

Supporting document 1

Risk and technical assessment report – Application A1109

Glutaminase from *Bacillus amyloliquefaciens* as a Processing Aid (Enzyme)

Executive summary

Application A1109 seeks approval to use the enzyme glutaminase, sourced from a chemically mutated strain of *Bacillus amyloliquefaciens* (strain GT), as a processing aid. The stated purpose of this enzyme is for the production of certain seasoning ingredients (e.g. yeast extract, hydrolysed vegetable proteins and hydrolysed animal proteins) or food products used as seasonings (e.g. soy sauce, miso, vinegar, fish sauce, etc.).

Glutaminase catalyses the conversion of L-glutamine present in these foods to L-glutamate, an important component of taste and quality in the foods to which glutaminase is added. The use of glutaminase to increase the glutamate content of these foods can be an alternative to use of chemicals (acid hydrolysis) or to external sources of glutamate (such as monosodium glutamate (MSG)), to form foods/food ingredients with high concentrations of glutamates.

The evidence presented to support the proposed uses provides adequate assurance that the enzyme, in the form and prescribed amounts, is technologically justified to be effective in achieving its stated purpose. The enzyme preparation meets international purity specifications for enzymes used in the production of food.

There were no public health and safety issues associated with the use of the enzyme preparation, containing glutaminase sourced from a chemically mutated strain of *B. amyloliquefaciens*, as a food processing aid on the basis of the following considerations:

- The production organism is not toxigenic or pathogenic and is not present in the final enzyme preparation used as the food processing aid. Further, *B. amyloliquefaciens* has a history of safe use as the production organism for a number of processing aids already permitted in the *Australia New Zealand Food Standards Code* (the Code).
- Glutaminase has a long history of safe use and although residual enzyme is expected to be present in the final food, it would be inactive and susceptible to digestion like any other dietary protein.
- Complete digestion of the enzyme in simulated digestive fluid suggests the enzyme is unlikely to be toxic.
- The enzyme preparation caused no observable adverse effects at the second-highest tested doses in a 13-week repeated dose toxicity study in rats. The NOAEL for the glutaminase concentrate was determined to be 0.6% w/w of the diet or 388 mg/kg bw/d (male rats) and 450 mg/kg bw/d (female rats).

- The enzyme was not genotoxic or mutagenic *in vitro*.
- Bioinformatic analysis indicated that the enzyme has no biologically relevant homology to known protein allergens.

Based on the reviewed toxicological data, it was concluded that in the absence of any identifiable hazard, an Acceptable Daily Intake (ADI) 'not specified' was appropriate. A dietary exposure assessment was therefore not required.

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1 Introduction

FSANZ received an application from Amano Enzyme Inc. Japan seeking approval for the enzyme glutaminase (EC 3.5.1.2) as a processing aid. The Applicant states that this enzyme will be used in the production of certain seasoning ingredients (e.g. yeast extract, hydrolysed vegetable proteins and hydrolysed animal proteins) or food products used as seasonings (e.g. soy sauce, miso, vinegar, fish sauce, etc.). The enzyme is sourced from a chemically mutated strain of *Bacillus amyloliquefaciens* strain NP, known as *B. amyloliquefaciens* strain GT2. Glutaminase sourced from this bacterium is by means of a fermentation process.

The Applicant proposes to use glutaminase to catalyse the conversion of L-glutamine to L-glutamate. L-glutamate enhances the taste of the foods to which the glutaminase is added. The use of glutaminase produced by *B. amyloliquefaciens* can be an alternative to use of chemicals (acid hydrolysis) or to external sources of glutamate (such as monosodium glutamate (MSG)), to form foods/food ingredients with high concentrations of glutamates.

1.1 Objectives of the Assessment

Currently, there are no permissions for the enzyme glutaminase from *B. amyloliquefaciens* or any other source in the Code. Therefore, any application to amend the Code to permit the use of this enzyme as a food processing aid requires a pre-market assessment.

The objectives of this risk assessment were to:

- determine whether the proposed purpose is clearly stated and that the enzyme achieves its technological function in the quantity and form proposed to be used as a food processing aid
- evaluate any potential public health and safety concerns that may arise from the use of glutaminase as a processing aid.

2 Food Technology Assessment

2.1 Characterisation of glutaminase (enzyme)

2.1.1 Identity of the enzyme

Information regarding the identity of the enzyme that was taken from the Application has been verified using an appropriate enzyme nomenclature reference (IUBMB, 2015). Additional information has also been included from this reference.

Generic common name:	Glutaminase
Systematic name:	L-glutamine amidohydrolase
Accepted IUBMB ¹ name:	Glutaminase
IUBMB enzyme nomenclature:	EC 3.5.1.2
C.A.S. number:	9001-47-2

¹ International Union of Biochemistry and Molecular Biology

Other names: glutaminase I; L-glutaminase; glutamine aminohydrolase
Reaction: L-glutamine + H₂O = L-glutamate + NH₃
Commercial name: Glutaminase SD-C100S

2.1.2 Enzymatic properties

The enzyme glutaminase belongs to the class of hydrolytic enzymes. Its catalytic characteristic is that it catalyses the hydrolysis of the γ -amido bond of L-glutamine to L-glutamate and ammonia (Nandakumar et. al., 2003).

Almost all living cells produce glutaminase, which plays a significant contributory role in cellular nitrogen metabolism. Glutaminase also has important pharmaceutical and industrial uses as an effective agent in the treatment of acute lymphocytic leukaemia and HIV, as an analytical agent, a biosensing agent, as a flavour enhancing agent and in the production of specialty chemicals such as threonine (Sathish & Prakasham, 2010). Microbial glutaminases have a long history of use and are used extensively in the food industry due to their role as flavour-enhancing agents (Sarada, 2013).

The glutaminase enzyme preparation that is the subject of this Application is used for the hydrolysis of the amino acid L-glutamine, which is naturally present in the starting food or ingredient, to L-glutamate, which is an important component of the quality and taste of the food products to which glutaminase is added (Figure 1). Such food products include seasoning ingredients (e.g. yeast extract, hydrolysed vegetable protein, and hydrolysed animal protein) and food products used as seasonings (e.g. soy sauce, miso, vinegar, and fish sauce).

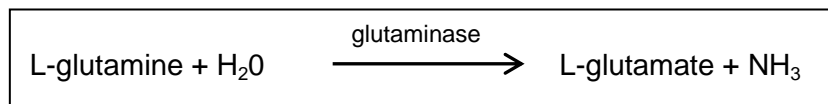


Figure 1: Reaction catalysed by glutaminase

The glutaminase enzyme preparation (powder) is active during the manufacture of the glutamine-containing food/food ingredient, with inactivation of the enzyme occurring either by temperature or pH changes. The Applicant recommends that inactivation be accomplished by changing the pH of the food so that it is lower than 5 or greater than 9, or by increasing the temperature above 60°C. This will ensure that the enzyme has no action or function in the final food product.

2.1.3 Physical properties

The commercial enzyme preparation, Glutaminase SD-C100S, is supplied as a light brown powder, comprising 91% (w/w) sodium chloride and 9% (w/w) glutaminase concentrate, and with approximately 86% Total Organic Solids (TOS).

The commercial enzyme preparation shows a glutaminase activity level of 110 glutaminase units (GTU)/gram. The maximum use level of the commercial preparation is 0.2% when used during the manufacture of seasoning ingredients/foods used as seasonings. Based on the dilution of the glutaminase concentrate with sodium chloride, this level of use would be equivalent to 0.018% of the glutaminase concentrate in the final food/food ingredient.

2.2 Production of the enzyme

The glutaminase concentrate is produced by the fermentation of *B. amyloliquefaciens* under standard culturing conditions.

The production steps can be summarised as a fermentation process, a filtration process, production of the glutaminase concentrate, and then formulation of the final commercial glutaminase preparation. The raw materials used in the production of Glutaminase SD-C100S are approved food ingredients, food additives, microbial nutrients, or permitted in the production of processing aids (FSANZ, 2014). The enzyme preparation is reported to be made according to Good Manufacturing Processes.

Once the fermentation is complete, the downstream processing steps consist of several filtration steps. The cultures are filtered twice using high pressure to remove the culture media. This is followed by ultrafiltration to concentrate the extracellular glutaminase, and then one final sterile filtration to remove any remaining bacteria. The resultant concentrate is then spray dried and blended to produce a powdered glutaminase concentrate.

To prepare the commercial enzyme preparation (Glutaminase SD-C100S), the glutaminase concentrate is diluted by the addition of sodium chloride. The Glutaminase SD-C100S preparation comprises 91% sodium chloride and 9% glutaminase concentrate. Some further details of the individual steps are provided in the Application.

2.2.1 Potential presence of allergens

Lactose (which may be produced from whey as a by-product of cheese and casein industries), defatted soybean, soybean oil and dextrin (which may be produced from wheat starch) comprise part of the fermentation medium in the preparation of the glutaminase enzyme. Milk products, soybean products and wheat products are identified as substances requiring declaration in paragraph 1.2.3—4(1)(d) of the revised Code.

2.3 Specifications

There are international specifications for enzyme preparations used in the production of food. These have been established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2016) and the Food Chemicals Codex (Food Chemicals Codex, 2015). These primary sources of specifications are listed in Schedule S3—2 of the revised Code. Enzyme preparations need to meet these enzyme specifications. Schedule 3 of the revised Code also includes specifications for heavy metals.

Table 1 provides a comparison of the product specifications with the international specifications established by JECFA as well as those detailed in the revised Code (as applicable).

Table 1: Product specifications for commercial glutaminase preparation compared to JECFA and Code specifications for enzymes

Analysis	Specifications		
	Product	JECFA	Australia New Zealand Food Standards Code
Lead (mg/kg)	≤2	≤ 5	≤2
Arsenic (mg/kg)	≤1	-	≤1
Mercury (mg/kg)	≤0.5	-	≤1
Cadmium (mg/kg)	≤0.5	-	≤1

Analysis	Specifications		
	Product	JECFA	Australia New Zealand Food Standards Code
Viable bacteria count (cfu/g)	$\leq 5 \times 10^4$	-	-
Coliforms (cfu/g)	≤ 30	≤ 30	-
<i>Salmonella</i> (in 25 g)	Negative	Absent	-
<i>E. coli</i>	Negative in 2.22 g	Absent in 25 g	-

The Application states that the glutaminase preparation contains no detectable antibiotic activity. Absence of antimicrobial activity is a requirement of the JECFA specifications for enzymes used in food processing.

The final enzyme preparation meets international and Code specifications for enzyme preparations used in the production of food.

2.4 Technological function of the enzyme

The enzyme glutaminase, sourced from *B. amyloliquefaciens*, is intended to be used as a processing aid in the production of certain seasoning ingredients (e.g. yeast extract, hydrolysed vegetable protein, and hydrolysed animal protein) or food products used as seasonings (e.g. soy sauce, miso, vinegar², and fish sauce). The enzyme hydrolyses the amino acid L-glutamine in the starting food or ingredient, to L-glutamate, an important flavour component.

Based on information provided by the Applicant, the glutaminase enzyme preparation has benefits that include a high glutamate yield, excellent thermal stability, and stability for at least 12 months when stored according to the recommended conditions.

There are a number of commercial methods available to increase the glutamate content of food products such as those mentioned above. Methods include natural fermentation, acid and base hydrolysis of glutamine-rich proteins, the addition of exogenous enzymes such as proteases, and a combination of these. All of these methods break down proteins in the starting material into individual amino acids, whereby glutamate is an essential element for the distinctive 'umami'³ flavour of these products.

A comparison of enzymatic hydrolysis using glutaminase against other methods for manufacturing certain food products indicates that there are a number of potential advantages. These include milder processing conditions (including milder temperatures), a desirable amino acid profile in the protein hydrolysates due to the specificity of the enzyme, minimal formation of unwanted by-products (such as of mono- and di-chloropropanols), and no neutralisation of the product required after the end of the hydrolysis (Pomeranz, 2013).

2.5 Food technology conclusion

The stated purpose of this glutaminase enzyme sourced from *B. amyloliquefaciens*, namely, for use as a processing aid in the production of certain seasoning ingredients (e.g. yeast extract, hydrolysed vegetable protein, and hydrolysed animal protein) or food products used as seasonings (e.g. soy sauce, miso, vinegar, and fish sauce), is clearly articulated in the Application.

² For example, East Asian black vinegar, which is an aged, fermented product made from rice, wheat, millet, sorghum or a combination, and can contain high concentrations of amino acids.

³ The 'fifth taste', a pleasant, savoury taste that is full, rounded and with a 'meaty' flavour.

The evidence presented to support the proposed uses provides adequate assurance that the enzyme, in the form and prescribed amounts, is technologically justified and has been demonstrated to be effective in achieving its stated purpose. The enzyme preparation meets international purity specifications.

3 Hazard Assessment

3.1 Background

3.1.1 Chemistry

Details of the chemistry of the glutaminase produced by *B. amyloliquefaciens*, including relevant physicochemical and enzymatic properties, and product specifications, are provided in the Food Technology Assessment (see Section 2).

3.1.2 Scope of the hazard assessment

The hazard of glutaminase sourced from *B. amyloliquefaciens* was evaluated by considering the:

- hazard of the production organism, including any history of safe use in food production processes
- hazard of the encoded protein, including potential allergenicity
- toxicity studies on the enzyme preparation intended for commercial use.

3.2 Hazard of the production organism – *B. amyloliquefaciens* strain GT2

The parental lineage of the production organism is classified as *B. amyloliquefaciens* strain NP. Strain GT2 is a mutant derived from the NP strain by eight rounds of chemical mutagenesis using *N*-methyl-*N*-nitrosoguanidine and selecting for enhanced glutaminase production. Strain GT2 is not genetically modified. This parental strain is neither pathogenic nor toxigenic.

It should be noted that the parent strain (NP) was previously classified as *Bacillus subtilis*, a species closely related to *B. amyloliquefaciens* and for which there is also an extensive history of use as a source organism for food products and processing aids in numerous jurisdictions, including Australia and New Zealand (FSANZ, 2014).

The source strain (GT2) is not listed on the American type culture collection (ATCC) or Deutsche Sammlung von Mikroorganismen und Zellkulturen – DSMZ (German Collection of Micro-organisms and Cell Cultures). The applicant provided additional information to establish the taxonomy of the source micro-organism (GT2) and the parent strain (NP). The analysis was performed by TNO Nutrition and Food Research. The microscopy analysis showed that both GT2 and NP were similar to the reference strain *B. subtilis* ATCC 13933, although all three strains could be differentiated. The biochemical analysis (API 50 CHB) could not satisfactorily identify GT2. The biochemical results for NP gave good identification to the *Bacillus* genus level but could not satisfactorily identify the strain. The results suggested that NP represents either *B. amyloliquefaciens* (37.1%), *B. licheniformis* (36.5%) or *B. subtilis* (22.3%). Results of the more definitive DNA hybridisation test confirmed the source microorganism (GT2) as *B. amyloliquefaciens* (95% homology to *B. amyloliquefaciens* LMG 9814T). DNA hybridisation results were not provided for the parent NP strain.

FSANZ has previously assessed *B. amyloliquefaciens* as a safe production organism for a number of food-grade enzymes. Subsection S18—4(5) of the revised Code permits the use of the following enzymes derived from *B. amyloliquefaciens* as food processing aids: α -acetolactate, α -amylase, β -amylase, β -glucanase, hemicellulose endo-1,4-xylanase, hemicellulose multicomponent enzyme, metalloproteinase, pullulanase and serine proteinase. There have been no reports of allergenicity associated with the use of *B. amyloliquefaciens* in Australia and New Zealand. Additionally, the applicant has reported that since beginning glutaminase production in strain NP in 1992 and strain GT2 in 1997, no adverse effects have been reported in workers exposed to either strain of *B. amyloliquefaciens*.

The European Food Safety Authority (EFSA, 2008) has listed *B. amyloliquefaciens* as a Qualified Presumption of Safety (QPS) organism provided there is evidence around the absence of emetic food poisoning toxins, surfactant activity and enterotoxic activity. The risk clarification derived by the European Community to protect workers exposed to biological agents classifies *B. amyloliquefaciens* in Group 1, meaning it is unlikely to cause human disease (EC, 2000). Additionally, The US FDA established in 1999 that carbohydrase and protease enzyme preparations derived from *B. amyloliquefaciens* or *B. subtilis* were considered Generally Recognized as Safe (GRAS) for use as a direct food additive (US FDA, 2009).

3.3 Hazard of the enzyme glutaminase

3.3.1 History of Use

Glutaminase concentrate has been used in Japan for many years in food processing and it is currently on the 'List of Existing Food Additives' published by the Ministry of Health and Welfare of Japan (MHLW, 2014). Glutaminase has been employed in the production of soy sauces since 1991 and in the production of miso since 1992 (Amano Enzyme, 2005). The use of glutaminase as a processing aid for the production of hydrolysed vegetable protein has been ongoing since 2003. Glutaminase enzymes sourced from *B. amyloliquefaciens* have a long history of use in Japan as they were first reported in the publicly available literature in 1988 (Shimizu et. al., 1991).

3.3.2 Bioinformatic analysis for potential allergenicity

The glutaminase sequence from *B. amyloliquefaciens* was compared via *in silico* analysis with known allergens in two databases, the Allergen Database for Food Safety (<http://allergen.nihs.go.jp/ADFS>) and Allergen Online (<http://www.allergenonline.com/databasefasta.shtml>). The sequences were compared to identify matches for 8-consecutive amino acids and an 80 amino acid sliding window (with 35% or higher identities within the 80 amino acid stretch). There were no hits (matches) in the search for 8 consecutive amino acids or for the 80 amino acid sliding window in either database. These results indicate that glutaminase does not share any significant amino acid sequence similarity with known food allergens and, therefore, is unlikely to be allergenic.

3.3.3 Bioinformatic analysis for potential toxicity

The applicant provided a bioinformatic study to examine the potential toxicity of the glutaminase enzyme using the Virulence Database, MvirDB (<http://mvirdb.llnl.gov/>), from the Lawrence Livermore National Laboratory, Livermore, California (A1109 Additional information (16 Nov 2015)). The database includes not only toxic proteins but all the enzymes expressed by drug resistant or pathogenic bacteria. The search was carried out using default parameters except for the E-value, which was changed from 0.5 to 0.2. The E-value is a measure of the similarity of sequences, the lower the E-value, the higher the congruity of the query amino acid sequence with the database sequence.

An E-value of zero would mean that there's an exact match for the amino acid sequence. The homology search revealed hits of approximately 32 to 33% identity between the glutaminase enzyme and two proteins from *Bacillus anthracis*, capD and gamma-glutamyltranspeptidase.

3.3.4 Enzyme stability to digestive degradation

The applicant provided a digestion test using simulated digestive fluid at pH1.2 and 2.0. The test was performed in accordance with the international validation method proposed by the International Life Science Institute (A1109 Additional information (16 Nov 2015)). The glutaminase enzyme was completely digested. Given the long history of safe use of both the production organism and enzyme, the enzyme is unlikely to present any public health and safety issues with regard to toxicity via oral ingestion.

3.4 Evaluation of toxicity studies of the enzyme product

3.4.1 Sub-chronic toxicity

Individual unpublished study

Appel, M.J (1999). Sub-chronic (13-week) oral toxicity study with Glutaminase in rats. TNO report V99.442. Lab: TNO Nutrition and Food Research Institute, Zeist, The Netherlands. Sponsor: DAIWA KASEI K.K. Osaka, Japan. **GLP:** OECD. **QA Statement:** Yes. **Test Guidelines:** OECD Guidelines for of Chemicals 408; Commission Directive 88/320/EEC, EEC Guidelines 87/302/EC, and the FDA Guidelines for testing of food additives (unpublished).

3.4.1.1 Range finding study (study 2054)

A range finding study was conducted over a 14 day period. Observations were made for weight, food conversion, water consumption, organ weight and relative organ weight.

Young albino Wistar rats (Charles River Deutschland, Sulzfeld, Germany) were used for this study; 5 males and 5 females for each treatment. The rats were about 4 weeks old at the start of the study and ranged in size from 126.8 to 158.3 g (mean 143.4 g) for males and from 88.3 to 125.7 g (mean 105.2 g) for females. Feed and water were provided *ad libitum*. The rats were fed a commercial rodent diet (Rat & Mouse No. 3 Breeding Diet, RM3) containing glutaminase (batch number GT70625L12) provided at constant concentrations of 0 (control), 0.2, 0.6 and 2.0% w/w of the diet.

At the high dose (2% w/w), there were significant decreases in mean body weight for males at all observation dates (days 3, 7, 10 and 14) and for females, but only at day 3. There were no other significant differences for any of the other observations. However, male and female rats of the high-dose group had a general trend of decreased food consumption during the first week of the study and decreased water consumption throughout the study.

3.4.1.2 Sub-chronic study (study 2055)

The sub-chronic study was conducted over 13 consecutive weeks. Clinical observations, growth, food consumption, food conversion efficiency, water consumption, ophthalmoscopy, haematology, clinical chemistry, urinalysis, organ weights, gross examination at autopsy and microscopic examination of various organs were used as criteria for disclosing possible harmful effects.

Young albino Wistar rats (source as above) were used for this study; 20 males and 20 females per treatment. The rats were about 4 weeks old at the start of the study and ranged in size from 139.3 to 190.6 g (mean 160.6 g) for males and 104.9 to 152.3 g (mean 124.3 g) for females. Feed and water were as described for the range finding study.

At the high dose (2.0% w/w), both male and female rats showed a significant decrease in body weight gain and feed consumption. The final mean weight was 5% lower for males and 9% lower for females compared to control animals. Transient decreases in water intake were also observed for the high-dose group.

Blood chemistry showed significant decreases in plasma alkaline phosphatase activity (males at mid and high dose and females at high dose), alanine aminotransferase activity (males at mid and high dose and females at high dose) and chloride level (females at mid-dose). For the high-dose group, there were significant decreases in the absolute brain weight (females), spleen (females), adrenals (females) and liver (males); and increases in the relative weights of the testes (males) and brain (females).

There were no treatment related changes/abnormalities observed in the ophthalmologic, urinalysis, haematological, gross pathology and histopathology tests. There were no deaths or abnormal clinical signs during this study.

Although there were changes in the growth of the rats in the highest dose group, these appear to be associated with decreased food consumption and thus may be related to palatability rather than toxicity. However, toxicity cannot be completely ruled out. Therefore, the NOAEL for this study was determined to be the mid-dose level (0.6% w/w). Based on body weight and reported daily intake of glutaminase concentrate, this would equate to 388 mg/kg/day body weight for male and 450 mg/kg/day body weight for female rats.

3.4.2 Genotoxicity

Individual unpublished studies

van Delft, J.H.M. (1998) Bacterial reverse mutation test with Glutaminase. Report number V98.442. Lab: TNO Nutrition and Food Research Institute, Zeist, The Netherlands. Sponsor: DAIWA KASEI K.K. Osaka, Japan. **GLP:** OECD. **QA Statement:** Yes. **Test Guidelines:** OECD Test Guideline 471; Commission Directive 88/320/EEC (unpublished).

Van Delft, J.H.M. and de Vogel, N. (1998) Chromosomal aberration test with Glutaminase in cultured Chinese hamster ovary cells. Report number V98.1202. Lab: TNO Nutrition and Food Research Institute, Zeist, The Netherlands. Sponsor: DAIWA KASEI K.K. Osaka, Japan. **GLP:** OECD. **QA Statement:** Yes. **Test Guidelines:** OECD Test Guideline 473; Commission Directive 88/320/EEC (unpublished).

The results of these unpublished *in vitro* studies are summarised in Table 2. Positive and negative (vehicle) controls were tested in each study and gave expected results. The reverse mutation study comprised three experiments involving either the plate incorporation method (solid medium) or liquid culture methods. The chromosomal aberration study comprised two experiments that involved two different exposure times and different dose concentrations. It is concluded that the glutaminase preparation did not induce gene mutations or chromosome aberrations in these assays.

Table 2: Summary of genotoxicity studies

Test	Test system	Test article	Concentration or dose range	Result
Bacterial reverse mutation (Ames test)	<i>Salmonella typhimurium</i> strains TA 98, 100, 1535 & 1537 <i>Escherichia coli</i> strain WP2 <i>uvrA</i>	Glutaminase concentrate derived from <i>B. subtilis</i> ¹ (Batch No. GT70625L12; 10.8 % purity) Water vehicle	Test 1 & Test 2: plate incorporation method (62-5000 µg/plate) Test 3: liquid culture method (62-5000 µg/mL)	Negative (±S9)

Test	Test system	Test article	Concentration or dose range	Result
Chromosomal aberration test	CHO K-1 cells from Chinese hamster ovaries	As above	Test 1: 0.05-30 µg/mL (+S9); 0.5-200 µg/mL (-S9) Test 2: 0.5-20 µg/mL (+S9); 0.25-60 µg/mL (-S9)	Negative (±S9)

1 = *B. subtilis* has been reclassified as *B. amyloliquefaciens* (see section 3.4)

3.4.3 Residual allergens and substances causing food intolerance from the culture medium

Soybean oil, defatted soybean, lactose and dextrin (which may be derived from wheat starch) comprise part of the fermentation medium in the preparation of the glutaminase enzyme. However, residual soy or wheat allergens and lactose that may cause food allergy are not expected to be present in the final commercial product. Glutaminase SD-C100S is proposed for use at 0.2% during manufacture of seasoning ingredients/foods used as seasonings and, as the product is 9% glutaminase, this is equivalent to 0.018% of the glutaminase concentrate in the seasoning. These seasonings (e.g. yeast extracts, soy sauce, miso, vinegar, fish sauce etc.) are typically added at low levels to other food products. As stated in Section 2.2, all the raw materials used in the production of Glutaminase SD-C100S are approved food ingredients, food additives, microbial nutrients, or permitted in the production of processing aids (FSANZ, 2014).

3.4.4 Residual enzymes from the production organism

During production, glutaminase is secreted by *B. amyloliquefaciens* during the fermentation process. However, there are other enzymes in the glutaminase concentrate and the final glutaminase preparation: α-amylase, protease and peptidase. Supporting studies (Appendix A) show that the peptidase activity was difficult to confirm and was below the detection limit and, therefore, the applicant has indicated it does not have a function in the manufacturing process. The applicant indicated via correspondence that the protease is a metalloproteinase. Both α-amylase and the protease (metalloproteinase) sourced from *B. amyloliquefaciens* are approved under Standard 1.3.3.

3.5 Hazard assessment conclusions

There are no public health and safety issues associated with the use of Glutaminase SD-C100S, containing glutaminase produced by *B. amyloliquefaciens*, as a food processing aid on the basis of the following considerations:

- The production organism is not toxigenic or pathogenic and is not present in the final enzyme preparation used as the food processing aid. Further, *B. amyloliquefaciens* has a history of safe use as the production organism for a number of processing aids already permitted in the Code.
- Glutaminase has a long history of safe use and although residual enzyme is expected to be present in the final food, it would be inactive and susceptible to digestion like any other dietary protein.
- Complete digestion of the enzyme in simulated digestive fluid suggests the enzyme is unlikely to be toxic.

- The enzyme preparation caused no observable adverse effects at the second-highest tested doses in a 13-week repeated dose toxicity study in rats. The NOAEL for the glutaminase concentrate was determined to be 0.6% w/w of the diet or 388 mg/kg bw/d (male rats) and 450 mg/kg bw/d (female rats).
- The enzyme was not genotoxic or mutagenic *in vitro*.
- Bioinformatic analysis indicated that the enzyme has no biologically relevant homology to known protein allergens.

Based on the reviewed toxicological data, it was concluded that in the absence of any identifiable hazard, an Acceptable Daily Intake (ADI) 'not specified' was appropriate. A dietary exposure assessment was therefore not required.

4 Conclusion

This risk assessment considered the technological suitability, potential hazard of the production microorganism and the potential hazard of the enzyme glutaminase.

It was concluded that the proposed use of the enzyme was technologically justified in the form and prescribed amounts, and was demonstrated to be effective. The evidence presented was sufficient to determine that no safety concerns with the production microorganism or the enzyme exist. Thus the enzyme glutaminase sourced from *B. amyloliquefaciens* is unlikely to pose any health risk when used as a food processing aid.

5 References

Amano Enzyme (2005) [unpublished]. *History of Uses [History of the Bacterial Strain for Glutaminase Production & History of Use of Glutaminase in Food] [Confidential]*. (Appendix 9). Shiga, Japan: Amano Enzyme Inc.

Appel MJ (1999) [unpublished]. *Sub-chronic (13-week) Oral Toxicity Study with Glutaminase in Rats*. (TNO Report V99.442). Prepared by Zeist, The Netherlands: TNO Nutrition and Food Research Institute for Osaka, Japan: Daiwa Kasei K.K., Sep. 1999.

EC (2000). Directive 2000/54/EC of the European Parliament and of the Council of 18 September 2000 on the protection of workers from risks related to exposure to biological agents at work (seventh individual directive within the meaning of Article 16(1) of Directive 89/391/EEC). *Off J Eur Communities* 43(L262):21–45. Available at: <http://new.eu-lex.europa.eu/legalcontent/EN/ALL/?uri=CELEX:32000L0054&qid=1380303475986>.

EFSA (2008) Opinion of the Panel on Biological Hazards on the maintenance of the list of QPS microorganisms intentionally added to food or feed. *The EFSA Journal* 923: 1-48.

Food Chemicals Codex 9th Edition (2015). The United States Pharmacopeia, United States Pharmacopeial Convention, Rockville, MD. <http://www.usp.org/food-ingredients/food-chemicals-codex>.

FSANZ (2014). *Australia New Zealand Food Standards Code*. Canberra, Australia / Wellington, NZ: Foods Standards Australia New Zealand (FSANZ), Australian Government ComLaw. Available at: <http://www.foodstandards.gov.au/code/Pages/default.aspx> [See Standards 1.3.1, 1.3.3., 1.3.4 (Some dated 2013); Code updated to 2014].

IUBMB (2015) EC 3.5.1.2. http://www.enzyme-database.org/query.php?name=3.5.1.2&search=search_all&display=show_all&order=ec_num&nr=50.

JECFA (2006) General specifications and considerations for enzyme preparations used in food processing. <http://www.fao.org/docrep/009/a0691e/A0691E03.htm>.

MHLW (2014). List of existing food additives [compiled and published by the Ministry of Health and Welfare on April 16, 1996]. Japan Food Chemical Research Foundation (JFCRF), Tokyo, Japan. <http://www.ffcr.or.jp/zaidan/FFCRHOME.nsf/pages/listexst>. add [Effective from January 30, 2014, Last update: 06/30/2014].

Nandakumar R, Kazuaki Y, Wakayama M, Moriguchi M (2003) Microbial glutaminase: biochemistry, molecular approaches and applications in the food industry. *Journal of Molecular Catalysis B: Enzymatic* 23(2003): 87-100.

Pomeranz Y, Meloan C.E (1994) *Food analysis: theory and practice* (3rd Edn). Chapman & Hall Inc. New York.

Sarada KV (2013) Production and applications of L-Glutaminase using fermentation technology. *Asia Pacific Journal of Research* 1(VIII).

Sathish T, Prakasham RS (2010) Enrichment of glutaminase production by *Bacillus subtilis* RSP-GLU in submerged cultivation based on neural network – genetic algorithm approach. *Journal of Chemical Technology & Biotechnology* 85:50-58.

Shimizu Y, Ueyama A, Goto K (1991). [Purification and characterization of glutaminase from *Bacillus subtilis* GT strain]. *Nippon Jozo Kyokaishi [J Brew Soc Jpn]* 86(6):441-446.

US FDA (1999). *Carbohydrase and Protease Enzyme Preparations Derived From Bacillus Subtilis or Bacillus Amyloliquefaciens; Affirmation of GRAS Status as Direct Food Ingredients (21 CFR Part 184) [Docket No. 84G-0257]*. (US Federal Register, vol 64, no 68). Rockville (MD): US Food and Drug Administration (US FDA), Division of Dockets Management, pp. 19887-19895. Available at: <http://www.fda.gov/ohrms/dockets/98fr/042399a.txt> [April 23, 1999].

van Delft JHM (1998) [unpublished]. *Bacteria Reverse Mutation Test with Glutaminase [Confidential]*. (TNO Report V98.442). Prepared by Zeist, The Netherlands: TNO Nutrition and Food Research Institute for Osaka, Japan: Daiwa Kasei K.K., Jul. 10, 1998.

van Delft JHM, de Vogel M (1998) [unpublished]. *Chromosomal Aberration Test with Glutaminase in Cultured Chinese Hamster Ovary Cells [Confidential]*. (TNO Report V98.1202). Dec. 22, 1998.