29, SEQ ID NO: 31, SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 37, SEQ ID NO: 39, SEQ ID NO: 41, SEQ ID NO: 43, SEQ ID NO: 45, SEQ ID NO: 47, SEQ ID NO: 49, SEQ ID NO: 51, SEQ ID NO: 53, SEQ ID NO: 55, SEQ ID NO: 57, SEQ ID NO: 59, SEQ ID NO: 61, SEQ ID NO: 63, SEQ ID NO: 65, SEQ ID NO: 67, SEQ ID NO: 69, SEQ ID NO: 71, or SEQ ID NO: 73.

Specifically, the variant polypeptide of the present disclosure may have or comprise an amino acid sequence having at least 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% or more homology or identity to the sequence numbers, or may consist of the amino acid sequence, or may essentially consist of the amino acid sequence.

In one embodiment, among the variant polypeptides of the present disclosure, the variant polypeptide comprising the single substitution of the amino acid corresponding to position 92 of the amino acid sequence of SEQ ID NO: 1 with tryptophan may be SEQ ID NO: 3; the variant polypeptide comprising the single substitution of the amino acid corresponding to position 208 of the amino acid sequence of SEQ ID NO: 1 with alanine or methionine may be SEQ ID NO: 5 or SEQ ID NO: 7, respectively; the variant polypeptide comprising the single substitution of the amino acid corresponding to position 380 of the amino acid sequence of SEQ ID NO: 1 with histidine, threonine, or alanine may be SEQ ID NO: 9, SEQ ID NO: 11, or SEQ ID NO: 13, respectively; the variant polypeptide comprising the substitution of the amino acid corresponding to position 381 of the amino acid sequence of SEQ ID NO: 1 with phenylalanine, serine, alanine, or

of a sucrose synthase, a polynucleotide encoding the same, and a vector comprising the same.

Specifically, the sucrose synthase of the present disclosure may have, comprise, consist of, or essentially consist of an amino acid sequence of SEQ ID NO: 131; or an amino acid sequence having at least 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% or more homology or identity thereto.

Specifically, the polynucleotide encoding the sucrose synthase of the present disclosure may have, comprise, consist of, or essentially consist of a polynucleotide sequence of SEQ ID NO: 132; or a polynucleotide sequence having at least 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% or more homology or identity thereto.

Still another aspect of the present disclosure provides a method of producing a steviol glycoside, the method comprising the step of culturing, in a medium, the microorganism comprising any one or more of the variant polypeptide of the present disclosure, the polynucleotide encoding the variant polypeptide, and the vector comprising the polynucleotide.

Specifically, the steviol glycoside may be any one or more of rebaudioside D and rebaudioside M.

The variant polypeptide, polynucleotide, vector, and microorganism are as explained in other aspects.

As used herein, the term “culturing” refers to growing a microorganism in appropriately adjusted environmental conditions. The culturing procedure may be performed according to appropriate media or culture conditions known in the art. Such a culturing process may be easily adjusted and used by a person skilled in the art according to the selected microorganism. Specifically, the culturing may be in a batch type, a continuous type, and/or a fed-batch type, but is not limited thereto.

Enzymes

Recombinant *Corynebacterium* strains expressing each of the complex improved enzymes prepared as in Example 4-1 were prepared by the same transformation method as in Example 2, and the activity assessment using the same was performed in the same manner as in Example 3-1, and the results are shown in FIG. 3.

As a result, as shown in FIG. 3, it was confirmed that all the prepared complex improved enzymes have remarkably superior enzyme activities, compared to the wild-type UGT-B.

Example 5. Assessment of RebDM Synthesis Activity of Improved Enzymes (2)

Additionally, the RebDM synthesis activities of the improved enzymes were assessed in a different way. Four types of *E. coli* BL21(DE3) strains were each prepared, into which for the expression of four types of enzymes (UGT-A, UGT-B_WT, UGT-B_M21, and RSS), each vector prepared by cloning pET28-α with the gene (SEQ ID NOS: 130, 127, 128, or 132) encoding the enzyme was introduced. Each strain was inoculated into 5 ml of LB medium and cultured at 37°C and 200 rpm for 8 hours, and then added to 2 L of LB medium to perform a main culture. After culturing at 37°C and 200 rpm until absorbance of the culture reached 0.4 at 600 nm, 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induce mass-expression of the enzyme. After adding IPTG, the culture was performed at 20°C and 120 rpm for about 16 hours. The culture was centrifuged at 6000 x g for 20 minutes at 4°C to obtain the cells, and the soluble proteins each for the four enzymes mentioned above were extracted through ultrasonication, and each enzyme was purified using Ni-NTA affinity chromatography.

The purified enzyme and the raw material (SG90, Haigen) comprising stevioside and RebA as the main components were used to perform the enzymatic reaction at 40˚C and pH 8.0 to 9.0 under conditions 1 to 3 as shown in Table 6 below.

[Table 6]

Composition	Condition 1	Condition 2	Condition 3
SG90 raw material concentration	3%	5%	10%
UDP concentration	10 mM		
Sucrose concentration	30%		
Na_2HPO_4 (pH8.0)	100 mM		
SS concentration (mg/ml)	0.2	0.3	0.4
UGT-A concentration (mg/ml)	1.0	2.0	4.0
UGT-B_WT or M21 concentration (mg/ml)	0.2	0.4	0.8

Then, after enzyme inactivation at 100˚C for 20 minutes, the production amounts of RebD, RebM, and RebDM (a mixture of RebD and RebM) when UGT-B_WT and UGT-B_M21 were added were compared through HPLC analysis in the same manner as Example 2, respectively.

In the case of the reaction solution to which UGT-B_M21 enzyme was added under conditions 1 to 3, the RebDM amount was 100%, 100%, and 90.8%, respectively, and the RebM amount was 89.5%, 83.8%, and 52.1%, respectively, after 72 hours of reaction, and the results are shown in Table 7 below.

[Table 7]

Raw material% (w/v)	UGT-B	24-hr reaction			48-hr reaction			72-hr reaction		
		RebD	RebM	RebDM	RebD	RebM	RebDM	RebD	RebM	RebDM
3%	WT	5.2%	18.3%	23.5%	5.4%	24.3%	29.7%	5.9%	32.2%	38.1%
	M21	**28.5%**	**43.9%**	**72.5%**	**25.8%**	**68.3%**	**94.1%**	**10.5%**	**89.5%**	**100.0%**
5%	WT	6.6%	19.3%	25.9%	8.4%	22.8%	31.3%	8.4%	36.9%	45.3%
	M21	**36.7%**	**43.0%**	**79.7%**	**32.5%**	**64.4%**	**96.9%**	**16.2%**	**83.8%**	**100.0%**
10%	WT	7.2%	16.7%	23.9%	9.7%	16.6%	26.3%	8.9%	24.0%	32.9%