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# Safety evaluation of the food enzyme β-amylase from genetically modified *Bacillus licheniformis* strain NZYM-JA

EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF), Vittorio Silano, Claudia Bolognesi, Laurence Castle, Jean-Pierre Cravedi, Paul Fowler, Roland Franz, Konrad Grob, Rainer Gürtler, Trine Husøy, Sirpa Kärenlampi, Wim Mennes, Maria Rosaria Milana, André Penninks, Andrew Smith, Maria de Fátima Tavares Poças, Christina Tlustos, Detlef Wölfle, Holger Zorn, Corina-Aurelia Zugravu, Andrew Chesson, Boet Glandorf, Lieve Herman, Klaus-Dieter Jany, Francesca Marcon, Davor Želježic, Margarita Aguilera-Gómez, Magdalena Andryszkiewicz, Zoltán Divéki, Natália Kovalkovičová and Karl-Heinz Engel

## Abstract

The food enzyme considered in this opinion is a β-amylase (4-α-D-glucan maltohydrolase; EC 3.2.1.2), produced with genetically modified *Bacillus licheniformis* strain NZYM-JA by Novozymes A/S (Denmark). The β-amylase food enzyme is intended to be used in starch processing for the production of glucose syrups. Since the residual amounts of total organic solids (TOS) in glucose syrups after filtration and purification during starch processing were considered negligible, no dietary exposure was calculated. Toxicological tests made with the food enzyme under application indicated that there was no concern with respect to genotoxicity, mutagenicity or systemic toxicity. The allergenicity was evaluated by searching for similarity of the amino acid sequence to those of known allergens; no match was found. The Panel considers that there are no indications for allergic reactions. Based on the genetic modifications performed, the manufacturing process, the compositional and biochemical data provided, the findings in the toxicological studies and allergenicity assessment, the Panel concludes that this food enzyme does not give rise to safety concerns under the intended conditions of use.



**Keywords:** food enzyme, β-amylase, 4-α-D-glucan maltohydrolase, EC 3.2.1.2, *Bacillus licheniformis*, genetically modified microorganism

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**Correspondence:** fip@efsa.europa.eu

**Panel members:** Claudia Bolognesi, Laurence Castle, Jean-Pierre Cravedi, Karl-Heinz Engel, Paul Fowler, Roland Franz, Konrad Grob, Rainer Gürtler, Trine Husøy, Sirpa Kärenlampi, Wim Mennes, Maria Rosaria Milana, André Penninks, Maria de Fátima Poças, Vittorio Silano, Andrew Smith, Christina Tlustos, Detlef Wölfle, Holger Zorn, Corina-Aurelia Zugravu.

**Note:** The full opinion will be published in accordance with Article 12(3) of Regulation (EC) No 1331/2008 once decision on confidentiality will be received from the European Commission. The following information has been provided under the confidentiality framework and has been redacted awaiting the decision of the Commission: monitoring of the production strain; manufacturing process and raw materials used; and genetic modifications.

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# Table of contents

# 1. Introduction

Article 3 of the Regulation (EC) No 1332/2008[1] provides definition for 'food enzyme' and 'food enzyme preparation'.

'Food enzyme' means a product obtained from plants, animals or microorganisms or products thereof obtained by a fermentation process using microorganisms: (i) containing one or more enzymes capable of catalysing a specific biochemical reaction and (ii) added to food for a technological purpose at any stage of the manufacturing, processing, preparation, treatment, packaging, transport or storage of foods.

'Food enzyme preparation' means a formulation consisting of one or more food enzymes in which substances such as food additives and/or other food ingredients are incorporated to facilitate their storage, sale, standardisation, dilution or dissolution.

Before January 2009, food enzymes other than those used as food additives were not regulated or were regulated as processing aids under the legislation of the Member States. On 20 January 2009, Regulation (EC) No 1332/2008 on food enzymes came into force. This Regulation applies to enzymes that are added to food to perform a technological function in the manufacture, processing, preparation, treatment, packaging, transport or storage of such food, including enzymes used as processing aids. Regulation (EC) No 1331/2008[2] established European Union (EU) procedures for the safety assessment and the authorisation procedure of food additives, food enzymes and food flavourings. The use of a food enzyme shall be authorised only if it is demonstrated that:

- it does not pose a safety concern to the health of the consumer at the level of use proposed,
- there is a reasonable technological need, and
- its use does not mislead the consumer.

All food enzymes currently on the EU market and intended to remain on that market, as well as all new food enzymes, shall be subjected to a safety evaluation by the European Food Safety Authority (EFSA) and approval via an EU Community list.

The 'Guidance on submission of a dossier on a food enzyme for evaluation' (EFSA, 2009a) lays down the administrative, technical and toxicological data required.

## 1.1. Background and Terms of Reference as provided by the requestor

### 1.1.1. Background as provided by the European Commission

Only food enzymes included in the EU Community list may be placed on the market as such and used in foods, in accordance with the specifications and conditions of use provided for in Article 7 (2) of Regulation (EC) No 1332/2008 on food enzymes. According to this Regulation, a food enzyme that falls within the scope of Regulation (EC) No 1829/2003[3] on genetically modified food and feed should be authorised in accordance with that Regulation as well as under this Regulation.

An application has been introduced by Novozymes A/S for authorisation of the food enzyme β-amylase obtained by fermentation with the genetically modified *Bacillus licheniformis* NZYM-JA.

Following the requirements of Article 12.1 of Commission Regulation (EU) No 234/2011[4] implementing Regulation (EC) No 1331/2008[2], the Commission has verified that the application falls within the scope of the food enzyme Regulation and contains all the elements required under Chapter II of that Regulation.

[1] Regulation (EC) No 1332/2008 of the European Parliament and of the Council of 16 December 2008 on Food Enzymes and Amending Council Directive 83/417/EEC, Council Regulation (EC) No 1493/1999, Directive 2000/13/EC, Council Directive 2001/112/EC and Regulation (EC) No 258/97. OJ L 354, 31.12.2008, p. 7–15.

[2] Regulation (EC) No 1331/2008 of the European Parliament and of the Council of 16 December 2008 establishing a common authorisation procedure for food additives, food enzymes and food flavourings. OJ L 354, 31.12.2008, p. 1–6.

[3] Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed. OJ L 268, 18.10.2003, p. 1–23.

[4] Commission Regulation (EU) No 234/2011 of 10 March 2011 implementing Regulation (EC) No 1331/2008 of the European Parliament and of the Council establishing a common authorisation procedure for food additives, food enzymes and food flavourings. OJ L 64, 11.3.2011, p. 15–24.

### 1.1.2. Terms of Reference

The European Commission requests EFSA to carry out the safety assessment on the food enzyme β-amylase obtained with the genetically modified strain of *Bacillus licheniformis* NZYM-JA in accordance with Article 17.3 of Regulation (EC) No 1332/2008 on food enzymes.

## 1.2. Information on existing authorisation and evaluations

The applicant indicated that this food enzyme has not been evaluated by authorities in the EU.

# 2. Data and methodologies

## 2.1. Data

The applicant has submitted a dossier in support of the application for authorisation of the food enzyme β-amylase produced with a genetically modified *B. licheniformis* (strain NZYM-JA). The food enzyme is intended to be used in starch processing for the production of glucose syrups.[5]

## 2.2. Methodologies

The assessment was conducted in line with the principles described in the EFSA Guidance on transparency in the scientific aspects of risk assessment (EFSA, 2009b) and following the relevant Guidances from the EFSA Scientific Committee.

The current guidance on the submission of a dossier for safety evaluation of a food enzyme (EFSA, 2009a) has been followed by the EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF) for the evaluation of the application with the exception of the exposure assessment, which was carried out in accordance to the methodology described in the CEF Panel statement on the exposure assessment of food enzymes (EFSA CEF Panel, 2016).

# 3. Assessment

## 3.1. Technical data

### 3.1.1. Identity of the food enzyme

| | |
|---|---|
| IUBMB nomenclature: | β-amylase |
| Systematic name: | 4-α-D-Glucan maltohydrolase |
| Synonyms: | Saccharogen amylase, glycogenase, 1,4-α-D-glucan maltohydrolase |
| IUBMB No: | EC 3.2.1.2 |
| CAS No: | 9000-91-3 |
| EINECS No: | 232-566-1. |

### 3.1.2. Chemical parameters

The β-amylase produced with the genetically modified strain *B. licheniformis* NZYM-JA consists of a single polypeptide of 515 amino acids. The 57.6 kDa molecular mass of the protein was calculated based on the amino acid sequence. The protein homogeneity of the food enzyme was investigated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis for three commercial batches. The apparent molecular mass based on the SDS-PAGE pattern is about 58 kDa (equivalent to the calculated molecular mass). The gel presented showed the same pattern in all samples, consisting of one main protein band and a number of much weaker bands.

The food enzyme was tested for other enzyme activities in the food enzyme product, i.e. α-amylase, glucoamylase, lipase and protease, which were below the limits of detection. No other enzymatic side activities have been reported by the applicant.

Data on the chemical parameters of the food enzyme have been provided for three commercial food enzyme batches and one batch to be used for toxicological tests (Table 1). The average total organic solids (TOS) of the three commercial food enzyme batches was 10.9% (w/w); the values ranged from 10.5% to 11.3%.

[5] European Commission working document describing the food processes in which food enzymes are intended to be used – not yet published at the time of adoption of this opinion.

The average enzyme activity/TOS ratio of the three food enzyme batches for commercialisation was 101.3 β-amylase Units/mg TOS (BAMU/mg TOS); the values ranged from 95.4 to 105.7 BAMU/mg TOS (Table 1).

**Table 1:** Compositional data of the food enzyme

| Parameter | Units | Batches | | | |
|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4[(a)] |
| β-Amylase activity | BAMU/g batch[(b)] | 11,100 | 10,300 | 11,600 | 9,544 |
| Protein | % | 9.3 | 8.7 | 9.5 | NA[(c)] |
| Ash | % | 0.4 | 0.5 | 0.4 | 1.2 |
| Water | % | 89.1 | 88.7 | 88.3 | 87.3 |
| Total organic solids (TOS)[(d)] | % | 10.5 | 10.8 | 11.3 | 11.5 |
| β-Amylase activity/mg TOS | BAMU/mg TOS | 105.7 | 95.4 | 102.7 | 83.0 |

(a): Batch used for the toxicological testings.
(b): BAMU/g batch: β-amylase Units (see Section 3.1.3).
(c): NA: Not Analysed.
(d): TOS calculated as 100% – % water – % ash.

The applicant provided analytical data on the three commercial batches and the batch used for toxicological studies, demonstrating that the content of lead (< 0.5 mg/kg) was below the specification for lead (≤ 5 mg/kg) as laid down in the general specifications and considerations for enzymes used in food processing (FAO/WHO, 2006). Furthermore, the levels of arsenic, cadmium and mercury were below their respective levels of detection (As: 0.3 mg/kg; Cd and Hg: 0.05 mg/kg). No antimicrobial activity was detected in any of these batches (FAO/WHO, 2006).

The food enzyme complies with the microbiological criteria as laid down in the general specifications and considerations for enzymes used in food processing (FAO/WHO, 2006), which stipulate that *Escherichia coli* and *Salmonella* species are absent in 25 g of sample and total coliforms should not exceed 30 colony forming units (CFU) per gram.

The applicant has provided information on the identities of the antifoam agents used and method for analysis. Taking into account the nature and properties of the antifoam agents, the manufacturing process and the quality assurance system implemented by the applicant, the Panel considers their use as of no safety concern.

The Panel considered the compositional data provided for the food enzyme as sufficient.

### 3.1.3. Properties of the food enzyme

The β-amylase catalyses the hydrolysis of 1,4-α-glycosidic linkages in starch and successively releases maltose units from the non-reducing ends of the chains.

The β-amylase activity is quantified based on the hydrolysis of the maltohexaose substrate to form maltose and maltotetraose and is expressed in BAMU/g. The analytical principle is based on the reaction between maltotetraose and lactose-oxidase and $O_2$, which produces hydrogen peroxide. This, in turn, in the presence of peroxidase, activates the oxidative condensation of 4-aminoantipyrine (AA) and *N*-ethyl-*N*-sulfopropyl-*m*-toluidine (reaction conditions: 37°C, pH 5.5; reaction time: 260 s) to form a purple product which is determined spectrophotometrically at 540 nm. Activity is calculated based on a standard with a known enzymatic activity.

The temperature profile of the β-amylase was measured between 30°C and 95°C at pH 5.0. The optimum temperature for activity was 60°C with virtually all activity lost by 80°C. The pH profile measured between pH 2.0 and 10.0 at 60°C showed optimum activity between pH 5.0 and 8.0. The thermostability of the β-amylase was tested at temperatures from 20°C to 95°C after an incubation period of 30 min at pH 5.0. The β-amylase stability decreases rapidly after 60°C showing no residual activity at 70°C. The activity itself was measured under standard assay conditions.

### 3.1.4. Information on the source material

#### 3.1.4.1. Information related to the genetically modified microorganism

The β-amylase production strain *B. licheniformis* NZYM-JA is deposited in the DSMZ with the deposit number [redacted]

### 3.1.4.2. Characteristics of the recipient or parental microorganisms

The parental strain [redacted] was identified as *B. licheniformis* by 16S rRNA sequence analysis.

*B. licheniformis* has been recommended for Qualified Presumption of Safety (QPS) status, with the qualification that the absence of acquired antibiotic resistance genes and toxigenic activity are verified for the specific strain used (EFSA BIOHAZ Panel, 2017a,b). The identity of the parental strain has been confirmed and has been shown to have no cytotoxic activity in Chinese hamster ovary cells (Pedersen et al., 2002) and VERO cells. Additionally it was demonstrated that the strain did not react with antibodies against *Bacillus cereus* enterotoxins by using commercial antibody-based kits. [redacted] Consequently, the parental strain is presumed to be safe for production purposes.

The recipient strain, *B. licheniformis* [redacted], has been developed from the parental strain [redacted]

[redacted]

### 3.1.4.3. Characteristics of the donor organisms

[redacted]

### 3.1.4.4. Description of the genetic modification process

The production strain *B. licheniformis* NZYM-JA was developed from the recipient strain [redacted]

[redacted]

#### 3.1.4.5. Safety aspects of the genetic modifications

[redacted]

### 3.1.5. Manufacturing process

The food enzyme is manufactured with food safety procedures based on Hazard Analysis and Critical Control Points (HACCP), in accordance with current Good Manufacturing Practice (GMP) and, when produced in the EU, in accordance with the Food Hygiene Regulation (EC) No 852/2004[6].

The food enzyme is produced by a pure culture in a contained, submerged, fed-batch fermentation system with conventional process controls in place. The identity and purity of the culture are checked at each transfer step from frozen vials until the end of fermentation.

The downstream processing includes recovery, purification, concentration and stabilisation. The food enzyme produced is recovered from the fermentation broth after biomass separation via press filtration. The liquor is then filtered to remove the remaining microorganisms, and concentrated by ultrafiltration, which removes the low-molecular-weight material.

Subsequently, the food enzyme concentrate is formulated and commercialised as a liquid or a solid preparation. To this end, the concentrated food enzyme solution is stabilised by the addition of preservatives [redacted], and finally subjected to polish- and germ-filtration.

The absence of the production strain in the product was demonstrated in [redacted]

No recombinant DNA was detected in three independent batches in triplicate [redacted]

The Panel considered the information provided on the raw materials and the manufacturing process as sufficient.

### 3.1.6. Safety for the environment

Neither the production strain nor its recombinant DNA was detected in the final product. Accordingly, no environmental risk assessment is required (EFSA GMO Panel, 2011).

### 3.1.7. Reaction and fate in food

The β-amylase catalyses the hydrolysis of 1,4-α-glycosidic linkages of starch (amylose and amylopectin), and successively releases maltose units from the non-reducing ends of the chains, resulting in the production of limit dextrins and maltose.

[6] Regulation (EC) No 852/2004 of the European Parliament and of the Council of 29 April 2004 on the hygiene of food additives. OJ L 226, 25.6.2004, p. 3–21.

The β-amylase is specific in its action and is not known to catalyse reactions other than the hydrolysis of starch. These reaction products, i.e. maltose and the remaining oligosaccharides are naturally present in starch-containing foods.

The information and data provided indicate that the β-amylase is removed during processing under the intended conditions of use (starch processing: filtration, carbon treatment and ion exchange).

The food enzyme was tested for the presence of other enzyme activities, i.e. α-amylase, glucoamylase, lipase and protease, which were below the limits of detection. No other side activities have been reported by the applicant.

#### 3.1.8. Case of need and intended conditions of use

As proposed by the applicant, the food enzyme is intended for use in starch processing for the production of glucose syrups containing maltose (glucose syrup with high maltose content, glucose syrup containing maltose and dried glucose syrup containing maltose), at an intended use level of up to 98.7 mg TOS/kg starch.

In starch processing, the β-amylase is added after the liquefaction during the saccharification step in order to convert liquefied starch into maltose-rich glucose syrups.

According to the applicant, the food enzyme is used at the minimum amount necessary to achieve the desired reaction according to GMP. The use level applied by a food manufacture in practice depends on the particular process.

### 3.2. Dietary exposure

For this application, the intended use of this β-amylase is to hydrolyse starch in order to produce maltose-containing glucose syrups. Experimental data on the significant removal (> 99%) of protein in the course of this process have been provided (Documentation provided to EFSA n. 4). The Panel considered this evidence as sufficient to conclude that the presence of residual amounts of TOS after the purification steps applied during the production of glucose syrups, i.e. filtration, ion exchange chromatography, carbon treatment and crystallisation, is negligible. Consequently, no exposure was calculated.

### 3.3. Toxicological data

The following toxicity data have been provided by the applicant: a bacterial reverse mutation test, an *in vitro* micronucleus assay and a repeated dose 90-day oral toxicity study in rodents. All the toxicological studies were performed with batch 4 (Table 1), having higher ash content and a lower enzyme activity per g food enzyme and per mg TOS in comparison to the reference batches.

#### 3.3.1. Bacterial reverse mutation test

A bacterial reverse mutation assay (Ames test) was performed according to the OECD Test Guideline 471 (OECD, 1997) and following Good Laboratory Practice (GLP) in four strains of *Salmonella* Typhimurium (TA1535, TA1537, TA98 and TA100) and *E. coli* WP2uvrA pKM 101, in the presence or absence of metabolic activation (S9-mix) applying a 'treat and plate' assay using six different concentrations (0, 156, 313, 625, 1,250, 2,500 and 5,000 μg TOS/mL) of the food enzyme using appropriate positive controls and water as a negative control. Upon treatment with the food enzyme, there was no increase in revertant colony numbers. Therefore, the Panel concluded that the food enzyme did not induce gene mutations under the conditions employed in this study.

#### 3.3.2. *In vitro* micronucleus assay

The *in vitro* micronucleus assay was carried out according to the OECD Test Guideline 487 (OECD, 2010) and following GLP. Human peripheral whole blood cultures were exposed to the food enzyme for a short treatment (3 h + 18 h recovery) in the presence and absence of S9 mix and a continuous treatment (24 h + 24 h recovery) without S9 mix using a concentration range from 500 to 5,000 μg TOS/mL. No biological relevant increase in the frequency of micronuclei (MN) was observed after treatment with the test article at the concentrations analysed. The Panel concluded that the food enzyme β-amylase did not induce MN under the test conditions employed.

### 3.3.3. Repeated dose 90-day oral toxicity study in rodents

A repeated dose 90-day oral toxicity study was performed according to the OECD test guideline 408 (OECD, 1998) and following GLP. Groups of 10 male and ten female Sprague–Dawley (Crl:CD(SD)) rats received daily via gavage for 90 days dose levels of 0 (reverse osmosis water as vehicle), 10%, 33% and 100% of the food enzyme in a final dose volume of 10 mL/kg body weight (bw)corresponding to 0, 120, 396 and 1,199 mg TOS/kg bw per day.

No treatment-related death or effects on clinical signs, general appearance and behaviour, sensory reactivity responses, grip strength and motor activity were observed. A statistically significant increase of overall weight gain and feed intake was observed in males but was no dose related and therefore considered of no toxicological importance.

All other intergroup differences of haematology and clinical chemistry parameters from controls were minor, confined to one sex or lacked dose-relationship and can be attributed to normal biological variation.

Variation in neurobehaviour parameters showed no consistent association with consumption of the food enzyme by either sex.

The Panel concluded that the no-observed-adverse-effect-level (NOAEL) was the highest concentration tested, which corresponds to 1,199 mg TOS/kg bw per day.

## 3.4. Allergenicity

The potential allergenicity of β-amylase produced with the genetically modified *B. licheniformis* strain NZYM-JA was assessed by comparing its amino acid sequence with those of known allergens according to the scientific opinion on the assessment of allergenicity of genetically modified (GM) plants and microorganisms and derived food and feed of the Scientific Panel on Genetically Modified Organisms (EFSA GMO Panel, 2010). Using higher than 35% identity in a window of 80 amino acids as the criterion, no match was found.

β-Amylase from *B. licheniformis* is not described as a potential allergen and no food allergic reactions to this food enzyme have been reported, so there is no evidence for potential allergenicity of this food enzyme.

According to the information provided, substances or products that may cause allergies or intolerances (Regulation EU 1169/2011)[7] are used as raw materials [redacted] in the media fed to the microorganisms in the course of the production of the enzyme. However, the proteins will be digested during the fermentation process and consumed by the microorganisms for cell growth, cell maintenance and production of enzyme protein. In addition, the microbial biomass and fermentation solids will be removed. Therefore, potentially allergenic residues of these foods employed as protein sources are not expected to be present.

Taken together, the Panel considers that there are no indications for allergic reactions, and therefore, this β-amylase produced with the genetically modified *B. licheniformis* (strain NZYM-JA) is not of safety concern.

## Conclusions

Based on the genetic modifications performed, the manufacturing process, the compositional and biochemical data provided, the findings in the toxicological studies and allergenicity assessment, the Panel concludes that this food enzyme does not give rise to safety concerns under the intended conditions of use.

## Documentation provided to EFSA

1) Dossier "Beta-amylase produced by a genetically modified strain of *Bacillus licheniformis* (strain NZYM-JA)". January 2015. Submitted by Novozymes A/S (Denmark).
2) Summary reports on technical data, toxicological data and on the genetic modifications were delivered by Hylobates Consulting/BiCT (Rome, Italy) on 5 August 2016, FoBiG GmbH

[7] Regulation (EU) No 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers, amending Regulations (EC) No 1924/2006 and (EC) No 1925/2006 of the European Parliament and of the Council, and repealing Commission Directive 87/250/EEC, Council Directive 90/496/EEC, Commission Directive 1999/10/EC, Directive 2000/13/EC of the European Parliament and of the Council, Commission Directives 2002/67/EC and 2008/5/EC and Commission Regulation (EC) No 608/2004. OJ L 304, 22.11.2011, p. 18–63.

(Freiburg, Germany) on 15 August 2016 and by the Technical University of Denmark (Søborg, Denmark) on 17 November 2016, respectively.
3) Additional information was received from Novozymes A/S on February 2017.
4) Additional information on "Food enzyme removal during the production of cereal based distilled alcoholic beverages" and "Food enzyme carry/over in glucose syrups". February 2017. Provided by the Association of Manufacturers and Formulators of Enzyme Products.

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## Abbreviations

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|---|---|
| AA | 4-aminoantipyrine |
| BAMU | β-amylase Units |
| bw | body weight |
| CAS | Chemical Abstracts Service |

| | |
|---|---|
| CEF | EFSA Panel on Food Contact Material, Enzymes, Flavourings and Processing Aids |
| CFU | colony forming unit |
| DSMZ | Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany |
| EC | Enzyme Commission |
| EINECS | European Inventory of Existing Commercial Chemical Substances |
| FAO | Food and Agricultural Organization |
| GLP | Good Laboratory Practice |
| GM | genetically modified |
| GMO | genetically modified organism |
| GMP | Good Manufacturing Practice |
| HACCP | Hazard Analysis and Critical Control Points |
| IUBMB | International Union of Biochemistry and Molecular Biology |
| JECFA | Joint FAO/WHO Expert Committee on Food Additives |
| MN | micronuclei |
| NOAEL | no-observed-adverse-effect-level |
| OECD | Organisation for Economic Cooperation and Development |
| PCR | polymerase chain reaction |
| QPS | Qualified Presumption of Safety |
| rRNA | ribosomal ribonucleic acid |
| SDS-PAGE | sodium dodecyl sulfate-poly acrylamide gel electrophoresis |
| TOS | total organic solids |
| WHO | World Health Organization |