FOOD STANDARDS
Australia New Zealand
Te Mana Kounga Kai – Ahitereiria me Aotearoa

3 November 2023
268-23

Supporting document 1

Risk and technical assessment – Application A1268

Steviol glycosides produced by bioconversion using new enzymes produced by GM *Escherichia coli*

Executive summary

This application from Manus Bio Inc. seeks FSANZ's approval for the use of genetically modified *Escherichia coli* K-12 to manufacture steviol glycosides (rebaudioside M and rebaudioside I). Both rebaudioside M and rebaudioside I are to be used as intense sweeteners in foods and are approved for that purpose in the Australia New Zealand Food Standards Code.

The *E. coli* strain has been genetically modified to produce the following enzymes used in production of the steviol glycosides:

1. Uridine triphosphate (UTP)-glucose-1-phosphate uridylyltransferase (EC 2.7.7.9) produced by GM *Escherichia* coli K-12, containing the gene for UTP-glucose-1-phosphate uridylyltransferase from *Bifidobacterium bifidum*
2. Uridine diphosphate (UDP)-Glucosyltransferase produced by GM *Escherichia coli* K-12, containing the gene for UDP-glucosyltransferase from *Oryza sativa* (rice)
3. Sucrose synthase (EC 2.4.1.13) produced by GM *Escherichia coli* K-12, containing the gene for sucrose synthase from *Glycine max* (soybean).

The three enzymes are technologically justified for their use to produce steviol glycosides by the bioconversion method of production, consistent with the JECFA Specification (Framework for) Steviol Glycosides, and are appropriately considered processing aids. The processing and purity steps undertaken ensure residual protein and residual DNA of the microorganisms and enzymes is removed and not in the final purified steviol glycosides.

All three enzymes are used together in the production of the Applicant's rebaudioside M. Only enzymes 1 and 3 – as listed above – are used together in the production of the Applicant's rebaudioside I.

The production organism *E. coli* strain K-12 has a long history of safe use. The derived strains which produce rebaudiosides M and I are neither pathogenic nor toxigenic and do not present a food safety risk. Analysis of the GM production strain confirmed the insertion and stability of the inserted genes.

No public health and safety concerns were identified in the assessment of any of the three

enzymes. The enzymes have a history of safe use for steviol glycoside production. For all three enzymes, the inserted genetic material is from a species with a long history of safe use either as a supplement (*Bifidobacterium bifidum*) or as a food (rice, soybean). Recent bioinformatics searches were conducted by comparing the amino acid sequences of the three enzymes to those of known toxins and known allergens. No homologies of concern were identified in these searches.

Based on the reviewed data it is concluded that in the absence of any identifiable hazard, an Acceptable Daily Intake (ADI) 'not specified' is appropriate for all three enzymes.

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1 Introduction

The purpose of this assessment is to consider the safety of the three enzymes proposed to be used in the production of two steviol glycosides, rebaudioside M and rebaudioside I. It is not to assess the safety of the individual steviol glycosides or whether such steviol glycosides are equivalent to those produced by other methods of production. This is because FSANZ has already assessed the safety of all steviol glycosides present in the *Stevia* leaf and, provided they comply with the relevant specifications and method of manufacture, permitted their use.

JECFA has recently completed a Specification (Framework for) Steviol Glycosides (JECFA framework) within monograph 26 (2021) of JECFA specifications (FAO and WHO 2021). This includes the four methods of production including annex 3 -Enzyme modified steviol glycosides - which is the method of production used for this application. The reason for the assessment of the enzymes is that they are either not listed within this JECFA specification or are derived from different sources to those listed.

FSANZ is updating the list of JECFA specification monographs to include monographs 25 and 26 as part of the current Proposal P1061 (Code Maintenance Proposal 2023)[1] which is being managed concurrently with this application but is likely to be gazetted before the completion of the assessment of this application.

1.1 Objectives of the assessment

The objectives of this risk and technical assessment were to:

- determine whether the proposed purpose is a solely technological purpose (function) and that the enzymes achieve their technological purpose as a processing aid in the quantity and form proposed to be used
- evaluate potential public health and safety concerns that may arise from the use of these enzymes, produced by a GM microorganism, as a processing aid. Specifically by considering the:
  - history of use of the gene donor and production microorganisms
  - characterisation of the genetic modification(s), and
  - safety of the enzymes.

2 Food technology assessment

2.1 Identity of the enzymes and manufacturing process

The application seeks permission for the use of three enzymes in the manufacture of steviol glycosides rebaudioside M and rebaudioside I by the bioconversion (enzymatic conversion) method of production. This form of manufacture is also called ‘enzyme modified’ and captured by Annex 3 – Enzyme modified steviol glycosides - of the JECFA framework within monograph 26 (2021) of JECFA specifications. This contains the definition for enzyme modified steviol glycosides as followed: a process in which steviol glycosides that have been extracted from the leaves of *Stevia rebaudiana* Bertoni undergo enzymatic conversion of major steviol glycosides to minor ones.

FSANZ has already assessed a number of applications using enzyme bioconversion method

[1] P1061 Code Maintenance Proposal 2023 (foodstandards.gov.au)

of manufacture (A1157, A1272, A1176 and A1183) so the manufacturing process summary builds on earlier assessments.

The relevant enzymes are (numbered to assist in their identification for later discussion):

1. Uridine triphosphate (UTP)-glucose-1-phosphate uridylyltransferase (EC 2.7.7.9) produced by GM *Escherichia* coli K-12, containing the gene for UTP-glucose-1-phosphate uridylyltransferase from *Bifidobacterium bifidum*
2. Uridine diphosphate (UDP)-Glucosyltransferase produced by GM *Escherichia coli* K-12, containing the gene for UDP-glucosyltransferase from *Oryza sativa* (rice)
3. Sucrose synthase (EC 2.4.1.13) produced by GM *Escherichia coli* K-12, containing the gene for sucrose synthase from *Glycine max* (soybean).

The first stage of the manufacturing process involves the preparation of the processing aids, being the three enzymes listed above, by fermentation. The processing aids are produced by a GM strain of *E.coli K-12* containing the genes of the listed enzymes sourced from *Bifidobacterium bifidum, Oryza sativa* (rice) and *Glycine max* (soybean). The two enzymes UDP-glucosyltransferase and sucrose synthase sourced from different microorganisms have been approved for the production of steviol glycosides by the bioconversion method of production and are listed within Schedule 18. UTP-glucose-1-phosphate uridylyltransferase (EC 2.7.7.9) has not been approved in the Code for this purpose.

As noted in later sections each of the enzymes have been protein engineered.

Information on the three enzymes used to produce rebaudioside M and rebaudioside I is provided below.

The applicant has confirmed that all three enzymes are used together in the production of the Applicant's rebaudioside M. Only enzymes 1 and 3 – as listed above – are used together in the production of the Applicant's rebaudioside I.

UTP-glucose-1-phosphate uridylyltransferase (EC 2.7.7.9) [2]

Source (strain):	*Escherichia coli* K-12 containing the UTP-glucose-1-phosphate uridylyltransferase gene from *Bifidobacterium bifidum*
Other names:	UDP glucose pyrophosphorylase; glucose-1-phosphate uridylyltransferase; UDPG phosphorylase; UDPG pyrophosphorylase; uridine 5'-diphosphoglucose pyrophosphorylase; uridine diphosphoglucose pyrophosphorylase; uridine diphosphate-D-glucose pyrophosphorylase; uridine-diphosphate glucose pyrophosphorylase
EC Number:	2.7.7.9
Systematic Name:	UTP:alpha-D-glucose-1-phosphate uridylyltransferase
Reaction:	UTP + alpha-D-glucose 1-phosphate = diphosphate + UDP-glucose

UDP-glucosyltransferase enzyme

Source (strain):	*Escherichia coli* K-12 containing the UDP-glucosyltransferase gene from *Oryza sativa* (rice)

[2]. EC 2.7.7.9 (qmul.ac.uk)

Common: Glucosyltransferase

EC Number: Not yet fully classified by the IUBMB

Systematic Name: UDP-glucose β-D-glucosyltransferase

CAS Number: 9033-07-2

Sucrose synthase enzyme (EC 2.4.1.13)[3]

Source (strain): *Escherichia coli* K-12 containing the sucrose synthase gene from *Glycine max* (soybean).

Common: Sucrose synthase

Other names: UDP glucose-fructose glucosyltransferase; sucrose synthetase; sucrose-UDP glucosyltransferase; sucrose-uridine diphosphate glucosyltransferase; uridine diphosphoglucose-fructose glucosyltransferase

EC Number: 2.4.1.13

Systematic Name: NDP-glucose:D-fructose 2-α-D-glucosyltransferase

Reaction: NDP-α-D-glucose + D-fructose = NDP + sucrose

CAS Number: 9030-05-1

2.2 Specifics of the enzymatic reaction

The information regarding the specifics of the enzyme reactions relating to UDP-glucosyltransferase and sucrose synthase were provided within the supporting documents for Applications A1157, A1172, A1176 and A1183 (FSANZ 2018, FSANZ 2019a, FSANZ 2019b, FSANZ 2020). This will not be repeated here. The enzyme UTP-glucose-1-phosphate uridylyltransferase is used to increase the supply of uridine diphosphate glucose, which is a precursor required for the glycosylation of steviol glycosides as part of the bioconversion process to produce the final steviol glycosides.

2.3 Specification for identity and purity for the enzymes

The enzymes are produced and used in-situ during the production of the final steviol glycosides. Therefore the purity of the specific enzyme preparations is not relevant for this application. Details of the specifications of the final steviol glycosides are provided in the application indicating they comply with the relevant JECFA specifications for steviol glycosides. Information is also provided confirming that the source microorganisms are not found in the final steviol glycosides. That is, the processing and purification steps undertaken ensure any residual protein or residual DNA from the microorganisms and enzymes is removed and not in the final purified steviol glycosides.

2.4 Food technology conclusion

The method of production of steviol glycosides using bioconversion is a well-known and

[3] EC 2.4.1.13 (qmul.ac.uk)

understood method of production which has been assessed by FSANZ for a number of earlier applications. This method is now also part of JECFA's updated specifications for steviol glycosides, being annex 3 of the JECFA framework. The enzymes used in the current method of production are similar to those listed in the JECFA specification, but they are sourced from different sources so needed their own assessment. Two of the three enzymes used in the bioconversion method of production, being UDP-glucosyltransferase and sucrose synthase have been assessed and approved for the production of steviol glycosides by FSANZ. They are also listed in the JECFA framework but sourced from different microorganisms. The third enzyme has not been assessed before nor is it listed with the JECFA specification, but its use is similar to the other two enzymes where it assists the bioconversion process to produce the specific steviol glycosides relevant to this application.

The three enzymes are technologically justified for their use to produce steviol glycosides by the bioconversion method of production, consistent with the JECFA framework.

All three enzymes are used together in the production of the Applicant's rebaudioside M. Only enzymes 1 and 3 – as listed earlier – are used together in the production of the Applicant's rebaudioside I.

These three enzymes have a technological purpose during the manufacturing process, so they are appropriately considered processing aids.

The purity of the final steviol glycosides comply with the relevant criteria of JECFA specifications for steviol glycosides.

3 Safety assessment

The objectives of this safety assessment are to evaluate any potential public health and safety concerns that may arise from the use of this enzyme system, produced by the specified strains of GM *E. coli*, for the production of the specified steviol glycosides.

Some information relevant to this section is Confidential Commercial Information (CCI), so full details cannot be provided in this public report.

3.1 History of use

3.1.1 Host organism

Escherichia coli is a normal inhabitant of the human colon. *E. coli* belongs to the family of *Enterobacteriaceae*. *E. coli* K-12 is the most commonly used laboratory model organism in microbial genetics and physiology research. This strain of *E.coli* does not normally colonise the human intestine and is non-pathogenic and non-toxigenic (EPA, 1997; NIH, 2019; EFSA, 2021). *E. coli* K-12 is a Biosafety Level 1 organism according to the NIH Guidelines (NIH, 2019) with a long history of safe use. In the biopharmaceutical industry approximately 30% of currently approved human recombinant therapeutic proteins in the United States are produced in *E. coli* K-12 (Huang et al. 2012; Jozala et al. 2016). The use of *E. coli* K-12 to produce food enzymes began in the 1980s (JECFA 1991). FSANZ has approved a number of applications with *E. coli* K-12 as the source production microorganism.

The parental strain, *E. coli* K-12 in this application was obtained from the Coli Genetic Stock Center (CGSC). The CGSC number and strain designation are 8003 and MGT655Fnr-, respectively (Guyer et al., 1981).

The production strains derived from *E. coli* K-12 were genetically modified to express specific

enzymes to improve the production of rebaudioside M and rebaudioside I. The production strain for rebaudioside I was derived from the production strain for rebaudioside M.
The identity of the final production strains were confirmed by alignment of the 16S rRNA consensus sequence with the parent strain.

In summary, the production strains derived from *E. coli* strain K-12 to produce rebaudioside M and rebaudioside I do not present a food safety risk.

3.1.2 Gene donor organisms

Three gene donor organisms, being *Bifidobacterium bifidum*, *Oryza sativa*, and *Glycine max*, contributed to the production of rebaudioside M in this application. The production strain was modified with coding for UTP -glucose-1-phosphate uridylyltransferase from *Bifidobacterium bifidum*, uridine diphosphate glucosyltransferases from *Oryza sativa*, and sucrose synthases from *Glycine max*.

Rice (*Oryza* spp.) is a significant caloric source for over half of the world's population and the cultivated species *Oryza sativa* is the most widely utilised, with thousands of cultivars being grown in over 100 countries. Rice has been found to be allergenic to some consumers (Yang et al., 2021). Unlike other food allergies, rice allergy is relatively uncommon (Nambu et al., 2006).

Soybean (*Glycine max*) is one of the most important legume crops and is the largest source of protein meal worldwide. Soybean is a source of food allergen affecting about 0.5% of the general population (Pi et al., 2021; Matsuo et al., 2020).

Bifidobacteria typically represent the most abundant bacteria of the human gut microbiota in healthy breastfed infants. Members of the *Bifidobacterium bifidum* species constitute one of the dominant taxa amongst these bifidobacterial communities. *Bifidobacterium bifidum* species have been described as probiotics due to their beneficial influences on human health (Turroni et al., 2019).

The incorporation of these modified genes into *E. coli* K-12 is not expected to involve other genetic material that will impart pathogenicity or toxicity to humans.

3.2 Characterisation of the genetic modification(s)

3.2.1 Description of DNA to be introduced and method of transformation

Multiple expression cassettes containing the genes encoding the steviol glycoside production enzymes, specified in Section 2.1, were introduced into the host organism. An expression cassette includes an open reading frame and any associated regulatory elements, e.g. sucrose synthase gene, a promoter and a terminator. The enzymes specified in Section 2.1 were protein engineered. Data provided by the applicant and analysed by FSANZ confirmed the identity of the enzymes.

Expression cassettes were inserted into the host's genome using homologous recombination. The methodology involved a series of gene deletion and overexpression events as well as the use of antibiotic-resistance selectable markers to enable positive selection. The final production strain has expression cassettes integrated at specific locus in the genome based on homologous recombination.

3.2.2 Characterisation of the inserted DNA

The final production organism was sequenced, identifying the site of integration and

confirming the presence of all inserted genes. The applicant provided whole genome sequencing data that confirmed the absence of antibiotic resistance selectable markers in the final production strain.

3.2.3 Genetic stability of the inserted gene

Data on the stability of the production strain provided by the applicant and analysed by FSANZ confirmed that the inserted genes have been stably integrated into the production strain's.

3.3 Safety of the enzymes

The purpose of the application is to include in the Code the following enzymes:

- UTP-glucose-1-phosphate uridylyltransferase (EC 2.7.7.9) produced by GM *E.coli* K-12, containing the gene for UTP-glucose-1-phosphate uridylyltransferase from *Bifidobacterium bifidum*
- Glucosyltransferase produced by GM *E. coli* K-12, with the genetic material sourced from *Oryza sativa* (rice)
- Sucrose synthase produced by GM *E. coli* K-12, with the genetic material sourced from *Glycine max* (soybean).

3.3.1 History of safe use

There are no current permissions in the Code for the use of UTP-glucose-1-phosphate uridylyltransferase. Two glucosyltransferases produced from GM *E. coli* K-12 are currently permitted in Schedule 18 of the Code, for the same purpose as the glucosyltransferase for which permission is sought. The genetic material was from *Solanum lycopersicum* (tomato plant) and *Stevia rebaudiana.* One sucrose synthase from GM *E.coli* K-12 is also permitted in the Code for the same purpose, with the inserted genetic material originating from *Arabidopsis thaliana* (thale cress or mouse-ear cress).

The specific enzymes that are the subjects of this application have been used for production of steviol glycosides for >24 months in the USA. It is also noted that all the donor organisms for the inserted genetic material (*Bifidobacterium bifidum, Oryza sativa*, *Glycine max*) have a history of safe human consumption.

3.3.2 Bioinformatic assessment of enzymes' toxicity

All reviewed protein sequences from the UniProtKB database (version released May 2022) annotated with the keyword 'toxin' that contain toxin were extracted. The sequence alignment program ClustalW 2.1 was used to align each toxin sequence to each of the enzyme sequences. The largest homology encountered was 16.5%, indicating that the homology of each of the three enzymes to any toxin sequence in the database is very low.

3.3.3 Evaluation of toxicity studies

Toxicity studies are not considered to be necessary because there is sufficient evidence of history of safe use of the host organism, the inserted genetic material, and the enzymes; and there is no significant homology between the amino acid sequences of any of the three enzymes and that of any known toxin.

3.3.4 Potential for allergenicity

Results of recent (2023) bioinformatics searches using the AllergenOnline[4] database (Version 21) were provided by the applicant. Searches included full-length alignment and homology using a sliding-window 80 amino acid search. No biologically significant matches were identified in any of the searches.

The applicant analysed three non-consecutive batches of each the steviol glycosides (Reb M or Reb I; ≥95% purity) to confirm that residual proteins were not present in the final products. Analysis was conducted using the bicinchoninic acid (BCA) assay, with a limit of detection (LOD) of 22.5 ppm on a w/v basis. All results were below the LOD, providing evidence that residual proteins were not present in the final steviol glycosides.

3.3.5 Assessments by other regulatory agencies

No assessments of the enzymes by other regulatory agencies were submitted or located. The applicant submitted a GRAS notification (GRN 1010) for Rebaudioside M produced by enzymatic conversion of stevia leaf extract to the US FDA. The FDA responded with a No Questions letter in January 2022. This does not constitute a regulatory assessment.

3.3.6 Discussion and conclusion of the safety assessment

No public health and safety concerns were identified in the assessment of any of the three enzymes that are the subjects of this application. The enzymes have a history of safe use for the production of steviol glycosides. The production organism is a strain of *E. coli* K-12, an organism with a long history of safe use as an enzyme production organism. For all three enzymes, the inserted genetic material is from a species with a long history of safe use either as a supplement (*Bifidobacterium bifidum*) or as a food (rice, soybean). Analysis of the GM production strain confirmed the insertion and stability of steviol glycoside biosynthesis genes. Recent bioinformatics searches were conducted by comparing the amino acid sequences of the three enzymes to those of known toxins and known allergens, using the UniProtKB and AllergenOnline databases respectively. No homologies of concern were identified in these searches.

4 Discussion

This application from Manus Bio Inc. seeks FSANZ's approval for the use of GM *Escherichia coli* strain K-12 to manufacture steviol glycosides (rebaudioside M and rebaudioside I). Both rebaudioside M and rebaudioside I are to be used as intense sweeteners in food and are approved for that purpose in the Food Standards Code.

The *E. coli* strain has been genetically modified to produce the following enzymes used in production of the steviol glycosides:

- Uridine triphosphate (UTP)-glucose-1-phosphate uridylyltransferase (EC 2.7.7.9) produced by GM *Escherichia* coli K-12, containing the gene for UTP-glucose-1-phosphate uridylyltransferase from *Bifidobacterium bifidum*
- Uridine diphosphate (UDP)-Glucosyltransferase produced by GM *Escherichia coli* K-12, containing the gene for UDP-glucosyltransferase from *Oryza sativa* (rice)
- Sucrose synthase (EC 2.4.1.13) produced by GM *Escherichia coli* K-12, containing the gene for sucrose synthase from *Glycine max* (soybean).

[4] AllergenOnline

The three enzymes are technologically justified for their use to produce steviol glycosides by the bioconversion method of production, consistent with the JECFA framework, and are appropriately considered processing aids.

The production strains derived from *E. coli* strain K-12 to produce rebaudioside M and rebaudioside I are neither pathogenic nor toxigenic and do not present a food safety risk. The GM production strain contains genes involved in the production of rebaudioside M and rebaudioside I. Analysis of the GM production strain confirmed the insertion and stability of the inserted genes.

No public health and safety concerns were identified in the assessment of any of the three enzymes that are the subjects of this application. The enzymes have a history of safe use for steviol glycoside production. The production organism has a long history of safe use as an enzyme production organism. For all three enzymes, the inserted genetic material is from a species with a long history of safe use either as a supplement (*Bifidobacterium bifidum*) or as a food (rice, soybean). Recent bioinformatics searches were conducted by comparing the amino acid sequences of the three enzymes to those of known toxins and known allergens. No homologies of concern were identified in these searches.

5 Conclusion

Based on the reviewed data it is concluded that in the absence of any identifiable hazard an Acceptable Daily Intake (ADI) 'not specified' is appropriate for all three enzymes.

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