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Te Mana Kounga Kai – Ahitereiria me Aotearoa

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Supporting document 1

Risk assessment – Application A1318

Steviol glycosides produced by enzymatic conversion using enzymes produced by GM *Escherichia coli* BL21

Executive summary

This application from Sichuan Ingia Biosynthetic Co. Ltd. seeks Food Standards Australia New Zealand's approval to permit the use of three new enzymes, sourced from a genetically modified (GM) strain of *Escherichia coli*, as processing aids for the enzymatic conversion of the steviol glycoside rebaudioside A (extracted from purified stevia leaf extract) to rebaudioside M. Rebaudioside M is used as an intense sweetener in foods and is already approved for that purpose (as a steviol glycoside) 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 rebaudioside M:

- sucrose synthase (EC 2.4.1.13), produced by GM *Escherichia coli* BL21, expressing the gene for sucrose synthase from *Arabidopsis thaliana*
- uridine diphosphate (UDP)-glucosyltransferase (91D2), produced by GM *Escherichia coli* BL21, expressing the gene for UDP-glucosyltransferase from *Stevia rebaudiana*
- uridine diphosphate (UDP)-glucosyltransferase (76G1), produced by GM *Escherichia coli* BL21, expressing the gene for UDP-glucosyltransferase from *Stevia rebaudiana*

The three enzymes are technologically justified for their use to produce rebaudioside M by the enzymatic conversion method of production, consistent with the JECFA framework for steviol glycosides specification, and are appropriately considered processing aids. The processing and purification steps undertaken ensure residual protein and residual DNA of the microorganisms and enzymes is removed and not in the final purified rebaudioside M.

No public health and safety concerns were identified in the assessment of the enzymes or production organism. 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 strain confirmed the insertion and stability of the genes involved in production of the three enzymes used to produce rebaudioside M.

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.

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 the steviol glycoside rebaudioside M. 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 Food Standards Australia New Zealand (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, has permitted their use.

1.1 Objectives of the assessment

The objectives of this risk and technical assessment were to:

- determine whether the proposed purpose is solely technological and that the enzymes achieve their technological purpose as processing aids 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 *Escherichia 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.

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 rebaudioside M by the enzymatic conversion method of production. This form of manufacture is also called 'enzyme modified' and is captured by Annex 3 – Enzyme modified steviol glycosides - of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) framework for steviol glycosides specifications within monograph 26 (2021) of JECFA specifications. This contains the definition for enzyme modified steviol glycosides as follows: 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.

Under this application, the process is multi step, in that the major steviol glycoside 'rebaudioside A' is first extracted from the purified stevia leaves (by hot water extraction). This stevia extract is then added directly to *E. coli* BL21 cells expressing the three enzymes used to convert rebaudioside A to the minor steviol glycoside, rebaudioside M. Rebaudioside M has a more favourable sensory characteristic compared to the major glycosides, as the taste profile is more reflective of sucrose.

FSANZ has already assessed several applications relating to the enzymatic conversion method of manufacture (A1157, A1272, A1176, A1183 and A1268) so the manufacturing process summary builds on earlier assessments.

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

1. Sucrose synthase (EC 2.4.1.13), produced by GM *Escherichia coli* BL21, expressing the gene for sucrose synthase from *Arabidopsis thaliana,*
2. Uridine diphosphate (UDP)-glucosyltransferase (91D2), produced by GM *Escherichia coli* BL21, expressing the gene for UDP-glucosyltransferase from *Stevia rebaudiana*

3. Uridine diphosphate (UDP)-glucosyltransferase (76G1), produced by GM *Escherichia coli* BL21, expressing the gene for UDP-glucosyltransferase from *Stevia rebaudiana*

The enzyme processing aids are produced by a GM strain of *E.coli* BL21 expressing the genes of the listed enzymes sourced from *Arabidopsis thaliana* and *Stevia rebaudiana*. All three enzymes, sourced from different plants, have been approved for the production of steviol glycosides by the enzymatic conversion method of production and are listed within Schedule 18.

None of the enzymes have been protein engineered[1].

Information on the three enzymes used to produce rebaudioside M from rebaudioside A is provided below.

Sucrose synthase enzyme (EC 2.4.1.13)[2]

Source (strain):	*Escherichia coli* BL21 containing the sucrose synthase gene from *Arabidopsis thaliana.*
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

UDP-glucosyltransferase enzymes

Source (strain):	*Escherichia coli* BL21 containing the UDP-glucosyltransferase gene from *Stevia rebaudiana*
Common:	Glucosyltransferase
EC Number:	Not yet fully classified by the IUBMB[3]
Systematic Name:	UDP-glucose β-D-glucosyltransferase
CAS Number:	9033-07-2

[1] Note, the application refers to the enzymes as protein engineered, however after FSANZ's assessment and discussion with the Applicant, it was agreed that none of the enzymes were to be classed as protein engineered. While the application still refers to protein engineered enzymes, this report and the A1318 CFS will not.
[2] EC 2.4.1.13 (qmul.ac.uk)
[3] An EC number for UDP-glucosyltransferase was incorrectly included for the draft variations prepared under applications A1157 and A1172. This will be corrected by FSANZ in a Code Maintenance Proposal.

2.2 Specifics of the enzymatic reaction

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.

2.3 Specification for identity and purity

The GM *E. coli* BL21 production strain generates the enzymes required for enzymatic conversion from rebaudioside A to rebaudioside M *in situ*. The purified rebaudioside A is added directly to a culture of the production strain containing the expressed sucrose synthase and UDP-glucosyltransferase enzymes. As the enzymes have not been purified and used as preparations separate from the production organism, specifications are not required.

Details of the final steviol glycosides specification (rebaudioside M) are provided in the application, indicating they are consistent with the relevant JECFA specification. Details of the specifications of the final steviol glycosides are consistent with the relevant JECFA specification. Information is also provided confirming that the source microorganisms are not present in the final steviol glycosides. That is, the processing and purification steps undertaken ensure any residual protein or DNA from the microorganisms are removed and not in the final purified rebaudioside M.

2.4 Food technology conclusion

The method of production of the steviol glycoside rebaudioside M using enzymatic conversion is a well-known and 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 framework of steviol glycosides specifications. The enzymes used in the current method of production are listed in the JECFA specification, but they are sourced from different microorganisms and not listed in the Code, so require assessment. The three enzymes sourced from different plants to produce steviol glycosides by have been assessed and approved previously FSANZ.

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

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

3 Safety assessment

The objectives of this safety assessment were to evaluate any potential 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 conversion of rebaudioside A to rebaudioside M.

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

BL21 is a laboratory strain widely used in biotechnology, molecular biology, and industrial applications. 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. BL21 contains many advantageous phenotypic traits, such as: reduced protease activity, which enhances protein stability during recombinant protein expression (Studier and Moffatt, 1986); efficient protein production under controlled conditions; and enhanced cell growth characteristics suitable for laboratory and industrial use (Rosano and Ceccarelli, 2014).

Because of its extensive industrial use, BL21 has previously been assessed by regulatory agencies, including the U.S. Food and Drug Administration (FDA) and the European Food Safety Authority (EFSA), for use in industrial processes under Good Manufacturing Practices (GMP). The use of BL21 as the production organism of food ingredients has been concluded to be 'generally recognised as safe' (GRAS) numerous times over the past decade (e.g. GRNs 485, 571, 876, 922, 923, 921, 925, 1015, and 1016).

BL21 is classified as non-pathogenic and is categorized as a Biosafety Level 1 (BSL-1) organism. Along with other laboratory strains of *E. coli,* including strains K-12, B, C, and their derivatives, BL21 is designated as a Risk Group 1 organism and is "not associated with disease in healthy adult humans" (National Institute of Health, 2024). The complete gene sequence of *E. coli* BL21 was published by Jeong et al. (2009), and comprehensive bioinformatics analyses of the organism are described by Studier et al. (2009). BL21 lacks the invasion factors, adhesion molecules and enterotoxins associated with virulence typically associated with pathogenic *E. coli* strains, such as enteropathogenic *E. coli* (EPEC) or Shiga toxin–producing *E. coli* (STEC) (Chart et al., 2000; Jeong et al., 2009; 2015; NCBI Genome Database, 2024). This includes reduced lipopolysaccharide (LPS) production because BL21 does not contain functional gene sequences encoding the O-antigen polysaccharide that typically coats the outer surface of *E. coli* cells (Jeong et al., 2009; Studier et al., 2009).

The *E. coli* BL21 production strain assessed here has been genetically modified to express sucrose synthase and UDP-glucosyltransferase (91D2 and 76G1) enzymes for the purpose of rebaudioside M production. The taxonomic identity of the production strain was confirmed by CCI data provided by the applicant. Furthermore, batch analyses demonstrate that the final product is absent of residual DNA and protein from the production organism.

Overall, no public health and safety concerns were identified for the use of *E. coli* BL21 as a production organism.

3.1.2 Gene donor organisms

The *E. coli* BL21 production strain is genetically modified with the sucrose synthase gene from *Arabidopsis thaliana* along with the UDP-glucosyltransferase 91D2 and UDP-glucosyltransferase 76G1 genes from *Stevia rebaudiana* Bertoni. The donor organisms' identities were confirmed with genomic data.

A. thaliana is a very popular model organism for plant biology and genetics research (Woodward and Bartel, 2018). *S. rebaudiana* Bertoni is widely grown for the manufacture of steviol glycosides and has a large body of evidence demonstrating it is safe for human consumption (Ahmad et al., 2020). Neither gene donor is associated with any known toxicity to humans.

3.2 Characterisation of the genetic modification(s)

3.2.1 Description of DNA to be introduced and method of transformation

The three genes encoding the enzymes for enzymatic conversion of rebaudioside A to rebaudioside M – sucrose synthase and UDP-glucosyltransferases 91D2 and 76G1 – were synthesised and introduced into a plasmid vector using standard molecular biology techniques. The recombinant plasmid was transformed into competent *E. coli* BL21 host cells using calcium chloride transformation. The plasmid also contains an ampicillin-resistance gene, allowing selection of successful transformants by growth on media containing ampicillin. Data provided by the applicant and analysed by FSANZ confirmed the identity of the enzymes.

3.2.2 Characterisation and genetic stability of the inserted DNA

Polymerase chain reaction (PCR) analyses were used to confirm the presence of the three target genes in the production strain, as well as their stability over 5 generations. In addition, 5 generations of the production strain were analysed by sodium dodecyl-sulfate polyacrylamide gel electrophoresis for expression of the sucrose synthase and UDP-glucosyltransferase (91D2 and 76G1) enzymes. The results of the analyses demonstrated the presence of the genes in the production strain and the stable expression of the three enzymes across multiple generations.

Although there is an ampicillin-resistance gene present in the final production strain, the applicant provided quantitative PCR data that confirmed the absence of the gene in the purified rebaudioside M extract.

3.3 Safety of the enzymes

The purpose of the application is to include in the Code, for the purpose of enzymatic conversion of rebaudioside A to rebaudioside M, the following enzymes:

- UDP-glucosyltransferases (sourced from *Escherichia coli* BL21 (DE3) containing the UDP-glucosyltransferase genes from *Stevia rebaudiana*
- sucrose synthase (EC 2.4.1.13) sourced from Escherichia coli BL21 (DE3) containing the sucrose synthase gene from *Arabidopsis thaliana*.

3.3.1 History of safe use

Schedule 18 of the Code currently includes “Uridine diphosphate (UDP) glucosyltransferase sourced from *Escherichia coli* K-12 containing the UDP glucosyltransferase gene from *Stevia rebaudiana*” and “Sucrose synthase (EC 2.4.1.13) sourced from *Escherichia coli* K-12 containing the gene for sucrose synthase from *Arabidopsis thaliana*”. The purpose of this application is to include *Escherichia coli* BL21 as a permissible source of these enzymes.

3.3.2 Bioinformatic assessment of enzymes’ toxicity

A recent (December 2024) BLAST search of the UniProt database (last updated April 2023) was conducted for each enzyme, using default parameters. No significant homology with any known toxin was found.

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 the enzymes and that of any known toxin.

3.3.4 Potential for allergenicity

The allergenicity potential of the enzymes was evaluated by searches of the AllergenOnline[4] database (Version 22, updated 25 May 2023). The searches were performed with the following criteria:

- Full-length sequence identity (with > 50% identity and E-value < 1x10-7)
- A sliding window of 80 amino acid sequences derived from the full-length amino acid sequence of the protein
- Eight amino acid exact matches

No significant identity matches were identified in the full-length sequence or 80-amino acid sliding window search that would be suggestive of an allergenic cross-reactive potential of these enzymes. In the eight amino acid search with UDP-glucosyltransferase, one match to alpha-actinin was identified. The usefulness of the exact match of eight contiguous amino acids has been debated, because this search strategy has a high 'false positive' result (Goodman et al., 2008; Ladics, 2019). FSANZ further notes that alpha-actinin is reported to be a respiratory allergen rather than a food allergen (An et al. 2013).

Under the proposed conditions of use of these enzymes for production of Rebaudioside M, FSANZ considers the allergenic potential to be low.

3.3.5 Assessments by other regulatory agencies

No assessments of the enzymes by other regulatory agencies were submitted or located.

3.3.6 Discussion and conclusion of the safety assessment

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. 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 strain confirmed the insertion and stability of the genes involved in production of the three enzymes used to produce rebaudioside M.

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.

[4] AllergenOnline

4 References

Ahmad J, Khan I, Blundell R, Azzopardi J, Mahomoodally MF (2020) Stevia rebaudiana Bertoni.: an updated review of its health benefits, industrial applications and safety. *Trends in Food Science and Technology, 100*:177-189.

An S, Shen C, Liu X, Chen L, Xu X, Rong M, Liu Z, Lai R (2013) Alpha-actinin is a new type of house dust mite allergen. PLoS One 8(12):e81377

Chart H, Smith HR, La Ragione RM, Woodward MJ (2000) An investigation into the pathogenic properties of Escherichia coli strains BLR, BL21, DH5alpha and EQ1. Journal of Applied Microbiology 89(6):1048-58. doi: 10.1046/j.1365-2672.2000.01211.x. PMID: 11123478.

Daegelen P, Studier FW, Lenski RE, Cure S, Kim JF (2009) Tracing ancestors and relatives of Escherichia coli B, and the derivation of B strains REL606 and BL21(DE3). Journal of Molecular Biology 394(4):634-43. doi: 10.1016/j.jmb.2009.09.022. PMID: 19765591.

Delbrück M, Luria SE (1942). Interference between bacterial viruses: I. Interference between two bacterial viruses acting upon the same host, and the mechanism of virus growth. Archives of Biochemistry 1, 111–141.

Escherich T (1885) Die Darmbakterien des Neugeborenen und Sauglings. Fortschritte der Medizin, 3, 515-522.

Goodman RE, Vieths S, Sampson HA, Hill D, Ebisawa M, Taylor SL, van Ree R (2008). Allergenicity assessment of genetically modified crops--what makes sense? Nature Biotechnology 26(1):73-81 [erratum, 26(2):241]. DOI:10.1038/nbt1343

Harper MS, Carpenter C, Klocke DJ, Carlson G, Davis T, Delaney B (2011) E. coli Lipopolysaccharide: acute oral toxicity study in mice. Food and Chemical Toxicology 49(8):1770-2. doi: 10.1016/j.fct.2011.04.025. PMID: 21554917.

Jeong H, Barbe V, Lee CH, Vallenet D, Yu DS, Choi SH, Couloux A, Lee SW, Yoon SH, Cattolico L, Hur CG, Park HS, Ségurens B, Kim SC, Oh TK, Lenski RE, Studier FW, Daegelen P, Kim JF (2009) Genome sequences of Escherichia coli B strains REL606 and BL21(DE3). Journal of Molecular Biology 394(4):644-52. doi: 10.1016/j.jmb.2009.09.052 PMID: 19786035.

Jeong H, Kim HJ, Lee SJ (2015) Complete Genome Sequence of Escherichia coli Strain BL21. Genome Announcements 3(2):e00134-15. doi: 10.1128/genomeA.00134-15. PMID: 25792055.

Ladics GS (2019). Assessment of the potential allergenicity of genetically-engineered food crops. Journal of Immunotoxicology16(1):43-53. DOI:10.1080/1547691X.2018.1533904.

National Institute of Health (2024). NIH Guidelines for Research Involving Recombinant or Synthetic Nucleic Acid Molecules (NIH Guidelines). Bethesda (MD): National Institutes of Health (NIH), Office of Science Policy. Available at: https://osp.od.nih.gov/wp-content/uploads/NIH_Guidelines.pdf

NCBI Genome Database. (2024). Escherichia coli BL21 Genome. Retrieved from

https://ncbi.nlm.nih.gov/nuccore/CP010816

Rosano GL, Ceccarelli EA (2014) Recombinant protein expression in Escherichia coli: advances and challenges. Frontiers in Microbiology 5:172. doi: 10.3389/fmicb.2014.00172. PMID: 24860555.

Studier FW, Daegelen P, Lenski RE, Maslov S, Kim JF (2009) Understanding the differences between genome sequences of Escherichia coli B strains REL606 and BL21(DE3) and comparison of the E. coli B and K-12 genomes. Journal of Molecular Biology 394(4):653-680. doi: 10.1016/j.jmb.2009.09.021. PMID: 19765592.

Studier FW, Moffatt BA (1986) Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. Journal of Molecular Biology, 189(1), 113-130.

Woodward AW, Bartel B (2018) Biology in Bloom: A Primer on the *Arabidopsis thaliana* Model System. Genetics 208(4):1337-1349. doi: 10.1534/genetics.118.300755. PMID: 29618591.

Zhang ZX, Nong FT, Wang YZ, Yan CX, Gu Y, Song P, Sun XM (2022) Strategies for efficient production of recombinant proteins in Escherichia coli: alleviating the host burden and enhancing protein activity. Microbial Cell Factories 21(1):191. doi: 10.1186/s12934-022-01917-y. PMID: 36109777.