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Safety evaluation of the food enzyme preparation isomaltulose synthase from *Serratia plymuthica* strain Z12A

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Abstract

The food enzyme isomaltulose synthase (sucrose glucosylmutase; EC 5.4.99.11) is produced with *Serratia plymuthica* strain Z12A by BENEOPalatinit GmbH. The food enzyme is used only in the form of an immobilised preparation of non-viable cells for the production of isomaltulose. Residual amounts of total organic solids (TOS) are removed by the purification steps applied during the production of isomaltulose consequently, dietary exposure was not calculated. Genotoxicity tests did not indicate safety concern. The systemic toxicity was assessed by means of a repeated dose 90-day oral toxicity study in rats. The Panel identified a no observed adverse effect level of 1,011 mg TOS/kg body weight (bw) per day, the highest dose tested. Similarity of the amino acid sequence of the enzyme to those of known allergens was searched and no match was found. The Panel considered that, under the intended conditions of use, the risk of allergic sensitisation and elicitation reactions by dietary exposure cannot be excluded, but the likelihood of such reactions to occur is considered to be low. Based on the data provided, the use of an immobilised food enzyme and the removal of TOS during the production of isomaltulose the Panel concluded that this food enzyme preparation does not give rise to safety concerns under the intended conditions of use.

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1. Introduction

Article 3 of the Regulation (EC) No 1332/2008¹ provides definition for 'food enzyme' and 'food enzyme preparation'.

'Food enzyme' means a product obtained from plants, animals or micro-organisms or products thereof including a product obtained by a fermentation process using micro-organisms: (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 1331/2008² established the 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:

- i) it does not pose a safety concern to the health of the consumer at the level of use proposed;
- ii) there is a reasonable technological need;
- iii) 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 food enzymes for safety evaluation' (EFSA CEF Panel, 2009) 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 European Union (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.

Five applications have been introduced by the Association of Manufacturers and Formulators of Enzyme Products (AMFEP) for the authorisation of the food enzyme consisting of Protease, Leucyl amino-peptidase, Oryzin and Aspergillopepsin I from *Aspergillus oryzae* and the companies "BENEO-Palatinit GmbH" for the authorisation of the food enzyme Isomaltulose synthase from *Protaminobacter rubrum* (strain Z12A), "Nagase (Europa) GmbH" for the authorisation of the food enzyme Chitinase from a genetically modified strain of *Streptomyces violaceoruber* (strain pChi), "Clasado Ingredients Ltd." for the authorisation of the food enzyme Beta-galactosidase from a genetically modified strain of *Escherichia coli* (strain BglA MCB3) and "Meiji Seika Pharma Co., Ltd" for the authorisation of the food enzyme consisting of Aspergillopepsin I and II from *Aspergillus niger var. macrosporus* (strain DBD-0406).

Following the requirements of article 12.1 of Regulation (EC) No 234/2011 implementing Regulation (EC) No 1331/2008, the Commission has verified that the five applications fall within the scope of the food enzyme Regulation and contain all the elements required under Chapter II of that Regulation and contain 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, pp. 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, pp. 1–6.

1.1.2. Terms of Reference

The European Commission requests the European Food Safety Authority to carry out the safety assessments on the food enzymes Protease, Leucyl amino-peptidase, Oryzin and Aspergillopepsin I from *Aspergillus oryzae*, Isomaltulose synthase from *Protaminobacter rubrum* (strain Z12A), Chitinase from a genetically modified strain of *Streptomyces violaceoruber* (strain pChi), Beta-galactosidase from a genetically modified strain of *Escherichia coli* (strain BglA MCB3) and Aspergillopepsin I and II from *Aspergillus niger* var. *macrosporus* (strain DBD-0406) in accordance with Article 17.3 of Regulation (EC) No 1332/2008 on food enzymes.

1.2. Interpretation of the Terms of Reference

The present scientific opinion addresses the European Commission's request to carry out the safety assessment of food enzyme isomaltulose synthase as food enzyme preparation from *Serratia plymuthica* (formerly *Protaminobacter rubrum*) strain Z12A.

The food enzyme is intended to be used in the production of isomaltulose syrups. The subsequently produced novel food after drying and food additive after hydrogenation is not covered by this assessment.

2. Data and methodologies

2.1. Data

The applicant has submitted a dossier in support of the application for authorisation of the food enzyme preparation isomaltulose synthase from *P. rubrum* (strain Z12A).

Additional information was requested from the applicant during the assessment process on 15 April 2019 and 2 March 2020 and was consequently provided (see 'Documentation provided to EFSA').

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, 2009) and following the relevant existing guidance's of EFSA Scientific Committees.

The current 'Guidance on the submission of a dossier on food enzymes for safety evaluation' (EFSA CEF Panel, 2009) has been followed 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

IUBMB nomenclature:	Isomaltulose synthase
Systematic name:	Sucrose glucosylmutase
Synonyms:	Isomaltulose synthetase; sucrose α -glucosyltransferase; trehalulose synthase, sucrose mutase
IUBMB No.:	EC 5.4.99.11
CAS No.:	159940-49-5

The isomaltulose synthase catalyses the transglycosylation of 1,2 glucosyl-fructose (sucrose), resulting in the generation of 6-O- α -D-glucopyranosyl- D-fructose (isomaltulose) and smaller amounts of mono-, di- and trisaccharides, mainly 1,1-glucosyl-fructose (trehalulose), glucose and fructose.³

The food enzyme preparation is used by the applicant for the production of isomaltulose.

3.1. Source of the food enzyme

The isomaltulose synthase is produced [REDACTED]⁴ by the bacterium *Serratia plymuthica* (formerly *Protaminobacter rubrum*) strain Z12A, which is deposited in the German Collection of Microorganisms and cell Cultures (DSMZ, Germany) with deposition number [REDACTED].⁵

³ Technical dossier/Table 3.

⁴ Technical dossier/Figure 1.

⁵ Technical dossier/Additional information October 2020/Annex 1.

The identity of the production strain is based on [REDACTED].⁶

S. plymuthica has been associated with occasional cases of infections (Carrero et al., 1995; Pascual Pérez et al., 2003). The main virulence factor of the genus *Serratia* is a haemolysin. Possible hazards associated with potential haemolytic activity in the food enzyme are considered in the toxicological studies (section 3.4.1).

[REDACTED] analysis of the production strain [REDACTED] revealed the presence of genetic sequences encoding for proteins involved in resistance to [REDACTED] and [REDACTED].⁷

The presence of an antimicrobial resistance (AMR) gene in the genome of the production strain is considered a hazard.

3.2. Production of the food enzyme preparation

The food enzyme preparation is manufactured according to the Food Hygiene Regulation (EC) No 852/2004⁸, with food safety procedures based on Hazard Analysis and Critical Control Points (HACCP) principles and in accordance with current good manufacturing practice (GMP).⁹

The production strain is grown as a pure culture using a typical industrial medium in a submerged, [REDACTED] fermentation system with conventional process controls in place. After completion of the fermentation, the solid biomass is harvested from the fermentation broth by centrifugation and the bacterial cells are killed [REDACTED].

[REDACTED]. The applicant provided information on the identity and analysis of the substances used to control the fermentation and in the subsequent downstream processing of the food enzyme.

The Panel considered that sufficient information has been provided on the manufacturing process and the quality assurance system implemented by the applicant to exclude issues of concern.

3.3. Characteristics of the food enzyme preparation

3.3.1. Properties of the food enzyme preparation

The isomaltulose synthetase consists of a single polypeptide of [REDACTED] amino acids with a calculated molecular mass of [REDACTED] kDa. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis showed a band migrating between the [REDACTED] kDa reference proteins, consistent with the calculated mass of the enzyme.¹¹ The SDS–PAGE gel showed the presence of a number of other bands, but no other activities were reported by the applicant.

The in-house determination of isomaltulose synthase activity is based on the transglycosylation of sucrose (reaction conditions: pH 5.5 and 20°C for 1 h). The enzymatic activity is determined by measuring the residual sucrose by high-performance liquid chromatography. One unit corresponds to the amount of enzyme needed for the conversion of 1 µmol sucrose to isomaltulose per minute.¹²

The temperature optimum of the immobilised enzyme is 30°C (pH 5.5), with no residual activity detectable at 50°C. The optimum pH is between 5 – 6.5 (20°C).¹³

⁶ Technical dossier/Additional information October 2019/Annex 1A, 1B.

⁷ Technical dossier/Additional information October 2019.

⁸ 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, pp. 3–21.

⁹ Technical dossier/p. 38 and Additional data October 2019.

¹⁰ Technical dossier/p. 36.

¹¹ Technical dossier/p. 17–20.

¹² Technical dossier/Annexes 16 and 17.

¹³ Technical dossier/Figures 4 and 5.

3.3.2. Chemical parameters

Data on the chemical parameters of the immobilised food enzyme preparation were provided for five batches used for the production of isomaltulose and two batches used for the toxicological testing (Table 1).¹⁴ The average total organic solids (TOS) content of the five batches used for the production of isomaltulose was 79.9 % and the average enzyme activity/TOS ratio is 1.1 U/mg TOS (Table 1).

Table 1: Compositional data provided for the dried food enzyme preparation

Parameter	Unit	Batches 1–5		6 ^(a)	7 ^(b)
		Mean	Range		
Isomaltulose synthase activity	U/g ^(c)	890.2	788–978	875	812
Protein	%	29.1	26.4–32.3	33.8	32.9
Ash	%	11.6	11.3–12.0	12.2	11.8
Water	%	8.5	6.3–10.6	10.5	7.3
Total organic solids (TOS)^(d)	%	79.9	78.1–81.7	77.3	80.9
Isomaltulose synthase activity/mg TOS	U/mg TOS	1.1	1.0–1.2	1.1	1.0

(a): Batch used for the bacterial reverse mutation test and *in vitro* mammalian cell micronucleus test.

(b): Batch used for the 90-day toxicity study.

(c): U: isomaltulose synthase units (see Section 3.3.1).

(d): TOS calculated as 100% – % water – % ash.

3.3.3. Purity

The highest lead content recorded amongst the five batches used for the production of isomaltulose was 0.55 mg/kg which complies with 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). In addition, the mean concentrations of arsenic and cadmium in the commercial batches were 0.12 mg/kg and 0.03 mg/kg, respectively.^{15,16} The Panel considered these concentrations as not of concern.

The total viable counts of aerobic bacteria in the five batches of the food enzyme preparation ranged from 400 up to 1.4×10^5 colony forming units (CFU) per gram.

The immobilised food enzyme preparation complies with the microbiological criteria (for total coliforms, *E. coli* and *Salmonella*)¹⁵ as laid down in the general specifications for enzymes used in food processing (FAO/WHO, 2006). No antimicrobial activity was detected in any of these batches¹⁷ (FAO/WHO, 2006).

The presence of mycotoxins (aflatoxin B1, B2, G1, G2, ochratoxin A, fumonisin B1, B2, B3, zearalenone, deoxynivalenol) was examined in five food enzyme preparation batches. All were below the limits of quantification (LoQs) of the applied analytical methods.^{15,18}

The amount of [REDACTED] found in the enzyme preparation was [REDACTED].¹⁵

3.3.4. Viable cells and DNA from the production strain

The absence of viable cells from the production strain was shown [REDACTED].¹⁷

DNA from the production strain is expected to be present in the food enzyme preparation.

3.4. Toxicological data

A battery of toxicological tests including a bacterial gene mutation assay (Ames test), an *in vitro* micronucleus assay, and a repeated dose 90-day oral toxicity study in rats has been provided. The

¹⁴ Technical dossier/Table 6 and Annexes 16, 17 and 18, and Additional data October 2019.

¹⁵ Technical dossier/Additional data October 2019.

¹⁶ LoQ: Pb = 0.02 mg/kg; As = 0.02 mg/kg; Cd = 0.02 mg/kg.

¹⁷ Technical dossier/p. 34.

¹⁸ LOQs: Aflatoxins (B1, B2, G1 and G2) = 0.1 µg/kg each; deoxynivalenol = 20 µg/kg; fumonisins (B1, B2 and B3) = 20 µg/kg each; ochratoxin A = 0.2 µg/kg; zearalenone = 10 µg/kg.

batches 6 and 7 (Table 1) were used for genotoxicity testing and 90-day toxicity study, respectively. Enzyme activities and TOS values in these two batches were comparable with those of the five batches used for the production of isomaltulose and are thus considered suitable as test items.

3.4.1. Genotoxicity

3.4.1.1. Bacterial reverse mutation test

A bacterial reverse mutation assay (Ames test) was performed according to Organisation for Economic Co-operation and Development (OECD) Test Guideline 471 (OECD, 1997a) and following Good Laboratory Practice (GLP).¹⁹ Four strains of *Salmonella* Typhimurium (TA 1535, TA 1537, TA 98, TA 100) and *E. coli* WP2uvrA were used in the presence or absence of metabolic activation (S9 mix), applying the standard plate incorporation method. Two experiments were carried out in triplicate plating using five different concentrations of the food enzyme preparation¹⁹ (62, 185, 556, 1,667 and 5,000 µg/plate, corresponding to ca. 47.9, 143.0, 429.8, 1,288.6 and 3,865.0 µg TOS/plate. No toxicity was observed at any concentration level of the test substance in any experiment. Upon treatment with the food enzyme preparation the numbers of the revertant colonies were comparable to the values observed in the vehicle control groups in all tester strain in the presence and absence of metabolic activation.

The Panel concluded that the food enzyme preparation did not induce gene mutations under the test conditions employed in this study.

3.4.1.2. *In vitro* mammalian cell micronucleus test

The *in vitro* micronucleus assay was carried out according to the OECD Test Guideline 487 (OECD, 2010) and following GLP.²⁰ Two separate experiments were performed in duplicate cultures of human peripheral whole blood lymphocytes. A dose range finding test was carried out with the food enzyme preparation from 52 to 2,000 µg/mL. No clear cytotoxicity was observed at any of the concentrations tested when compared to the concurrent negative control cultures, except slight cytotoxicity (16% reduction of the replication index) at the highest dose level of 2,000 µg/mL in the presence of metabolic activation. No haemolytic effects were reported. The food enzyme preparation was tested at 592, 889 and 1,333 µg/mL, corresponding to 458, 687 and 1,030 µg TOS/mL in the short-term treatment (4 h +20 h recovery time) in the presence and absence of S9 and at 800, 1,000 and 1,333 µg/mL, corresponding to 618, 773 and 1,030 µg TOS/mL in the long-term treatment (20 h +28 h recovery time) in the absence of S9 mix. No cytotoxicity, determined as a reduction of the proliferation index, was observed after treatments, neither in the presence nor in the absence of S9-mix. The frequency of binucleated cells with micronuclei (MNBN) was comparable to the negative controls at all doses tested.

The Panel concluded that the food enzyme preparation *S. plymuthica* Z12A, did not induce an increase in the frequency of MNBNs in cultured human peripheral blood lymphocytes, under the test conditions employed in this study.

3.4.2. Repeated dose 90-day oral toxicity study in rodents

The repeated dose 90-day oral toxicity study was performed according to OECD Test Guideline 408 (OECD, 1998), and following GLP. Groups of 10 female and 10 male Wistar Outbred rats (RccHanTM: WIST) received in the feed the food enzyme in doses corresponding to 291, 566 or 1,181 mg TOS/kg body weight (bw) per day for females and in doses corresponding to 251, 502 or 1,011 mg TOS/kg bw per day for males. Controls received the same diet with no food enzyme added.

No mortality was observed.

A few behavioural and clinical appearance deviations were observed occasionally in a few animals from different groups, at different time points during the study, without dose dependency and were therefore considered by the Panel to be not treatment related.

Clinical chemistry examination, revealed a slight but statistically significant decrease in plasma phospholipids in mid- and high-dose males. As there were no statistically significant differences in related clinical chemistry parameters and there was no indication of adverse effects on liver function,

¹⁹ Technical dossier, Annex 16.

²⁰ Technical dossier, Annex 17.

morphology or absolute weight, and the phospholipid values were within the historical control range from the laboratory the Panel considered this finding to be of no toxicological significance.

The relative liver to body weight of mid-dose males was statistically significantly lower as compared to controls but in light of no dose-dose response relationship, absence of morphological changes in the organ or statistically significant effect on the absolute weight the Panel considered this finding to be of no toxicological significance.

No other statistically significant effects were reported.

Overall, the Panel identified a no observed adverse effect level (NOAEL) at the highest dose tested corresponding to 1,011 mg TOS/kg bw per day.

3.4.3. Allergenicity

The potential allergenicity of the isomaltulose synthase produced with the *S. plymuthica* strain Z12A was assessed by comparing its amino acid sequence with those of known allergens according to the scientific opinion on the assessment of allergenicity of 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 sliding window of 80 amino acids as the criterion, no match was found.

No information is available on oral sensitisation or elicitation reactions of this isomaltulose synthase. In addition, no allergic reactions upon dietary exposure to any isomaltulose synthase have been reported in the literature.

According to the information provided, substances or products that may cause allergies or intolerances (██████████) are present in the media fed to the microorganisms. However, during the fermentation process, these products will be degraded and utilised by the microorganisms for cell growth, cell maintenance and production of enzyme protein. In addition, the microbial biomass and fermentation solids are removed. Taking into account the fermentation process and downstream processing, the Panel considered that potentially allergenic residues of these materials employed as protein sources are not expected to be present.

Quantifying the risk for allergenicity is not possible in view of the individual susceptibility to food allergens. Allergenicity can be ruled out only if the proteins are fully removed. In the production of isomaltulose, experimental data showed a significant removal (> 99%) of protein. However, traces of protein could be present in the isomaltulose syrups produced using the immobilised enzyme.

The Panel considered that, under the intended conditions of use, the risk of allergic sensitisation and elicitation reactions upon dietary exposure to this food enzyme preparation cannot be excluded but the likelihood of such reactions occurring is considered to be low.

3.5. Dietary exposure

3.5.1. Intended use of the food enzyme

The food enzyme is intended to be used in the form of an immobilised, non-viable cell preparation for the production of isomaltulose from sucrose in a continuous process. ██████████²¹

A flowchart depicting the manufacturing process steps of the isomaltulose has been provided.²² A sterilised solution of pure sucrose is mixed with the immobilised enzyme preparation and allowed to react. The resulting solution containing ██████████ isomaltulose is then separated from the immobilised cells by filtration, passed through ion-exchange columns, concentrated by evaporation and crystallised.

Potential leaching of food enzyme TOS from the immobilised cells was measured under manufacturing conditions ██████████.

██████████. Analysis of intermediate liquid isomaltulose after the different stages of purification and of the final dried isomaltulose for total nitrogen, conductivity ash, isomaltulose and other carbohydrate content revealed an efficient removal of potentially leaching food enzyme TOS from the final product. ██████████

²¹ Technical dossier/page 44.

²² Technical dossier/Figure 11 & Additional information October 2020/Annex 3.1.

24,25

Taken together, the Panel considered the evidence as sufficient to conclude that residual amounts of food enzyme TOS leaching from the immobilised enzyme preparation (including substances other than proteins such as formaldehyde) are removed by the purification steps applied to the production of the dried isomaltulose.

Absence of DNA from the production strain was demonstrated in three batches of isomaltulose

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3.5.2. Dietary exposure estimation

The technical information and experimental data provided on the removal of food enzyme TOS during production of dried isomaltulose from sucrose were considered by the Panel as sufficient to exclude this process from the exposure estimation (Annex B in EFSA CEF Panel, 2016). Consequently, a dietary exposure was not calculated.

Conclusions

The food enzyme preparation consists of a non-viable, non-genetically modified microorganism containing an antimicrobial resistance gene, which is considered a hazard.

Under the very specific intended conditions of use described by the applicant, and based on the evidence showing the removal of TOS during the production of isomaltulose, and absence of DNA in the isomaltulose, the Panel concluded that the identified hazard associated with the food enzyme isomaltulose synthase produced with *Serratia plymuthica* strain Z12A will not result in a risk.

4. Recommendation

According to the applicant, the food enzyme preparation isomaltulose synthase produced with *Serratia plymuthica* strain Z12A is used solely for in-house production of isomaltulose. The applicant stated that this proprietary enzyme is not for sale to third parties.

The applicant should ensure that during the manufacture of the food enzyme preparation and of the isomaltulose by using this food enzyme preparation, no DNA is released into the environment.

Documentation provided to EFSA

- 1) Dossier 'Isomaltulose synthase – Enzyme Preparation *Protaminobacter rubrum* Z12A'. February 2015. Submitted by Beneo-Palatinit GmbH.
- 2) Additional information, October 2019. Submitted by Beneo-Palatinit GmbH.
- 3) Additional information, October 2020. Submitted by Beneo-Palatinit GmbH.

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²³ Additional information October 2020/pp. 3–7. & Annex 3.

²⁴ LoQ = 0.3 mg/kg.

²⁵ Technical dossier/Additional information October 2019/Annex 6.

²⁶ Technical dossier/Additional information October 2020/Annex 2.

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Abbreviations

bw	body weight
CAS	Chemical Abstracts Service
CEF	EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids
CFU	colony forming units
EINECS	European Inventory of Existing Commercial Chemical Substances
FAO	Food and Agricultural Organization of the United Nations
GLP	Good Laboratory Practices
GMP	Good Manufacturing Practices
IUBMB	International Union of Biochemistry and Molecular Biology
LOQ	limit of quantification
MNBN	binucleated cells with micronuclei
OECD	Organisation for Economic Cooperation and Development
SDS–PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
TOS	total organic solids
WHO	World Health Organization