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Supporting document 1

Risk and technical assessment – Application A1357

Steviol glycosides (rebaudioside M and rebaudioside D) produced by enzymatic conversion using enzymes produced by *Escherichia coli* BL21

Executive summary

Adorvia Biotechnology Co Ltd (a subsidiary of Abiochem Biotechnology Co Ltd) has applied to amend the Australia New Zealand Food Standards Code (the Code) to permit the use of steviol glycosides with a high rebaudioside M (Reb M) or rebaudioside D (Reb D) content produced by enzymatic conversion, as a food additive. The process uses three enzyme processing aids derived from genetically modified (GM) strains of *Escherichia coli* BL21. The enzymes are sucrose synthase and two uridine diphosphate (UDP)-glucosyltransferases as follows:

- sucrose synthase (EC 2.4.1.13) produced by *E. coli* BL21, containing the gene for sucrose synthase from *Methylocaldum szegediense*
- β-1,2-glucosyltransferase (EC 2.4.1.-) produced by *E. coli* BL21, containing the gene for β-1,2-glucosyltransferase from *Solanum tuberosum*
- β-1,3-glucosyltransferase (EC 2.4.1.-) produced by *E. coli* BL21, containing the gene for β-1,3-glucosyltransferase from *Stevia rebaudiana*.

Steviol glycosides are currently permitted in the Code as an intense sweetener food additive at maximum permitted levels in a number of food categories and at Good Manufacturing Practice levels in tabletop sweeteners.

Substances used as food additives must comply with any relevant identity and purity specifications listed in Schedule 3 of the Code. Schedule 3 contains a specification for steviol glycosides prepared from the leaves of *Stevia rebaudiana* Bertoni, which includes Reb M and Reb D, produced by enzymatic conversion (section S3—35). However, this specification does not cover the applicant's particular method of enzymatic conversion using their specified enzymes.

Schedule 18 of the Code contains a number of permissions for enzymes used as processing aids in the production of different steviol glycosides, including the following sourced from *E. coli* BL21:

- sucrose synthase (EC 2.4.1.13) sourced from *E. coli* BL21 containing the gene for sucrose synthase from *Arabidopsis thaliana,* to produce Reb M

- UDP-glucosyltransferase sourced from *E. coli* BL21 containing the gene for UDP-glucosyltransferase from *Stevia rebaudiana,* to produce Reb M.

However, the enzymes which are the subject of this application are not currently permitted processing aids for the production of Reb M and Reb D in Schedule 18. This is because two of the enzymes are produced by GM strains of *E. coli* BL21 that contain genes sourced from donor organisms that are not included in existing permissions. While a permission does exist for the third enzyme, that permission is limited to its use in the production of Reb M. Therefore, FSANZ has carried out a risk assessment to determine whether there are any public health and safety concerns associated with the applicant's steviol glycosides preparations produced using the specified enzymes as processing aids.

FSANZ concluded the three enzymes are technologically justified to produce Reb M and Reb D by enzymatic conversion and are appropriately considered processing aids. The processing and purification steps undertaken ensure residual protein and residual DNA of the production microorganisms and enzymes are below quantifiable and detectable levels, respectively. Dietary exposure to any of the enzymes through use of the final product is negligible.

No public health and safety concerns were identified in the assessment of the enzymes or production organisms. The host organism is a strain of *E. coli* BL21, an organism with a long history of safe use as an enzyme production organism. Analysis of the production strains confirmed the presence and stability of the genes involved in production of the three enzymes used to produce Reb M and Reb D.

The enzymes have a history of safe use for steviol glycoside production. 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.

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1 Introduction

Adorvia Biotechnology Co Ltd (a subsidiary of Abiochem Biotechnology Co Ltd) applied to Food Standards Australia New Zealand (FSANZ) to amend the Australia New Zealand Food Standards Code (the Code) to permit the use of steviol glycosides with a high rebaudioside M (Reb M) or rebaudioside D (Reb D) content produced by enzymatic conversion using enzymes derived from genetically modified (GM) strains of *Escherichia coli* BL21. The enzymes are sucrose synthase and two uridine diphosphate (UDP)-glucosyltransferases, namely, β-1,2-glucosyltransferase and β-1,3-glucosyltransferase.

The above enzymes act as processing aids converting the naturally occurring steviol glycosides in an extract of leaves from the *Stevia rebaudiana* Bertoni plant, for example, the 'major glycoside' rebaudioside A, to 'minor glycosides' Reb M and Reb D, which have more favourable sensory characteristics.

Schedule 3 contains a specification for steviol glycosides prepared from the leaves of *S. rebaudiana* Bertoni, which includes Reb M and Reb D, produced by enzymatic conversion (section S3—35). However, this specification does not cover the applicant's particular method of enzymatic conversion using their specified enzymes.

In addition, the applicant's proposed enzymes are not listed as approved enzymes for the production of steviol glycosides in Schedule 18. Two of the proposed enzymes are produced by GM strains of *E. coli* BL21 that contain genes sourced from donor organisms that are not included in existing permissions. While a permission does exist for the third enzyme, a UDP-glucosyltransferase produced from *E. coli* BL21 containing the gene for UDP-glucosyltransferase from *S. rebaudiana*, that permission is limited to use in the production of Reb M.

1.1 Objectives of the assessment

The objectives of this risk and technical assessment are to:

- determine whether the proposed purpose of the enzymes is solely technological and that they achieve this purpose as processing aids during the production of Reb M and Reb D
- evaluate public health and safety concerns that may arise from the use of these enzymes, produced by GM strains of *E. coli* BL21, expressing steviol glycoside enzymatic conversion genes as processing aids. 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.

This report provides some general information regarding the identity, and physical and chemical properties of Reb M and Reb D. It does not assess the safety of these steviol glycosides or whether they are equivalent to those produced by other methods of production. This is because FSANZ has previously assessed safety of steviol glycosides present in the stevia leaf and, provided they comply with the relevant specifications and method of manufacture, concluded that they are safe.

The application does not request:

- changes to the purity (≥95% steviol glycosides) or other parameters listed in the specification for steviol glycosides produced by enzymatic conversion in Schedule 3 and, specifically, paragraph S3—35(4) of the Code

- an extension of use of steviol glycosides to food groups additional to those that are currently permitted to contain steviol glycosides in Schedule 15 – Substances that may be used as food additives
- increases to the maximum permitted levels at which steviol glycosides are currently permitted to be added to those food groups in accordance with Schedule 15.

Therefore, these factors have not been considered in the assessment.

2 Food technology assessment

2.1 Identity of the enzyme processing aids

The applicant provided information regarding the identity of the 3 enzymes used in the manufacture of Reb M and Reb D by enzymatic conversion. This information has been verified, where available, using the IUBMB enzyme nomenclature reference database (McDonald et al. 2009). Details of the identity of the enzymes are provided below.

Table 1: Identity of enzymes used in steviol glycoside production

Sucrose synthase enzyme		
Accepted name:	Sucrose synthase	
Systematic name:	NDP-α-D-glucose:D-fructose 2-α-D-glucosyltransferase	
Other names:	UDP glucose-fructose glucosyltransferase; sucrose synthetase; sucrose-UDP glucosyltransferase; sucrose-uridine diphosphate glucosyltransferase; uridine diphosphoglucose-fructose glucosyltransferase	
IUBMB enzyme nomenclature:	EC 2.4.1.13	
Reaction:	NDP-α-D-glucose + D-fructose = NDP + sucrose	
CAS number:	9030-05-1	
Function:	Catalyses the formation of uridine diphosphate glucose (UDPG)	
Source:	*Escherichia coli* BL21 containing the gene for sucrose synthase from *Methylocaldum szegediense*	
Uridine diphosphate (UDP)-glucosyltransferase enzymes		
Accepted name:	β-1,2-glucosyltransferase	β-1,3-glucosyltransferase
IUBMB enzyme nomenclature:*	EC 2.4.1.-	EC 2.4.1.-
Function:	Catalyses the addition of glucose to steviol glycosides	
Source:	*Escherichia coli* BL21 containing the gene for:	
	β-1,2-glucosyltransferase from *Solanum tuberosum*	β-1,3-glucosyltransferase from *Stevia rebaudiana*\

*β-1,2-glucosyltransferase and β-1,3-glucosyltransferase are not yet fully classified by the IUBMB and fourth-level EC numbers have not been assigned. Both enzymes are therefore appropriately classified as EC 2.4.1.

2.2 Identity of the food additive

Steviol glycosides (INS 960) are a group of naturally occurring sweet compounds derived from the leaves of *Stevia rebaudiana* Bertoni. More than 60 steviol glycoside molecules have been identified in the stevia leaf extract, all of which share the same steviol backbone structure (Figure 1). Individual steviol glycosides differ in the sugar moieties attached to this

backbone (R_1 and R_2), including glucose, rhamnose, xylose, fructose and deoxyglucose, which may be attached in various combinations, quantity and orientation (FAO/WHO 2023).

The technological purpose of steviol glycosides is as an intense sweetener. Under Schedule 14 of the Code, intense sweeteners are used to replace the sweetness normally provided by sugars in food without contributing significantly to their available energy. As such, steviol glycosides are commonly used in reduced-energy and no-added-sugar foods.

Schedule 15 of the Code lists permissions for steviol glycosides at maximum permitted levels in a number of food classes and at Good Manufacturing Practice (GMP) levels in tabletop sweeteners.

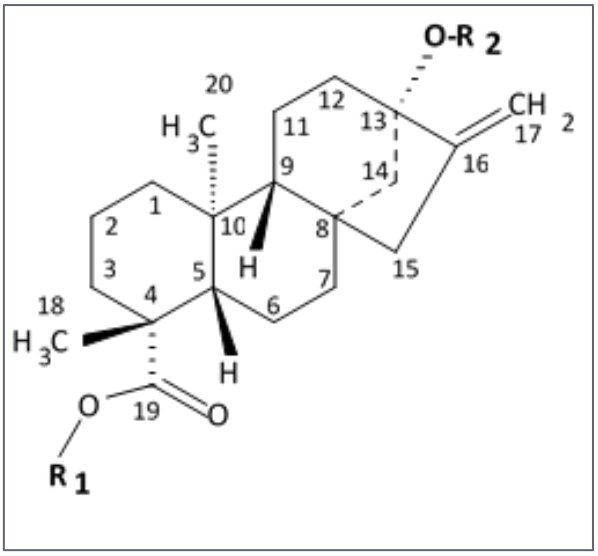


Figure 1: Chemical backbone structure for steviol glycosides

For this application, steviol glycosides extracted from *S. rebaudiana* Bertoni leaves undergo enzymatic conversion to produce Reb M or Reb D and a total steviol glycoside content of ≥95%. Further information regarding the identity of the steviol glycosides is provided in Table 2.

Table 2: Identity of Reb M or Reb D

	Rebaudioside M	Rebaudioside D
Chemical name:	13-[(O-b-D-Glucopyranosyl-(1,2)-O-[b-D-glucopyranosyl-(1,3)]-b-D-glucopyranosyl)oxy]-kaur-16-en-18-oic acid (4')-O-b-D-glucopyranosyl-(1,2)-O-[b-D-glucopyranosyl-(1,3)]-b-D-glucopyranosyl ester	13-[(O-β-D-glucopyranosyl-(1→2)-O-[β-D-glucopyranosyl-(1→3)]-β-D-glucopyranosyl)oxy]-, 2-O-β-D-glucopyranosyl-β-D-glucopyranosyl ester
Common names:	Steviol glycosides, enzyme-modified steviol glycosides, enzymatically produced steviol glycosides, steviol glycosides by enzyme modification	
Synonyms:	Reb M	Reb D
Chemical formula:	$C_{56}H_{90}O_{33}$	$C_{50}H_{80}O_{28}$
Molecular weight:	1291	1129
CAS number:	1220616-44-3	63279-13-0

2.3 Manufacturing process

The manufacturing process encompasses production of the enzymes followed by production of the two minor glycosides Reb M or Reb D by enzymatic conversion, in an *in vitro* multi-enzyme cascade reaction.

2.3.1 Production of the enzyme processing aids

The 3 enzymes used to produce Reb M and Reb D are sucrose synthase and two UDP-glucosyltransferases as follows:

1. sucrose synthase (EC 2.4.1.13) produced by *E. coli* BL21, containing the gene for sucrose synthase from *M. szegediense*

2. β-1,2-glucosyltransferase (EC 2.4.1.-) produced by *E. coli* BL21, containing the gene for β-1,2-glucosyltransferase from *S. tuberosum*

3. β-1,3-glucosyltransferase (EC 2.4.1.-) produced by *E. coli* BL21, containing the gene for β-1,3-glucosyltransferase from *S. rebaudiana*.

Enzymes from microorganisms are typically produced by controlled fermentation followed by removal of the production microorganism, purification and concentration of the enzyme. In this instance, for each individual enzyme, the production strain is fermented to obtain the fermentation broth. Once fermentation is complete, the fermentation broth is concentrated by filtration and homogenised to release the intracellular enzymes. The enzymes are isolated from the fermentation biomass by several filtration steps to remove impurities, resulting in a clear liquid enzyme product, ready for use in the enzymatic conversion process which follows.

2.3.2 Production of the food additive

Following production and isolation, the enzymes are added to a mixture of purified rebaudioside A powder (a naturally occurring steviol glycoside extracted from *S. rebaudiana* Bertoni leaves) and sugar, with sugar acting as a glucose donor. The subsequent enzymatic reactions that take place generate a steviol glycoside mixture containing high levels of the desired rebaudiosides.

The enzymatic conversion process is described in the application and summarised as follows:

1. β-1,2-glucosyltransferase catalyses the transfer of a glucose molecule from uridine diphosphate glucose (UDPG) or adenosine diphosphate glucose (ADPG) (activated donor molecules) to rebaudioside A, forming Reb D

2. β-1,3-glucosyltransferase catalyses the transfer of another glucose molecule from UDPG or ADPG to Reb D, producing Reb M

3. Sucrose synthase regenerates the glucose sources (i.e. uridine diphosphate (UDP) and adenosine diphosphate (ADP) to UDPG and ADPG, respectively) in a reversible reaction enabling the enzymatic conversion process to continue until the desired levels of Reb D or Reb M are achieved in the steviol glycoside mixture.

Further details regarding the enzyme reactions that occur in the formation of rebaudiosides are available in the supporting documents for Applications A1157 and A1172 (FSANZ 2018;

FSANZ 2019a).

Following attainment of the required rebaudioside levels, the reaction mixture undergoes a series of filtration and purification steps. The result is a steviol glycosides product that meets established identity and purity specifications for steviol glycosides produced by enzymatic conversion (see section 2.4 of this report).

The applicant states that all materials and processing aids used in the manufacture of Reb M and Reb D are food-grade and comply with relevant United States Pharmacopeial Convention Food Chemicals Codex (FCC) or other internationally recognised standards.

Further information on the manufacturing process, including the raw materials and processing aids used, have been provided in Appendix A of the application. This information is deemed confidential commercial information (CCI) under section 114 of the *Food Standards Australia New Zealand Act 1991* (FSANZ Act) and can only be disclosed under certain circumstances in accordance with that Act. This information has been evaluated by FSANZ but cannot be disclosed in this report.

2.3.3 Stability of the food additive

Table B.1.3-1 of the application presents data on the shelf-life stability of steviol glycosides with a high Reb M content under the accelerated conditions study protocol (40 ± 2 °C; relative humidity: 75 ± 5%) over a 3-month storage period. The full study report has been provided in CCI Appendix E of the application.

No significant losses or sensory changes are reported and microbial parameters meet the relevant specifications. The applicant states these results indicate the Reb M preparation tested would have a similar shelf-life stability profile as other high-purity steviol glycoside preparations currently on the marketplace. Shelf-life stability studies on Reb D were not provided in the application but were not considered necessary on the basis of the similarity in the chemical structure and that it has been previously established that steviol glycosides are sufficiently thermally and hydrolytically stable for use in foods, under normal conditions of processing and storage (JECFA 2007).

2.4 Specifications for identity and purity

2.4.1 Specifications for the enzyme processing aids

There are international general specifications for enzyme preparations used in the production of food, established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in its Compendium of Food Additive Specifications and in the FCC (14th edition), referenced in section S3—2 of Schedule 3 of the Code. Enzymes used as processing aids need to meet either of these specifications, or a relevant specification in section S3—3 of Schedule 3.

Schedule 3 of the Code includes specifications for arsenic and heavy metals (section S3—4) if they are not already detailed within specifications in sections S3—2 or S3—3.

The applicant provided analytical data for 3 batches of each of the 3 enzymes (section B.5.2.2 and Appendix F of the application). Table 3 provides a comparison of the overall results of those analyses with international specifications established by JECFA and the FCC, as well as those in the Code. Based on those results, the enzymes conform to recommended purity criteria.

The applicant states the enzymes are food-grade quality and are used exclusively by Adorvia Biotechnology Co Ltd in the manufacture of steviol glycosides with a high Reb M or Reb D

content.

Table 3: Analysis of 3 batches of the 3 enzymes compared to JECFA, Food Chemicals Codex, and Code specifications for enzymes

Test parameters	Test results	Specifications		
		JECFA	Food Chemicals Codex	The Code – section S3—4
Sucrose synthase				
Lead (mg/kg)*	Compliant with Code	≤5	≤5	≤2
Arsenic (mg/kg)	Compliant with Code	-	-	≤1
Cadmium (mg/kg)	Compliant with Code	-	-	≤1
Mercury (mg/kg)	Compliant with Code	-	-	≤1
Coliforms (cfu/g)	<10	≤30	≤30	-
***Salmonella* (in 25 g)**	ND	Absent	Negative	-
***Escherichia coli* (in 25 g)**	ND	Absent	-	-
Antimicrobial activity	Negative	Absent	-	-
β-1,2-glucosyltransferase				
Lead (mg/kg)*	Compliant with Code	≤5	≤5	≤2
Arsenic (mg/kg)	Compliant with Code	-	-	≤1
Cadmium (mg/kg)	Compliant with Code	-	-	≤1
Mercury (mg/kg)	Compliant with Code	-	-	≤1
Coliforms (cfu/g)	<10	≤30	≤30	-
***Salmonella* (in 25 g)**	ND	Absent	Negative	-
***Escherichia coli* (in 25 g)**	ND	Absent	-	-
Antimicrobial activity	Negative	Absent	-	-
β-1,3-glucosyltransferase				
Lead (mg/kg)*	Compliant with Code	≤5	≤5	≤2
Arsenic (mg/kg)	Compliant with Code	-	-	≤1
Cadmium (mg/kg)	Compliant with Code	-	-	≤1
Mercury (mg/kg)	Compliant with Code	-	-	≤1
Coliforms (cfu/g)	<10	≤30	≤30	-
***Salmonella* (in 25 g)**	ND	Absent	Negative	-
***Escherichia coli* (in 25 g)**	ND	Absent	-	-
Antimicrobial activity	Negative	Absent	-	-

cfu = colony forming units; ND = not detected
*mean of three results
NB: for those results expressed as 'Compliant with Code' signed certificates of analysis were provided by the applicant indicating levels were 'Compliant' with the Australia New Zealand Food Standards Code Schedule 3, S3—4. Numerical values were not provided on the certificates of analysis for these parameters.

2.4.2 Specifications for the food additive

2.4.2.1 JECFA Framework for Steviol Glycosides

JECFA has prepared a Specifications Monograph (JECFA Framework for Steviol Glycosides), the latest version of which was published in FAO JECFA Monographs 31 (2023).

Annex 3 of the JECFA Framework describes enzyme-modified steviol glycosides as being produced through the enzymatic treatment of purified steviol glycosides extracted from *S. rebaudiana* Bertoni leaves. This manufacturing technique maximises the production of steviol glycosides that are not naturally present in high concentrations in the leaf extract, particularly Reb M and Reb D.

The JECFA Framework specifies that the enzymes used in this process, glucosyltransferase and sucrose synthase (EC 2.4.1.13), may be produced in non-toxigenic, non-pathogenic genetically modified *E. coli* strains containing genes from 4 listed donor organisms: *Acidithiobacillus caldus*, *Arabidopsis thaliana* and, of relevance to this application, *S. tuberosum* and *S. rebaudiana*.

The enzymatic conversion method described in the application is consistent with Annex 3 of the JECFA Framework. This is with the exception of one donor organism, *M. szegediense*, which is not currently listed in Annex 3.

2.4.2.2 The Code

Substances used as food additives must comply with any relevant identity and purity specifications listed in Schedule 3 of the Code – Identity and purity. There is an existing specification in Schedule 3 for steviol glycosides preparations obtained from the leaves of *S. rebaudiana* Bertoni and produced by enzymatic conversion (section S3—35). The specifications state that the preparation may contain different individual steviol glycosides, with total steviol glycosides present at levels not less than 95% (on the dried basis) (paragraph S3—35(3) and (4)).

2.4.2.3 Consistency of the applicant's product with relevant specifications

Table B.6.1-1 and Table B.6.1-2 of the application detail the product specifications for steviol glycosides with high Reb M and Reb D contents, respectively. Analytical data for 5 batches from non-consecutive lots of each steviol glycosides product are summarised in Table B.6.2.1-1. The certificates of analysis supporting these results are provided in Appendix G of the application.

Tables B.3.1-1 and B.3.1-2 of the application provide further details of the actual steviol glycoside distribution in 5 analysed batches of each steviol glycoside preparation. For the Reb M preparation, Reb M was analysed at levels ≥95%, with small amounts of rebaudioside B also present. For the Reb D preparation, Reb D was measured at levels ≥85%, together with Reb M at approximately 7% and small amounts or other rebaudiosides, to make up a product comprising total steviol glycosides ≥95%.

Analytical results indicate that the steviol glycoside preparations of Reb M and Reb D are consistent with the assay and purity specifications set out in Annex 3 of the JECFA Framework, and sections S3—35 and S3—4 of the Code (Table 4).

Table 4: Analysis of 5 non-consecutive batches of steviol glycosides with high Reb M and high Reb D content compared to JECFA and Code specifications

Analysis	Test results	Specifications		
		JECFA	The Code – section S3—35	The Code – section S3—4
Description	White to off-white powder	White to light yellow powder	White to light yellow powder	-
Purity (%) steviol glycosides	≥ 99	≥ 95	≥ 95	-
Solubility	Pass	Very slightly soluble to freely soluble in water	Freely soluble in water	-
pH (1% solution)*	5.7	4.5-7.0	4.5-7.0	-
Total ash (%)*	0.02	≤1	<1	-
Loss on drying*	4.6	≤6	≤6	-
Residual methanol (mg/kg) [#]	ND	≤200	≤200	-
Residual ethanol (mg/kg)*	330	≤5000	≤5000	-
Lead (mg/kg)	<0.2	≤1.0	≤1.0	-
Arsenic (mg/kg)	<0.015	≤1.0	≤1.0	-
Cadmium	<0.015	-	-	≤1.0
Mercury	<0.07	-	-	≤1.0
Total (aerobic) plate count (CFU/g)	<10	≤1000	-	-
Yeast and mould (CFU/g)	<10	≤200	-	-
***Escherichia coli* (MPN/g)**	<3	Negative in 1 g	-	-
***Salmonella* sp.**	ND	Negative in 25 g		

MPN = most probable number; CFU = colony forming units; ND = not detected
[#]Not measured in Reb D samples
*mean of 10 results

While there is an existing specification in Schedule 3 for steviol glycosides, including Reb M and Reb D, produced by enzymatic conversion (section S3—35), the existing specification does not cover the applicant's particular method of enzymatic conversion using their specified enzymes. Specifically, the specification does not include steviol glycosides produced using UDP-glucosyltransferases from *E. coli* BL21 containing the UDP-glucosyltransferase gene from *S. tuberosum.* Nor does it include sucrose synthase from *E. coli* BL21 containing the sucrose synthase gene from *M. szegediense.* While the specification lists steviol glycosides produced using a UDP-glucosyltransferase from *E. coli* BL21 containing the UDP-glucosyltransferase gene from *S. rebaudiana,* the specification is for the production of Reb M only. Therefore, section S3—35 will require amendment to include the production of steviol glycosides using these enzymes and their sources (with concomitant amendments to Schedule 18).

2.5 Food technology conclusion

The enzymatic conversion method of production of steviol glycosides has been previously assessed by FSANZ (Applications A1157 (FSANZ 2018), A1172 (FSANZ 2019a), A1176 (FSANZ 2019b) and A1183 (FSANZ 2020)) and is described in Annex 3 of the JECFA Framework (2023).

As noted in section 2.4 of this report, while there are existing permissions in the Code to use UDP-glucosyltransferases and sucrose synthase from several sources for the production of different rebaudiosides, those that are the subject of this application are not currently listed in the Code and therefore require assessment.

The enzymes have been assessed as technologically justified for their use to produce steviol glycosides by the enzymatic conversion method of production, consistent with JECFA, FCC and Code specifications. They perform their technological purpose during the manufacturing process and are therefore appropriately considered processing aids.

The resulting product, Reb M or Reb D, is consistent with assay and purity specifications and it meets its technological purpose as an intense sweetener. The applicant's product is stable under accelerated conditions (40 ± 2 °C; relative humidity: 75 ± 5%) over a 3-month storage period and, as such, it would have a similar shelf-life stability profile as other high-purity steviol glycoside preparations currently in the marketplace.

3 Safety assessment

The objectives of this safety assessment were to evaluate any public health and safety concerns that may arise from the use of the enzymes produced by the specified strain of GM *E. coli* BL21 for the enzymatic production of Reb M and Reb D.

Some information relevant to this section is CCI under section 114 of the FSANZ Act and has been evaluated by FSANZ but cannot be disclosed in this public report.

3.1 Source microorganism

The production strains for the enzymes β-1,2-glucosyltransferase, β-1,3-glucosyltransferase, and sucrose synthase are derivatives of *Escherichia coli* BL21 (DE3), which is a direct derivative of *E. coli* BL21 with minimal and well-characterised divergence. Accordingly, FSANZ's microbiological assessment will focus on the safety of the parent strain, *E. coli* BL21.

E. coli is a facultatively anaerobic, gram-negative, rod-shaped bacterium found in the gut of mammals (Guerra et al. 2019). Some *E. coli* strains can be pathogenic to humans causing a wide range of diseases, which can be fatal (Guerra et al. 2019; Mir and Kudva 2019). However, there are strains of *E. coli*, termed safe strains, that are used in research and industry specifically because of their inability to cause disease in humans (Bauer et al. 2008). BL21 is one of these safe strains and lacks well-recognised pathogenic mechanisms associated with disease (Chart et al. 2000; Jeong et al. 2009; Studier et al. 2009; Jeong et al. 2015; NCBI Genome Database 2024). Accordingly, it is classified as non-pathogenic and categorised as a Biosafety Level 1 and Risk Group 1 organism (Bauer et al. 2008; Pinske et al. 2011).

BL21 is a laboratory strain widely used in biotechnology, molecular biology, and industrial applications (Pinske et al. 2011; Kim et al. 2017). Originally developed for its utility as an expression host, BL21 has a long history of safe use in research and production of recombinant proteins and other bioproducts. Given its extensive industrial use, BL21 has been assessed by multiple regulatory agencies, including the US Food and Drug Administration (FDA), the European Food Safety Authority, and FSANZ (Applications A1190, A1318, and A1339), for use in industrial processes under GMP. The use of BL21 as the production organism for food ingredients has been concluded to be 'generally recognised as safe' (GRAS) in multiple FDA GRAS Notices, including GRNs 485, 571, 876, 922, 923, 921,

925, 1015 and 1016.

The taxonomic identity of the production strains was confirmed by whole-genome sequencing and bioinformatic analysis. In addition, batch analyses demonstrate that the final product does not contain viable cells from the production organisms.

The microbiology assessment identified no public health and safety concerns regarding the use of *E. coli* BL21-derived strains as production organisms.

3.2 Gene donor organisms

The source of the β-1,2-glucosyltransferase gene is *S. tuberosum* (potato), a widely consumed staple crop. The gene for β-1,3-glucosyltransferase is from *S. rebaudiana*, commonly known as honey leaf, sweet leaf or sweet-herb (Peteliuk et al. 2021). The source of the gene for sucrose synthase is *M. szegediense*, a thermophilic methanotrophic bacterium (Delherbe et al. 2024).

3.3 Characterisation of the genetic modification to the production strain

3.3.1 Description of the DNA to be introduced and the method of transformation

The genes encoding β-1,2-glucosyltransferase, β-1,3-glucosyltransferase and sucrose synthase were synthesised *in vitro* based on the amino acid sequences from the respective source organisms (section 3.2 of this report). Three separate production strains were created in *E. coli* BL21 using standard molecular biology techniques, each expressing one of the three enzymes involved in the enzymatic conversion of Reb A to Reb D and Reb M.

Data provided by the applicant and analysed by FSANZ confirmed the identity of the enzymes.

3.3.2 Characterisation and genetic stability of the inserted DNA

FSANZ reviewed data provided by the applicant which confirmed the genetic stability of each of the production strains. Relevant analyses also confirmed the absence of recombinant DNA in the final products.

3.4 Safety of the enzymes used to produce the steviol glycosides

The applicant conducted appropriate literature searches but located no new information that would justify a change to the existing Acceptable Daily Intake for steviol glycosides, expressed as steviol, of 0-4 mg/kg bw/day. The objective of this safety assessment is to evaluate any public health and safety concerns that may arise from the use of enzymes that are not already approved for production of steviol glycosides. The enzymes are the applicant's sucrose synthase, β-1,2-glucosyltransferase, and β-1,3-glucosyltransferase.

3.4.1 History of safe use

The applicant's sucrose synthase, β-1,2-glucosyltransferase, and β-1,3-glucosyltransferase have been used in commercial production with no reported adverse reactions.

In addition, permissions already exist in the Code for the use of these enzymes in the production of different steviol glycosides, including for the following sourced from *E. coli* BL21:

- sucrose synthase (EC 2.4.1.13) sourced from *E. coli* BL21 containing the gene for sucrose synthase from *A. thaliana*, to produce Reb M

- UDP-glucosyltransferase sourced from *E. coli* BL21 containing the gene for UDP-glucosyltransferase from *S. rebaudiana*, to produce Reb M.

However, there are currently no permissions in the Code for the new enzymes proposed by the applicant. This is because two of the gene donor organisms for the enzymes are different and, for the third (relating to *S. rebaudiana*), the existing permission for use covers the production of Reb M only.

3.4.2 Bioinformatics concerning homology with toxins

The application included results of sequence alignment queries of the amino acid sequences of all three enzymes against the curated database of venom proteins and toxins maintained by UniProt[1]. Structural homology/similarity criteria were: identity >40%[2], E-value[3] <0.001, bit-score[4] >40. No significant similarity to any toxins was identified from the sequence homology searches for any of the three enzymes.

3.4.3 Bioinformatics concerning homology with allergens

Results were provided of sequence alignment queries of the amino acid sequences of all three enzymes against the AllergenOnline[5] database, version 24 (26 January 2026). The sequence homology searches for each enzyme were:

1. a “sliding window” of 80–amino acid sequences derived from each full-length amino acid sequence of the food enzyme (minimum identity cut-off = 35%);
2. an 8–amino acid exact match; and
3. the full-length amino acid sequence (FASTA36; E-value cut-off = 1 and maximum alignments of 20).

No matches with >35% identity were found for any of the three enzymes.

3.4.4 Absence of residual protein in the finished product

The application includes results of analysis of five representative batches of each steviol glycoside product for the presence of residual protein. Residual protein could be indicative of the presence of the enzymes, breakdown products of the enzymes, and/or other proteins from the production organism, in the final product. The analyses were conducted using a validated bicinchoninic acid protein assay. Protein levels in all analysed batches were below the limit of quantification, supporting the conclusion that exposure to any of the enzymes through use of the final product will be negligible or low.

3.4.5 Safety assessments by other regulatory agencies

No safety assessments by other regulatory agencies are available. As previously noted in section 2.4.2 of this report, the enzymes are consistent with those set out in Annex 3 of the JECFA Framework for Steviol Glycosides with the exception of one donor organism, *M. szegediense*, which is not currently listed.

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[1] https://www.uniprot.org/

[2] percentage of identical residues in the sequence alignment

[3] the E-value is the number of hits that is expected to occur by chance in a database of a given size

[4] the bit score is a measure that normalizes raw sequence alignment scores to allow comparisons

[5] http://www.allergenonline.com/

5 Discussion

No public health and safety concerns were identified in the assessment of the enzymes that are the subjects of this application. The enzymes have a history of safe use in the production of steviol glycosides and show no relevant homology to known toxins or allergens. Levels of residual proteins in the final products were below the level of quantification. The production organism is a strain of *E. coli* BL21, an organism with a long history of safe use as an enzyme production organism. Analysis of the GM production strains confirmed the presence and stability of the genes involved in production of the three enzymes used to produce Reb M and Reb D.

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