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Assessment of safety: *In vitro* reverse mutation and *in vivo* acute oral toxicity tests of three biomass products from amino acid-producing *Corynebacterium glutamicum*

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ABSTRACT

Microbial fermentation has emerged as a pivotal process for sustainable production of essential goods and chemicals. *Corynebacterium glutamicum* is a proficient platform organism that contributes significantly to amino acid production through microbial fermentation. Despite its recognized safety, challenges persist in efficiently biosynthesizing natural products compared with other organisms. This study evaluated the safety of biomass products from bioengineered *C. glutamicum* through two different toxicological studies: a bacterial reverse mutation test (AMES test) and an acute oral toxicity test in rats. Three types of dried fermentation biomass products, each engineered for the enhanced production of specific amino acids (L-lysine, L-threonine, and L-tryptophan), were examined. The tests were conducted in compliance with Organization for Economic Co-operation and Development guidelines and revealed no mutagenicity or acute toxicity at the tested doses. These findings suggest the safety of biomass products from bioengineered *C. glutamicum* as potential feed materials, although further toxicity studies are recommended for comprehensive evaluation. This study underscores the importance of stringent safety assessments for advancing biotechnological applications and provides valuable insights into the potential utilization of microbial fermentation products in various industries. Moreover, this study highlights the significance of regulatory compliance and adherence to international standards to ensure the safety and efficacy of novel biotechnological products.

1. Introduction

Microbial fermentation plays an essential role in the sustainable production of a diverse array of goods, facilitating scalable and environmentally conscious manufacturing processes for essential food items and ingredients. In addition, microbial fermentation serves as a fundamental trigger for advancing the production of sustainable chemicals, natural products, and various food or feed additives, thereby broadening their reach and influence [1]. Notably, during the 1960s and the 1970s, microbial fermentation emerged as an important method for the production of various chemicals, including essential amino acids utilized in food and feed applications and industrial enzymes with extensive utility. Since then, amino acid production via microbial fermentation has become a prominent method in industrial microbiology.

Among the diverse microorganisms used, *Corynebacterium glutamicum* is a proficient platform organism for biotechnological production and serves as a pivotal process in industrial biotechnology [2–4]. In recent decades, this bacterium has become important for amino acid production, commencing with the synthesis of L-glutamate in the 1950s [5,6].

C. glutamicum holds a certification affirming its safety for use. In the United States, the Food and Drug Administration (FDA) designates *C. glutamicum* as a generally regarded as safe (GRAS) microorganism. Similarly, in the European Union, *C. glutamicum* is recognized as a qualified presumption of safety (QPS)-status microorganism, specifically endorsed for the production of various amino acids [7,8].

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Despite its pivotal role and recognized safety, *C. glutamicum* encounters challenges in efficiently biosynthesizing natural products compared with other organisms. Therefore, various gene expression control mechanisms have been refined to allow precise manipulation of the *C. glutamicum* genome [7,9,10]. Numerous strains of bioengineered *C. glutamicum* have been authorized to produce an array of food and feed materials and are extensively utilized in the bioindustry. Furthermore, there is an ongoing need to utilize the biomass of *C. glutamicum* itself as a protein source in feed materials.

In the present study, to evaluate the safety of the biomass of bioengineered *C. glutamicum* strains, two toxicological studies were conducted: a reverse mutation test (AMES test) and an acute oral toxicity test in rats. Three types of biomass products from bioengineered *C. glutamicum* were tested individually, and all test methods followed the Organization for Economic Co-operation and Development (OECD) guidelines for testing chemicals to comply with registration requirements.

2. Materials and methods

2.1. Test substances

Three types of dried fermentation biomass products derived from bioengineered *C. glutamicum* were used in this study: 1) L-lysine producing *C. glutamicum* strain (LYS), 2) L-threonine producing *C. glutamicum* strain (THR), and 3) L-tryptophan producing *C. glutamicum* strain (TRP). Each strain was bioengineered to enhance the yields of the respective target amino acids. To facilitate toxicological assessment, the fermentation liquid from each inactivated *C. glutamicum* strain was filtered through a membrane filter. Subsequently, the filtered strain was concentrated by evaporation. The concentrated biomass was further dried to yield a non-viable biomass product, which constituted the material used in this study. The preparation process of the test material in this study was the same as that used for commercial biomass products.

2.2. Regulatory compliance

Tests were conducted according to the principles of good laboratory practice (GLP) outlined by the Organization for Economic Co-operation and Development (OECD) and in compliance with the GLP regulations for nonclinical laboratory studies stipulated by the Ministry of Food and Drug Safety of the Republic of Korea (Notification No. 2018–93) [11,12]. The bacterial reverse mutation test (AMES test) followed the OECD Test Guideline 471 [13], and the acute oral toxicity test in rodents was conducted in accordance with the OECD Test Guideline 420 [14].

The use of animals for acute oral toxicity tests was approved by the Institutional Animal Care and Use Committee (IACUC) of Biotoxtech Co., Ltd. This study was conducted in accordance with the Animal Protection Act of the Republic of Korea (approval no. 230013, 220674, and 220164). The number of animals used in this study was minimized to adhere to the mandates of the regulatory guidelines governing such tests and ensure the attainment of scientifically justifiable endpoints.

2.3. Bacterial reverse mutation test (AMES test)

2.3.1. Bacterial strains and reagents

Four histidine-requiring *Salmonella typhimurium* strains (TA98, TA100, TA1535, and TA1537) and one tryptophan-requiring *Escherichia coli* strain (WP2*uvr*A) were used in this study. To evaluate a specific test substance, TRP, all bacterial strains employed were histidine-requiring *S. typhimurium* strains (TA98, TA100, TA1535, TA1537, and TA102), to prevent false-positive results induced by tryptophan deficiency. Appropriate positive controls, including sodium azide (SA), 2-nitrofluorene (2-NF), 2-aminoanthracene (2-AA), 9-aminoanthracene (9-AA), 4-nitroquinoline N-oxide (4-NQO), and mitomycin C (MMC), were administered at suitable doses to assess the validity of the study for each strain.

2.3.2. Preparation of dosing formulation

Based on the preliminary solubility test results, either water for injection or phosphate buffered saline (PBS) was used as the vehicle for each test substance. All formulations were freshly prepared on the day of dosing. A predetermined amount of test substance was weighed and placed in a mortar. A small quantity of vehicle was added and mixed with the test substance using a pestle until a homogeneous suspension was obtained. The resulting mixture was transferred to a measuring tube and additional vehicle was added to reach the desired dosage level. The high-dose formulation was sequentially diluted to obtain lower dosage levels within the prescribed range.

2.3.3. Metabolic activation

To assess the mutagenicity of the test substance, all bacterial strains were exposed to it in the presence and absence of an appropriate metabolic activation system. A cofactor-supplemented post-mitochondrial fraction (S9) mixture was used for metabolic activation. The S9 fraction was derived from the livers of 7-week-old male Sprague-Dawley rats (Crl: CD [SD]) treated with enzyme-inducing agents, specifically phenobarbital and 5,6-benzoflavone. To prepare the S9-mix, frozen S9 and Cofactor A thawed and combined in a ratio of 1:9. The S9-mix was freshly prepared immediately before use.

2.3.4. Dose-range-finding study

A dose-range-finding study was conducted to establish a high dose for the primary study. The high dose of the test substance was determined as 5000 μg/plate, as stipulated by the test guideline. Subsequently, the high dose was subjected to sequential dilution, employing a geometric ratio of 4 to generate five lower dose levels: 1250, 313, 78.1, 19.5, and 4.88 μg/plate. Negative and positive control groups were also established and the study was conducted using methods and conditions identical to those employed in the primary study. Each dose level in the dose-range-finding study was evaluated using two plates.

2.3.5. Primary study

The primary study was conducted according to the pre-incubation method described in the guideline (OECD 471). The high dose of the test substance in the primary study was also determined as 5000 μg/plate with a geometric ratio of 2 to generate four lower dose levels: 2500, 1250, 625, and 313 μg/plate. All the treatments were categorized into groups with and without metabolic activation. For each dose, three plates were used in the primary study and the treatments were administered in duplicate. Each plate was designated with an identification number denoting the bacterial strain, dose level, negative and positive controls, and the presence or absence of the S9 mix.

In the presence or absence of metabolic activation, 100 μL of each test substance, negative control, and strain-specific positive controls were dispensed into respective tubes. Subsequently sodium phosphate buffer (500 μL of 0.1 mol/L, pH 7.4) or S9 mix was added accordingly, followed by the addition of 100 μL of strain suspension. The mixtures underwent incubation in a shaking water bath set at 37℃ and 90 rpm for 20 min. After incubation, 2 mL of warmed top agar suitable for either *S. typhimurium* or *E. coli* was added to bacterial strains (TA98, TA100, TA102, TA1535, TA1537, and WP2*uvr*A). The mixtures were thoroughly mixed using a vortex mixer and poured onto minimal glucose agar plates, allowing them to solidify at room temperature.

Upon solidification of the top agar, the plates were inverted and placed in an incubator (DK-LI020-P, Daiki scientific, Republic of Korea) set at 37℃ for 48 h. After incubation, the number of revertant colonies was automatically counted using a colony counter (ProtoCOL3; Synbiosis, UK). The number of revertant colonies was manually enumerated when automatic counting was deemed inaccurate. Any precipitation of the test substance was visually inspected and recorded at the time of

application and colony counting. The background lawn was assessed using a stereoscopic microscope (45-fold magnification, SZ61, Olympus, Japan). The detection of growth inhibition was based on a reduction in the number of revertant colonies or a decrease in or clearance of the background lawn relative to the negative control group.

2.4. Acute oral toxicity test

2.4.1. Animals and husbandry

A total 11 of seven-week-old SD (Crl:CD) rats were procured from Orient Bio Inc. (Republic of Korea). Upon arrival, the rats underwent clinical examination, and their body weights were recorded. Following a quarantine acclimation period of 3 d, clinical signs and changes in body weight were monitored to assess the health status of all rats. Subsequently, 10 healthy rats with body weights close to the mean were randomly assigned to four groups: two groups consisting of one rat each for the sighting study and two groups consisting of four rats each for the primary study.

Throughout the test period, encompassing both the quarantine acclimation and observation phases, all rats were individually housed in stainless-steel wire mesh cages measuring 260 mm × 350 mm × 210 mm (W × D × H). Environmental conditions were meticulously regulated, maintaining temperatures within the range of 19.9–23.4℃ and relative humidity at 45.0–59.4 %. The air within the cages was changed 10–15 times per hour to ensure cleanliness and freshness. A 12-hour light/dark cycle was enforced, with an illumination intensity ranging from 150 to 300 lx. The rats were provided *ad libitum* access to pelleted rodent chow feed (Envigo RMS, Inc., USA) and filtered/UV-irradiated public tap water to meet their nutritional and hydration requirements.

2.4.2. Dosing

The dried fermentation biomass of *C. glutamicum* was weighed and suspended in sterile water for irrigation. The dosing formulation was freshly prepared on the day of administration, immediately before dosing. Individual dose volumes were calculated for each rat based on their respective body weights recorded immediately before dosing, while they were in a fasted state, at a dose volume of 10 mL/kg body weight.

Rats were orally dosed via gastric intubation using disposable syringes equipped with intubation tubes. The animals were subjected to an overnight fast for approximately 16 h prior to administration. Feed was provided to rats 4 h after dosing.

2.4.3. Group assignment, dose levels, and observations

Two sighting studies and one primary study were conducted for each test substance. In the first sighting study, a starting dose of 300 mg/kg of the test substance was administered to one animal. Subsequent dose levels were determined based on observations of mortality and clinical signs in animals for 3 d following administration at a dose of 2000 mg/kg body weight in the first sighting study. For the primary study, the test substance was administered to four female rats in accordance with OECD guidelines 420.

2.4.4. Observations (body weights, clinical signs, and necropsy)

The body weight of each rat was recorded once on the day of dosing, prior to administration, and on days 2, 4, 8, and 15 (day of necropsy). Weight changes were documented and calculated throughout the study. At the end of the test, all surviving rats were weighed and euthanized.

Observations regarding clinical signs, including type, severity, onset time, recovery, and mortality, were conducted for all rats at 0.5, 1, 2, 4, and 6-h post-dosing on day 1. Subsequently, daily assessments were performed for 14 d (days 2–15).

On the day of necropsy (day 15), all the rats were anesthetized via CO_2 gas inhalation, followed by exsanguination from the abdominal aorta. Comprehensive gross postmortem examinations were conducted for all study subjects. All gross pathological changes were meticulously recorded for each animal, and if any abnormal signs were observed, a microscopic examination of the organs was performed.

3. Results

3.1. Bacterial reverse mutation test (AMES test)

3.1.1. Dose range-finding study

No growth inhibition attributable to any test substance was observed at any dose level ranging 4.88–5000 μg/plate in all strains, both in the absence and presence of metabolic activation (S9) (Table 1). Although precipitation of each test substance was evident at dose levels of 313 μg/plate or higher under the same conditions, the counting of revertant colonies was not impeded. Therefore, for the primary study, the highest dose was set at 5000 μg/plate, with sequential dilutions achieved by applying a geometric ratio of 2 to generate lower dose levels (2500, 1250, 625, and 313 μg/plate).

3.1.2. Primary mutagenicity study

In the two primary mutagenicity studies conducted for each test substance, the mean number of revertant colonies was consistently less than twice that of the negative control group across all dose levels and strains in both the absence and presence of metabolic activation (S9). Conversely, in the strain-specific positive control group, the mean number of revertant colonies for each strain exhibited a marked increase of more than twice that in the negative control group.

No growth inhibition attributable to any test substance was observed at any dose in any strain in the absence or presence of metabolic activation (Table 2). However, precipitation of each test substance was evident at dose levels of 313 μg/plate or higher under the same conditions.

3.2. Acute oral toxicity test

3.2.1. Mortality and clinical signs

No deaths occurred among animals administered 300 and 2000 mg/kg of the test substance throughout the study period. Furthermore, no clinical abnormalities were observed in any animal.

3.2.2. Body weights

Throughout the study, normal body weight gain was observed in all animals at all concentrations (Table 3). For one test substance (TRP), a decrease in body weight was noted in only one animal (-0.5 g) on day 15 at a dose of 2000 mg/kg. However, it was not considered a test substance-related effect, as it was a minor and temporary decrease observed in only one animal, and the decreased body weight fell within the historical range of the test facility (data not shown). Additionally, no clinical signs or morphological abnormalities were observed during necropsy.

3.2.3. Necropsy findings

No abnormal gross findings were observed in any of the animals administered 300 and 2000 mg/kg. All animals appeared normal during gross necropsy; therefore, a microscopic examination was not performed.

4. Discussion

Toxicological assessment is a crucial step in the advancement and utilization of a novel compound. It is standard practice to conduct genotoxicity examinations on both chemicals and different substances to gauge their capability to interact with nucleic acids and cause permanent harm or mutations, even at low concentrations [15].

The *in vitro* AMES test serves as an initial screening tool for assessing the genotoxic potential of a substance, particularly its ability to induce point mutations, which involve the substitution, addition, or deletion of

Table 1
Number of revertant colonies per plate in the absence and presence of metabolic activation (S9): dose range-finding study.

Strain	Dose (μg/plate)	LYS		THR		TRP	
		- S9	+ S9	- S9	+ S9	- S9	+ S9
TA98	0	18.5 ± 1.5	24.0 ± 1.0	20.5 ± 0.5	27.5 ± 0.5	15.5 ± 0.5	32.0 ± 1.0
	4.88	16.0 ± 0.0	23.5 ± 0.5	20.0 ± 2.0	28.0 ± 2.0	15.5 ± 0.5	32.5 ± 1.5
	19.5	19.5 ± 1.5	24.5 ± 1.5	20.5 ± 1.5	26.5 ± 0.5	14.0 ± 1.0	28.5 ± 0.5
	78.1	15.0 ± 1.0	23.0 ± 1.0	19.0 ± 1.0	26.5 ± 1.5	17.0 ± 1.0	28.0 ± 1.0
	313	17.0 ± 1.0	21.5 ± 1.5	20.5 ± 0.5	27.0 ± 1.0	15.0 ± 0.0	25.0 ± 1.0
	1250	13.5 ± 0.5	24.5 ± 0.5	17.5 ± 1.5	28.5 ± 1.5	13.0 ± 1.0	26.0 ± 1.0
	5000	16.5 ± 1.5	26.0 ± 1.0	17.5 ± 0.5	30.0 ± 1.0	13.5 ± 0.5	25.5 ± 0.5
	PC[a]	615.5 ± 5.5	407.5 ± 10.5	640.5 ± 16.5	241.0 ± 12.0	588.0 ± 23.0	362.5 ± 12.5
TA100	0	115.5 ± 0.5	118.0 ± 2.0	100.5 ± 0.5	122.0 ± 2.0	115.0 ± 2.0	135.5 ± 2.5
	4.88	117.0 ± 2.0	119.0 ± 2.0	101.5 ± 0.5	127.0 ± 3.0	122.5 ± 2.5	128.0 ± 2.0
	19.5	118.5 ± 0.5	126.0 ± 3.0	100.5 ± 1.5	126.0 ± 1.0	121.5 ± 1.5	131.0 ± 2.0
	78.1	121.0 ± 3.0	129.0 ± 3.0	102.5 ± 0.5	124.0 ± 2.0	128.0 ± 2.0	129.0 ± 1.0
	313	120.0 ± 3.0	128.0 ± 2.0	105.5 ± 0.5	123.0 ± 3.0	117.0 ± 2.0	132.0 ± 2.0
	1250	120.0 ± 2.0	124.5 ± 0.5	104.5 ± 0.5	127.0 ± 2.0	120.5 ± 0.5	129.5 ± 1.5
	5000	121.5 ± 0.5	121.0 ± 2.0	102.5 ± 0.5	128.0 ± 2.0	126.5 ± 1.5	134.5 ± 3.5
	PC	645.5 ± 35.5	812.5 ± 36.5	591.0 ± 7.0	788.0 ± 38.0	685.5 ± 32.5	1059.5 ± 52.5
TA1535	0	10.5 ± 0.5	11.5 ± 1.5	16.5 ± 1.5	9.5 ± 1.5	11.5 ± 0.5	10.5 ± 0.5
	4.88	8.0 ± 0.0	14.0 ± 1.0	17.0 ± 0.0	10.0 ± 0.0	10.5 ± 0.5	12.0 ± 0.0
	19.5	10.0 ± 0.0	10.5 ± 0.5	14.5 ± 0.5	9.5 ± 0.5	13.0 ± 1.0	10.0 ± 1.0
	78.1	8.5 ± 0.5	9.5 ± 0.5	15.0 ± 1.0	10.5 ± 2.5	9.5 ± 0.5	13.0 ± 1.0
	313	8.0 ± 1.0	8.0 ± 0.0	16.5 ± 0.5	10.5 ± 1.5	11.5 ± 0.5	12.0 ± 0.0
	1250	10.5 ± 0.5	8.5 ± 0.5	17.5 ± 0.5	10.5 ± 0.5	11.0 ± 0.0	11.0 ± 1.0
	5000	7.0 ± 0.0	9.5 ± 0.5	16.5 ± 0.5	9.0 ± 1.0	10.0 ± 1.0	10.0 ± 0.0
	PC	584.5 ± 26.5	156.0 ± 7.0	521.0 ± 12.0	152.5 ± 7.5	625.5 ± 8.5	191.5 ± 2.5
TA1537	0	9.0 ± 0.0	15.0 ± 1.0	10.5 ± 0.5	12.5 ± 0.5	10.0 ± 0.0	11.5 ± 0.5
	4.88	9.5 ± 0.5	10.5 ± 1.5	9.0 ± 2.0	13.5 ± 0.5	10.5 ± 0.5	12.0 ± 0.0
	19.5	8.0 ± 0.0	11.5 ± 1.5	10.0 ± 2.0	13.0 ± 1.0	10.5 ± 0.5	11.0 ± 0.0
	78.1	7.0 ± 1.0	9.5 ± 0.5	10.5 ± 1.5	13.5 ± 0.5	12.0 ± 1.0	11.5 ± 0.5
	313	9.5 ± 0.5	11.5 ± 1.5	10.0 ± 1.0	15.0 ± 0.0	9.5 ± 0.5	13.0 ± 0.0
	1250	10.5 ± 0.5	12.5 ± 0.5	9.5 ± 1.5	15.0 ± 1.0	8.5 ± 0.5	11.5 ± 0.5
	5000	8.0 ± 0.0	10.5 ± 0.5	10.0 ± 1.0	16.0 ± 1.0	8.5 ± 0.5	12.5 ± 0.5
	PC	583.0 ± 16.0	179.5 ± 6.5	498.5 ± 11.5	156.5 ± 2.5	660.0 ± 20.0	162.5 ± 7.5
TA102 or WP2*uvr*A	0	25.5 ± 0.5	24.0 ± 1.0	23.0 ± 1.0	23.0 ± 1.0	225.0 ± 4.0	302.5 ± 2.5
	4.88	25.0 ± 1.0	24.0 ± 0.0	24.5 ± 2.5	23.5 ± 2.5	226.5 ± 2.5	305.5 ± 4.5
	19.5	26.5 ± 0.5	26.0 ± 1.0	23.5 ± 0.5	22.5 ± 0.5	220.0 ± 1.0	301.0 ± 4.0
	78.1	25.0 ± 1.0	26.5 ± 1.5	23.0 ± 2.0	25.0 ± 1.0	219.5 ± 0.5	318.5 ± 1.5
	313	25.5 ± 0.5	27.0 ± 1.0	22.0 ± 1.0	22.5 ± 1.5	211.5 ± 1.5	320.5 ± 2.5
	1250	27.5 ± 0.5	25.5 ± 0.5	24.0 ± 2.0	25.0 ± 2.0	181.0 ± 3.0	255.5 ± 4.5
	5000	29.0 ± 1.0	29.0 ± 1.0	23.0 ± 2.0	22.0 ± 2.0	157.5 ± 2.5	231.5 ± 3.5
	PC	727.0 ± 20.0	563.0 ± 20.0	745.5 ± 55.5	587.5 ± 6.5	1769.0 ± 16.0	1215.0 ± 54.0

S. typhimurium TA98 (-S9:2-NF, 5.0 μg/plate; +S9:2-AA, 1.0 μg/plate), *S. typhimurium* TA100 (-S9: SA, 1.5 μg/plate; +S9:2-AA, 2.0 μg/plate), *S. typhimurium* TA1535 (-S9: SA, 1.5 μg/plate; +S9:2-AA, 3.0 μg/plate), *S. typhimurium* TA1537 (-S9:9-AA, 80.0 μg/plate; +S9:2-AA, 3.0 μg/plate), *S. typhimurium* TA102 (-S9: MMC, 0.5 μg/plate; +S9:2-AA, 10.0 μg/plate), *E. coli* WP2*uvr*A (-S9:4-NQO, 0.3 μg/plate, +S9:2-AA, 10.0 μg/plate).

[a] PC, positive control.

one or more DNA base pairs. Although primarily employed to identify revertant mutations in bacterial strains, the AMES test is also applicable to detect the mutagenicity of various environmental samples, including drugs, dyes, cosmetics, pesticides, and other soluble substances in liquid suspensions [16]. In the present study, histidine-auxotrophic strains of *S. typhimurium*, including TA100, TA98, TA1535, and TA1537, along with the tryptophan-auxotrophic strain *E. coli* WP2 *uvr*A, were used as described previously [17]. However, for one specific test substance, TRP, a histidine-requiring *S. typhimurium* TA102, was used instead of *E. coli* WP2 *uvr*A to exclude false-positive results due to tryptophan deficiency. None of the test substances increased the number of revertant colonies across all strains, indicating that the three biomass products used in this study did not induce reverse mutations.

Acute toxicity assessments predominantly focus on the median lethal dose (LD_{50}) test, complemented by alternative methods that are consistent with the principle of reducing, refining, or replacing animal usage in toxicity testing (3 R principle). Acute toxicity commonly pertains to adverse changes that manifest promptly or shortly following exposure to a substance, whether in a single instance or over a brief duration, or as adverse effects arising shortly after the administration of a single or multiple doses within a 24-hour period. We employed a fixed-dose procedure to assess the LD_{50} values of the test substances. In all three acute toxicity tests, no mortality or morbidity was observed in any of the animals up to the dose limit (2000 mg/kg). Furthermore, in accordance with OECD TG420 and the Globally Harmonized System (GHS) for the classification of chemicals, each test substance (LYS, THR, and TRP) was classified as GHS category 5 or remained unclassified.

In summary, based on the results of both the reverse mutation tests and acute oral toxicity tests in rats, it can be concluded that biomass products derived from bioengineered *C. glutamicum* used in this study do not induce reverse mutations in cells and do not demonstrate acute toxicity in rats. Collectively, these findings suggested that the three biomass products of bioengineered *C. glutamicum* are safe for use as feed materials.

CRediT authorship contribution statement

So-Young Kim: Writing – review & editing, Resources. **Yang Hee Kim:** Writing – review & editing, Supervision, Conceptualization. **Jiyeon Kim:** Investigation. **Ji-Eun Park:** Writing – original draft, Investigation, Data curation. **Hyo-Jin An:** Methodology, Formal analysis. **MiNa Baek:** Investigation. **Joon Young Jung:** Writing – review & editing. **Chan-Sung Park:** Methodology, Formal analysis.

Table 2
Number of revertant colonies per plate in the absence and presence of metabolic activation (S9).

(a) 1st primary study							
Strain	Dose (μg/plate)	LYS		THR		TRP	
		- S9	+ S9	- S9	+ S9	- S9	+ S9
TA98	0	16.3 ± 0.9	29.0 ± 2.2	17.0 ± 1.6	27.0 ± 1.6	18.3 ± 1.2	29.3 ± 1.7
	313	17.0 ± 0.8	23.7 ± 1.2	16.7 ± 0.5	25.3 ± 1.2	18.7 ± 1.2	28.7 ± 1.9
	625	17.0 ± 2.2	25.3 ± 0.5	15.0 ± 0.8	28.0 ± 0.8	17.7 ± 1.2	27.7 ± 1.2
	1250	15.7 ± 2.1	27.0 ± 2.2	15.0 ± 1.6	28.0 ± 0.8	16.3 ± 0.9	29.0 ± 0.8
	2500	17.0 ± 0.8	29.3 ± 0.9	16.0 ± 1.6	28.7 ± 1.2	17.0 ± 1.4	27.0 ± 1.4
	5000	19.3 ± 1.2	31.0 ± 2.2	14.7 ± 0.5	31.0 ± 1.6	17.7 ± 1.2	27.3 ± 0.9
	PC[b]	702.7 ± 12.3	358.0 ± 4.9	664.0 ± 17.7	424.7 ± 26.7	589.3 ± 40.5	375.3 ± 60.9
TA100	0	107.0 ± 2.2	127.3 ± 2.6	108.0 ± 4.1	131.0 ± 4.9	106.3 ± 1.2	132.3 ± 2.1
	313	108.0 ± 2.9	125.3 ± 2.1	113.0 ± 4.9	125.7 ± 1.2	106.0 ± 2.9	133.0 ± 2.8
	625	107.0 ± 2.2	123.3 ± 2.1	112.0 ± 3.3	128.0 ± 4.1	100.7 ± 3.1	140.7 ± 2.5
	1250	107.0 ± 2.2	117.3 ± 5.0	111.0 ± 4.1	129.0 ± 4.9	101.3 ± 2.6	138.3 ± 2.9
	2500	108.0 ± 2.2	113.7 ± 2.6	111.0 ± 3.3	125.7 ± 2.5	110.3 ± 3.3	144.7 ± 3.1
	5000	110.3 ± 2.9	110.0 ± 3.7	109.0 ± 4.9	130.0 ± 4.1	102.3 ± 1.7	148.7 ± 2.6
	PC	661.7 ± 5.6	812.7 ± 9.4	670.0 ± 14.7	797.7 ± 34.1	694.0 ± 34.6	930.3 ± 38.9
TA1535	0	10.3 ± 1.9	11.7 ± 1.2	10.0 ± 0.8	9.7 ± 0.5	11.7 ± 0.9	10.0 ± 0.8
	313	10.3 ± 0.5	9.0 ± 0.8	10.7 ± 0.5	9.3 ± 1.2	10.3 ± 0.9	11.7 ± 1.2
	625	10.3 ± 0.5	10.7 ± 2.4	10.3 ± 0.5	9.3 ± 1.2	10.0 ± 0.8	11.3 ± 1.2
	1250	12.3 ± 1.7	12.0 ± 1.6	10.0 ± 1.6	7.7 ± 0.5	10.7 ± 0.9	10.7 ± 1.2
	2500	10.7 ± 2.1	12.0 ± 0.8	10.3 ± 1.7	9.3 ± 1.2	13.3 ± 0.9	12.0 ± 1.6
	5000	9.0 ± 0.8	13.7 ± 1.7	9.7 ± 0.5	7.0 ± 0.8	11.0 ± 1.4	10.3 ± 0.5
	PC	559.3 ± 19.7	153.0 ± 13.5	549.0 ± 19.6	179.3 ± 7.8	567.0 ± 48.2	183.7 ± 13.5
TA1537	0	10.3 ± 1.2	16.0 ± 0.8	9.3 ± 0.5	18.0 ± 1.6	8.7 ± 0.9	13.7 ± 0.5
	313	10.0 ± 0.8	17.7 ± 1.7	8.0 ± 0.8	19.0 ± 1.6	8.7 ± 0.5	13.0 ± 0.8
	625	8.7 ± 1.2	16.7 ± 0.5	7.0 ± 1.6	18.3 ± 1.2	8.0 ± 0.8	14.0 ± 1.4
	1250	8.7 ± 0.9	18.7 ± 1.2	8.0 ± 0.8	18.3 ± 1.2	7.7 ± 0.5	12.0 ± 0.8
	2500	8.0 ± 0.8	19.3 ± 1.2	7.7 ± 1.2	19.0 ± 0.8	8.7 ± 0.5	14.0 ± 0.8
	5000	6.7 ± 1.2	17.7 ± 0.9	9.3 ± 1.2	22.0 ± 1.6	7.7 ± 0.5	16.3 ± 0.5
	PC	615.0 ± 24.3	169.3 ± 5.9	598.0 ± 23.4	169.0 ± 9.0	544.3 ± 20.2	179.0 ± 14.4
TA102 or	0	27.7 ± 2.5	28.0 ± 0.8	31.0 ± 1.6	38.3 ± 1.7	227.0 ± 2.2	314.3 ± 1.2
WP2*uvr*A	313	29.3 ± 1.2	30.0 ± 1.4	26.0 ± 1.6	35.0 ± 1.6	222.7 ± 2.4	323.3 ± 2.1
	625	31.3 ± 1.2	29.3 ± 0.5	27.0 ± 0.8	38.0 ± 2.4	223.3 ± 3.4	279.7 ± 3.4
	1250	31.0 ± 2.2	27.3 ± 1.2	26.0 ± 2.4	36.0 ± 1.6	190.0 ± 2.4	269.3 ± 3.1
	2500	32.3 ± 1.2	28.3 ± 1.7	26.0 ± 1.6	34.0 ± 2.4	188.0 ± 2.2	260.0 ± 3.3
	5000	32.7 ± 0.9	31.0 ± 1.4	24.7 ± 0.5	33.0 ± 2.4	157.7 ± 2.9	244.3 ± 2.4
	PC	577.3 ± 17.6	513.0 ± 14.3	630.3 ± 53.9	501.7 ± 13.1	2356.0 ± 42.8	1127.0 ± 93.0
(b) 2nd primary study							
Strain[1]	Dose (μg/plate)	LYS		THR		TRP	
		- S9	+ S9	- S9	+ S9	- S9	+ S9
TA98	0	14.0 ± 2.2	27.0 ± 1.6	15.0 ± 0.8	29.0 ± 0.8	20.7 ± 0.5	30.7 ± 0.5
	313	16.0 ± 0.8	25.0 ± 0.8	14.0 ± 0.8	30.0 ± 1.6	18.3 ± 0.5	28.7 ± 0.5
	625	18.3 ± 1.7	25.0 ± 0.8	14.0 ± 1.6	29.7 ± 0.5	18.3 ± 0.9	28.3 ± 0.9
	1250	16.0 ± 0.8	26.0 ± 1.6	15.7 ± 0.5	31.0 ± 2.4	20.7 ± 0.5	30.3 ± 0.5
	2500	17.3 ± 1.7	26.7 ± 0.5	15.0 ± 1.6	33.0 ± 1.6	18.3 ± 0.5	28.3 ± 0.5
	5000	18.3 ± 1.2	32.0 ± 0.8	12.7 ± 0.5	34.0 ± 0.8	20.0 ± 0.8	28.3 ± 0.9
	PC[b]	652.0 ± 13.7	391.0 ± 11.5	624.7 ± 7.8	360.0 ± 9.4	576.3 ± 28.5	329.7 ± 18.2
TA100	0	105.0 ± 2.4	120.0 ± 2.4	118.0 ± 4.1	113.0 ± 3.3	107.7 ± 2.6	125.7 ± 3.3
	313	106.0 ± 3.3	118.0 ± 3.3	120.0 ± 4.1	108.0 ± 4.1	104.0 ± 0.8	129.7 ± 2.1
	625	106.0 ± 1.6	117.0 ± 2.4	120.0 ± 4.9	109.0 ± 4.1	106.0 ± 2.9	133.7 ± 2.6
	1250	108.0 ± 2.4	115.0 ± 3.3	122.0 ± 3.3	111.0 ± 4.9	104.0 ± 1.6	142.3 ± 2.1
	2500	109.0 ± 3.3	114.0 ± 3.3	121.0 ± 3.3	107.0 ± 2.4	103.7 ± 2.6	147.0 ± 2.9
	5000	109.0 ± 1.6	112.0 ± 4.1	120.0 ± 4.9	110.0 ± 4.1	105.0 ± 2.2	151.3 ± 2.6
	PC	683.7 ± 16.7	775.7 ± 53.9	633.7 ± 21.9	867.7 ± 21.3	742.0 ± 38.9	895.7 ± 31.2
TA1535	0	8.7 ± 0.5	13.0 ± 0.8	8.7 ± 0.5	12.7 ± 0.5	11.0 ± 0.8	11.0 ± 0.8
	313	9.0 ± 0.8	11.0 ± 1.6	11.0 ± 1.6	9.0 ± 0.8	11.7 ± 0.5	10.0 ± 0.8
	625	10.0 ± 1.6	12.0 ± 1.6	10.0 ± 1.6	10.7 ± 2.4	9.7 ± 0.5	11.7 ± 0.5
	1250	11.0 ± 0.8	14.0 ± 0.8	9.7 ± 0.5	12.0 ± 1.6	11.7 ± 0.5	10.3 ± 0.5
	2500	9.7 ± 0.5	15.0 ± 0.8	9.3 ± 0.5	15.0 ± 0.8	10.0 ± 0.8	10.3 ± 0.9
	5000	9.0 ± 0.8	14.7 ± 0.5	9.0 ± 0.8	14.7 ± 0.5	12.7 ± 0.5	9.7 ± 0.5
	PC	586.3 ± 11.3	171.0 ± 9.1	561.0 ± 13.4	171.0 ± 9.1	601.0 ± 14.9	166.7 ± 15.1
TA1537	0	10.7 ± 1.7	17.3 ± 0.5	11.0 ± 0.8	17.3 ± 0.5	9.3 ± 0.5	15.3 ± 0.5
	313	10.0 ± 0.0	17.7 ± 1.7	9.7 ± 0.5	17.7 ± 1.7	9.7 ± 0.5	13.3 ± 0.5
	625	6.7 ± 1.2	17.0 ± 0.8	8.7 ± 0.5	17.0 ± 0.8	9.0 ± 0.8	14.0 ± 1.4
	1250	7.3 ± 1.9	20.3 ± 1.2	8.0 ± 1.6	20.3 ± 1.2	8.7 ± 0.5	13.3 ± 0.5
	2500	9.0 ± 0.8	18.7 ± 0.9	9.0 ± 0.8	18.7 ± 0.9	8.3 ± 0.5	12.3 ± 0.5
	5000	7.3 ± 1.2	18.7 ± 0.5	7.7 ± 0.5	18.7 ± 0.5	7.7 ± 0.5	14.7 ± 0.5
	PC	530.0 ± 21.9	184.3 ± 6.0	538.3 ± 2.6	184.3 ± 6.0	604.3 ± 22.6	155.0 ± 10.4
TA102 or	0	28.3 ± 1.2	28.7 ± 1.2	28.0 ± 2.4	28.7 ± 1.2	225.3 ± 3.3	324.7 ± 3.3
WP2*uvr*A	313	28.3 ± 1.2	30.0 ± 2.2	25.0 ± 1.6	30.0 ± 2.2	223.7 ± 3.3	319.7 ± 0.9
	625	28.3 ± 0.5	28.7 ± 1.2	26.0 ± 1.6	28.7 ± 1.2	216.3 ± 3.1	284.3 ± 2.5
	1250	31.0 ± 2.2	29.3 ± 2.4	25.7 ± 0.5	29.3 ± 2.4	197.0 ± 3.6	264.7 ± 2.9

(*continued on next page*)

Table 2 (*continued*)

(b) 2nd primary study							
Strain[1]	Dose (μg/plate)	LYS		THR		TRP	
		- S9	+ S9	- S9	+ S9	- S9	+ S9
	2500	33.0 ± 0.8	31.0 ± 1.6	25.0 ± 1.6	31.0 ± 1.6	186.3 ± 3.3	254.0 ± 3.6
	5000	33.3 ± 0.5	33.3 ± 0.9	23.7 ± 0.5	33.3 ± 0.9	160.7 ± 2.5	242.0 ± 1.6
	PC	525.3 ± 39.3	470.7 ± 17.6	553.0 ± 15.7	470.7 ± 17.6	2137.7 ± 89.3	1044.0 ± 50.9

S. typhimurium TA98 (-S9:2-NF, 5.0 μg/plate; +S9:2-AA, 1.0 μg/plate), *S. typhimurium* TA100 (-S9: SA, 1.5 μg/plate; +S9:2-AA, 2.0 μg/plate), *S. typhimurium* TA1535 (-S9: SA, 1.5 μg/plate; +S9:2-AA, 3.0 μg/plate), *S. typhimurium* TA1537 (-S9:9-AA, 80.0 μg/plate; +S9:2-AA, 3.0 μg/plate), *S. typhimurium* TA102 (-S9: MMC, 0.5 μg/plate; +S9:2-AA, 10.0 μg/plate), *E. coli* WP2*uvr*A (-S9:4-NQO, 0.3 μg/plate, +S9:2-AA, 10.0 μg/plate).

[b] PC, positive control.

Table 3
Individual body weight data in Acute oral toxicity in rats.

(a) L-lysine producing *C. glutamicum* strain (LYS)						
Study	1st sighting study (n=1)	2nd sighting study (n=1)	Primary study (n=4)			
Dose (mg/kg bw)	300	2000	2000			
Animal no.	1	2	3	4	5	6
Body weight (g)						
Day 1	162.37	179.00	200.90	191.12	197.82	199.22
Day 2	183.32	204.75	221.83	214.08	215.16	217.75
Day 4	196.95	210.22	219.14	218.42	222.60	229.33
Day 8	207.06	228.46	236.88	231.61	239.00	254.85
Day 15	217.43	259.18	257.72	261.56	254.85	266.25
(b) L-threonine producing *C. glutamicum* strain (THR)						
Study	1st sighting study (n = 1)	2nd sighting study (n = 1)	Primary study (n = 4)			
Dose (mg/kg bw)	300	2000	2000			
Animal no.	1	2	3	4	5	6
Body weight (g)						
Day 1	204.90	206.97	220.51	218.77	220.15	229.41
Day 2	228.10	233.69	243.74	247.98	243.50	247.39
Day 4	239.21	246.97	257.12	256.59	257.65	264.77
Day 8	256.62	254.03	270.14	272.88	265.87	280.17
Day 15	273.51	271.07	275.37	281.65	285.96	293.61
(c) L-tryptophan producing *C. glutamicum* strain (TRP)						
Study	1st sighting study (n = 1)	2nd sighting study (n = 1)	Primary study (n = 4)			
Dose (mg/kg bw)	300	2000	2000			
Animal no.	1	2	3	4	5	6
Body weight (g)						
Day 1	185.37	189.11	199.11	200.97	202.19	186.49
Day 2	213.39	214.33	212.97	223.04	218.62	205.59
Day 4	221.93	216.34	219.03	224.11	227.50	206.06
Day 8	236.26	220.81	229.82	236.12	236.17	215.44
Day 15	248.53	225.01	239.76	242.77	243.04	214.96

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Ji-Eun Park, Jiyeon Kim, MiNa Baek, Joon Young Jung, So-Young Kim reports a relationship with CJ CheilJedang Corp that includes: employment. Yang Hee Kim reports a relationship with CJ CheilJedang Corp that includes: employment. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that has been used is confidential.

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