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Research article

# Production of safe cyanobacterial biomass for animal feed using wastewater and drinking water treatment residuals

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ABSTRACT

The growing interest in microalgae and cyanobacteria biomass as an alternative to traditional animal feed is hindered by high production costs. Using wastewater (WW) as a cultivation medium could offer a solution, but this approach risks introducing harmful substances into the biomass, leading to significant safety concerns. In this study, we addressed these challenges by selectively extracting nitrates and phosphates from WW using drinking water treatment residuals (DWTR) and chitosan. This method achieved peak adsorption capacities of 4.4 mg/g for nitrate and 6.1 mg/g for phosphate with a 2.5 wt% chitosan blend combined with DWTR-nitrogen. Subsequently, these extracted nutrients were employed to cultivate *Spirulina platensis*, yielding a biomass productivity rate of 0.15 g/L/d, which is comparable to rates achieved with commercial nutrients. By substituting commercial nutrients with nitrate and phosphate from WW, we can achieve a 18 % reduction in the culture medium cost. While the cultivated biomass was initially nitrogen-deficient due to low nitrate levels, it proved to be protein-rich, accounting for 50 % of its dry weight, and contained a high concentration of free amino acids (1260 mg/g), encompassing all essential amino acids. Both in vitro and in vivo toxicity tests affirmed the biomass's safety for use as an animal feed component. Future research should aim to enhance the economic feasibility of this alternative feed source by developing efficient adsorbents, utilizing cost-effective reagents, and implementing nutrient reuse strategies in spent mediums.

## 1. Introduction

With the rising consumption of meat and fish in people's diets, the cost of traditional animal feed production has increased and its

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environmental impact has intensified, leading to increased greenhouse gas emissions, land degradation, and significant consumption of scarce water resources [1,2]. These challenges underscore the urgent need for alternative feed sources that are not only economical but also of high quality, to supplement traditional feedstocks and meet the growing demand [2,3]. Numerous studies are exploring various alternative sources for animal feed, including plant leaves [4,5], insects [6,7], and microbes [8–10].

Microalgae serve as a versatile and sustainable resource with applications that span from lower-value biofuels to high-value pharmaceutical and nutritional products. These applications are driven by various cultivation strategies and differing compositional profiles [11–13]. Certain species of microalgae and cyanobacteria, such as *Chlorella*, *Scenedesmus*, and *Spirulina* (often referred to as blue-green algae), are gaining popularity as potential ingredients for animal feed due to their rich content of proteins, carbohydrates, lipids, minerals, vitamins, and pigments [14–17]. This trend is not only driven by their impressive nutritional profile and digestibility but also by their positive impact on the quality and yield of animal products [2,18–20]. The global market for microalgae is projected to reach approximately USD 6.5 billion, with products for aquaculture and animal feed expected to make up around USD 700 million of this sum [21].

Microalgae and cyanobacteria, owing to their high nutritional value, are increasingly being recognized as promising alternatives to traditional protein sources in both human and animal diets [20,22]. Specifically, strains like *Chlorella* and *Spirulina* are notable for their high protein content, making them potential renewable supplements for feed [2,3,23]. This innovation is crucial for animal feed, as microbial protein has the potential to replace 10–19 % of conventional proteins from crops and animals by 2050 [24,25]. Nonetheless, the prevailing production costs of microalgal protein remain higher than other traditional protein feedstocks [2,3,26].

To counteract these high production costs, researchers are exploring wastewater (WW) sources—such as those from meat, grain, dairy, and breweries—as potential cultivation media for microalgae and other microorganisms [24,26,27]. hese sources of WW are characteristically rich in essential elements like carbon, nitrogen, and phosphorus and generally contain fewer contaminants, like pathogens and heavy metals [24]. Hence, utilizing such WW sources opens up possibilities for producing appropriate amino acids for animal feeds [26,27]. However, employing biomass grown in different types of WW for animal feed can pose risks due to possible contamination with undesirable substances present in the WW. Mitigating these risks might necessitate additional processing steps, such as diluting with cleaner water or treatment with specific microbes to purify the biomass.

The central objective of our study is to develop a versatile method for the safe and nutritious production of cyanobacterial biomass from various types of WW, offering a diverse range of choices for water sources. Central to this endeavor is the use of adsorption techniques to recover specific nutrients, nitrate and phosphate, from WW, with the aim of producing biomass suitable for animal feed. Our approach to indirect WW utilization is categorized into three distinct phases.

1. **Extraction Phase**: Initially, we use drinking water treatment residuals (DWTR) in combination with chitosan to selectively extract nitrate and phosphate from WW. Notably, DWTR, a byproduct from water treatment facilities, is primarily composed of aluminum and iron salt flocculants along with various associated colloids [28].
2. **Regeneration Phase**: Next, we initiate the regeneration of the extracted nutrients by employing a desorption process in an alkaline solution, thereby priming them for reuse.
3. **Cultivation Phase**: Lastly, we utilize the reclaimed nutrients, complemented by additional essential nutrients, to cultivate the cyanobacterial biomass.

To validate the feasibility of the cultivated cyanobacterial biomass as a potential protein source for feed, we undertook rigorous evaluations. This included both in vitro and in vivo toxicity tests and thorough examinations of its compositional attributes, including its amino acid profile.

## 2. Materials and methods

### *2.1. Granulation of drinking water treatment residuals (DWTR) for nitrate and phosphate recovery*

Drinking water treatment residuals (DWTR) were obtained from a facility in Jinju, Gyeongsangnam-do, Republic of Korea, and was sampled in August 2017. The procured sample was converted into a powder form (referred to as DWTR-pristine, Table 1) and was subsequently calcined in a nitrogen atmosphere at 450 °C for 4 h to produce DWTR-nitrogen (Table 2).

To produce chitosan-encased DWTR-nitrogen gel beads (DWTR-CH), we combined various concentrations of DWTR-nitrogen with a chitosan solution in acetic acid. This combination was then dispensed dropwise into a sodium hydroxide solution. Following the

**Table 1**
XRF data of chemical composition of DWTR-pristine.

| Element/Oxide | Composition (wt. %) | Element/Oxide | Composition (wt. %) |
|---|---|---|---|
| $Al_2O_3$ | 48.30 | $K_2O$ | 1.61 |
| $SiO_2$ | 24.60 | MnO | 1.35 |
| $Fe_2O_3$ | 12.50 | $TiO_2$ | 0.63 |
| $SO_3$ | 4.89 | Br | 0.095 |
| Cl | 0.63 | CuO | 0.18 |
| $P_2O_5$ | 1.40 | ZnO | 0.21 |
| CaO | 3.47 | SrO | 0.046 |

neutralization and drying processes, as illustrated in Fig. 1, we evaluated the beads' nitrate and phosphate adsorption capacities. This was done by submerging 0.5 g of the material in a 50 mL solution containing 100 mg/L of either nitrate or phosphate at pH 3, and stirring the mixture for 24 h at 25 °C. Subsequently, we determined the adsorption rates. The recyclability of the DWTR adsorbent underwent testing across five cycles. Each cycle involved a 24-h adsorption period at pH 3 and a 24-h desorption period at pH 11, both conducted at 25 °C.

### 2.2. Preparation of a modified medium (MM) using real wastewater (WW)

We formulated a Modified Medium (MM), wherein nitrate and phosphate retrieved from wastewater (WW) supplanted commercial sources of nitrogen and phosphorus. This wastewater, as detailed in Table 3, originated from a livestock wastewater treatment facility in Sancheong, Gyeongsangnam-do, Republic of Korea, and was sampled in July 2018. Initially, we enabled the adsorption of nitrate and phosphate ions onto 250 g of DWTR-CH (0.25 wt%) from 100 L of WW at pH 3, over a span of 2 h. Subsequently, these ions were desorbed into 3.4 L of deionized water over another 2 h, with the solution adjusted to pH 11. Following this, the pH of the resulting solution was adjusted to 9 to establish the MM. Aside from the integrated nitrate and phosphate, this medium preserved nutrient concentrations consistent with those in the Control Medium (referred to as CM and modeled on the Zarrouk medium). Table 4 presents the compositions of both the CM and MM, along with specifics on the retrieved nitrate and phosphate.

### 2.3. Production of cyanobacterial biomass

In this study, we employed *Spirulina platensis* (PS-0056) provided by the Library for Marine Samples (LIMS). Initially, the inoculum was cultured in Zarrouk medium and subsequently transferred to 500 mL baffled flasks for experimentation. Each flask contained 400 mL of cyanobacterial culture and was maintained at a temperature of 20 °C with a light intensity of 100 μmol/$m^2$/s. All experiments were conducted in triplicate to assess the effectiveness of the Modified Medium (MM) in producing cyanobacterial biomass, comparing its performance to the Control Medium (CM). Our evaluation criteria considered various factors, such as dry cell weight, maximum quantum yield, composition, and amino acid profile.

### 2.4. Toxicity testing

#### 2.4.1. In vitro toxicity test

We used a previous method [29] to prepare a hot water extract from the *S. platensis* biomass. Briefly, 10 g/L of dried biomass was autoclaved in distilled water at 120 °C for 1 h. After centrifugation and filtration, the supernatant was freeze-dried and reconstituted in DMEM to achieve a concentration of 1000 μg/mL. This solution was further diluted as required in DMEM for subsequent experiments. We employed a modified MTT assay to assess cell viability. RAW 264.7 cells were cultured in DMEM supplemented with 10 % FBS and antibiotics in a 5 % $CO_2$ incubator maintained at 37 °C. Cells, at a concentration of $5 \times 10^4$ cells/well, were seeded onto a 96-well plate and incubated for 3 h at 37 °C. Following this, the *S. platensis* extract was added, and the incubation continued for another 24 h under the same conditions. After this period, MTT solution was introduced and the plate was incubated for an additional 4 h at 37 °C. Once the formazan had dissolved, we measured the optical density at both 450 nm and 570 nm using a microplate reader.

#### 2.4.2. In vivo toxicity test

The acute oral toxicity test was conducted in accordance with OECD guideline 420 [30], using a limit test because of the anticipated non-toxic nature of the *S. platensis* biomass. Seven-week-old Sprague Dawley rats were first acclimatized for a week under automatically controlled conditions, which included temperature (20–27 °C), humidity (50 ± 20 %), and a 12:12 light-dark cycle. After acclimatization, a single dose of 2000 mg/kg body weight of the biomass was administered orally to both male and female rats (five each) on day 0. Subsequent to dosing, individual observations were made at specified intervals (0, 1, 2, 3, 4, and 24 h) on the first day, and daily thereafter for 14 days, to monitor for mortality and signs of toxicity. Body weights were recorded on days 0 (pre-dosing), 1, 7, and 14. At the conclusion of the observation period, all rats underwent a necropsy, which consisted of a detailed visual inspection of all organs, post-euthanasia by $CO_2$ inhalation. This experiment was conducted in compliance with the guidelines set by the Korea Institute of Toxicology's IACUC (Institutional Animal Care and Use Committee) (AEC-20121211-0002), and the study was approved by the Animal Care and Use Protocol (Approval no. 1807-0004).

**Table 2**
XRF data of chemical composition of DWTR-nitrogen.

| Element/Oxide | Composition (wt. %) | Element/Oxide | Composition (wt. %) |
|---|---|---|---|
| $Al_2O_3$ | 52.60 | $K_2O$ | 1.45 |
| $SiO_2$ | 24.30 | MnO | 1.04 |
| $Fe_2O_3$ | 9.93 | $TiO_2$ | 0.53 |
| $SO_3$ | 4.20 | Br | 0.0618 |
| Cl | 0.35 | CuO | 0.122 |
| $P_2O_5$ | 1.30 | ZnO | 0.141 |
| CaO | 2.97 | SrO | 0.03 |

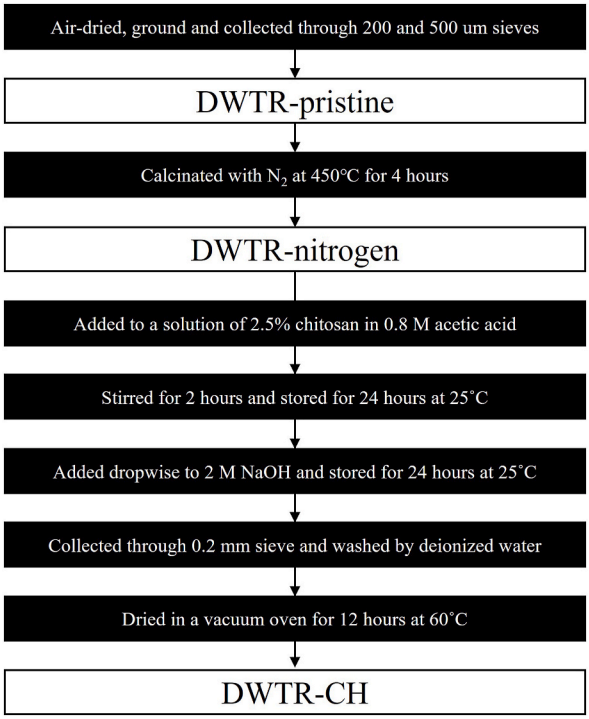


**Fig. 1.** Synthesis procedure of DWTR based adsorbents used in this study.

**Table 3**
Physicochemical properties of nitrification tank effluent of livestock wastewater treatment process.

| Component | | Component | |
|---|---|---|---|
| T-N (mg/L) | 297.7 | BOD (mg/L) | n.a. |
| T-P (mg/L) | 27.9 | Zn (mg/L) | 0.357 |
| $NH_3$ (mg/L) | 3.9 | Cu (mg/L) | 0.076 |
| $NO_2$ (mg/L) | 0.039 | Mn (mg/L) | 0.368 |
| $NO_3$ (mg/L) | 1029.6 | Cd (mg/L) | N.D. |
| $PO_4$ (mg/L) | 59.5 | Pb (mg/L) | N.D. |
| Organic phosphorus (mg/L) | n.d. | Fe (mg/L) | 1.205 |
| Color (degree) | 872 | Al (mg/L) | 0.05 |
| Hardness (mg/L) | 877 | F (mg/L) | 0.47 |
| Suspended solids (mg/L) | 11,546 | $Cl^-$ (mg/L) | 3456.1 |
| Total organic carbon (mg/L) | 286.7 | ABS (mg/L) | n.d. |
| $COD_{cr}$ (mg/L) | 598.6 | $SO_4$ (mg/L) | 390.5 |

Abbreviations: $COD_{Cr}$, chemical oxygen demand based on dichromate analysis; BOD, biological oxygen demand; T-N, total nitrogen, T-P, total phosphorus; ABS, anionic biosurfactant; n.a., not available; n.d., not detected.

**Table 4**
Initial nitrate and phosphate concentrations in the modified medium (MM) and control medium (CM).

| Component | MM | CM |
|---|---|---|
| $NO_3$ (mg/L) | 282.5 ± 1.4 | 2001.4 ± 3.7 |
| $PO_4$ (mg/L) | 180.0 ± 4.5 | 252.0 ± 3.0 |

### 2.5. Analytical methods

In this study, we used ion chromatography (930 Compact IC, Metrohm AG, Switzerland) to determine the nitrate and phosphate concentrations in filtered samples. The dry cell weight was estimated indirectly through spectrophotometry (UV-1800, Shimadzu, Japan) at 680 nm using equation $0.8722 \times OD_{680} - 0.2385$. The maximum quantum yield of photosystem II ($F_v/F_m$) was measured using an Aquapen C-100 (PSI, Czech Republic) after dark adaptation to ensure equilibrium in photosystem II. After cultivation, samples of

cyanobacterial biomass were centrifuged and subsequently lyophilized for analysis. To determine carbohydrates [31], resuspend 5–10 mg of dry biomass in 10 ml of water, sonicate for 10 min, then add 1 ml of 5 % phenol and 5 ml of sulfuric acid. Vortex the mixture and let it sit for 30 min. Measure the absorbance at 490 nm using a dextrose calibration curve ranging from 0 to 0.1 mg/ml. For protein determination [32], resuspend 5–10 mg of biomass in 1–5 ml of water, sonicate, then mix with sodium hydroxide. Heat the mixture, allow it to cool, and measure the absorbance at 750 nm using a bovine serum albumin calibration curve ranging from 0 to 1 mg/ml. To measure lipids [33], resuspend 5–10 mg of biomass in water, add sulfuric acid, heat, cool, then add phospho-vanillin reagent. Incubate and measure the absorbance at 530 nm using a canola oil calibration curve ranging from 0 to 0.8 mg/ml. The amino acid profile was assessed with an amino acid analyzer (Skyam S7130, Germany) equipped with UV-VIS detectors set at 400 nm and 570 nm [34].

## 3. Results and discussion

### 3.1. The impact of drinking water treatment residuals (DWTR) granulation on nitrate and phosphate adsorption

While most research has emphasized the direct application of drinking water treatment residuals (DWTR) in powder or cake form, there are considerable challenges associated with their recovery and reuse [35]. As such, identifying an effective granulation method is critical for the sustainable usage of DWTR. In this study, we examined the adsorption efficiencies of nitrate and phosphate across various DWTR-based adsorbents, as presented in Table 5. We observed a higher adsorption capacity for trivalent phosphate ions compared to monovalent nitrate ions in all investigated DWTR-based adsorbents. Following calcination under nitrogen, adsorption efficiencies increased approximately 3.1 times for nitrate and 1.1 times for phosphate at pH 3. Given the high content of $Al_2O_3$ and $Fe_2O_3$ in DWTR, which offer nitrate and phosphate adsorption sites, the increased adsorption could be attributed to an expanded surface area after calcination. This is in line with a previous study [36] that revealed enhanced adsorption kinetics due to a larger surface area achieved through calcination.

To capitalize on these increased adsorption efficiencies, we conducted granulation using a mixture of DWTR-nitrogen and chitosan. Chitosan is a biopolymeric adsorbent frequently used for pollutant removal due to its biocompatibility, non-toxicity, easy modification, and cost-effective production [37]. Although chitosan can exhibit weak mechanical and thermal stability, these limitations can be offset by modifying it with metals or clays [37,38]. We incorporated varying concentrations of DWTR-nitrogen into chitosan (ranging from 0.25 to 2.5 wt%) to produce DWTR-CH. Our findings indicated the highest nitrate adsorption efficiency at a 0.25 wt% concentration, which resulted in 3.8 and 1.2 times more nitrate adsorption compared to DWTR-pristine and DWTR-nitrogen, respectively. This could be attributed to the protonation of chitosan's amine functional group under acidic pH conditions, creating a positive charge that promotes nitrate adsorption through electrostatic interactions [39]. As the DWTR concentration in DWTR-CH increased (from 0.25 to 2.5 wt%), we observed a corresponding decrease in adsorbed nitrate. This suggests that a reduction in active sites on chitosan outweighs the increase in active sites on DWTR. Regarding phosphate adsorption, DWTR-nitrogen outperformed DWTR-CH, likely due to the coating of the active sorption site on DWTR [40]. Similarly, with nitrate, phosphate adsorption decreased as the incorporated concentration of chitosan in DWTR-CH increased, attributable to the diminishing active sites on DWTR.

To assess DWTR-CH's sustainability, we examined its recyclability at a 0.25 wt% concentration. During the second to fifth consecutive adsorption-desorption cycles, as depicted in Fig. 2, the average adsorption capacities for nitrate and phosphate consistently remained around 95 % and 96 %, respectively. This indicates that the DWTR-CH's morphology and surface properties endured despite multiple uses, making DWTR-CH at a 0.25 wt% concentration a promising candidate for further studies owing to its superior adsorption and recyclability.

### 3.2. Growth of S. platensis using recovered wastewater nutrients

The growth responses of *S. platensis* in the control medium (CM, Zarrouk medium) and the modified medium (MM) are depicted in Fig. 3a–d and were observed to be comparable throughout the culture period. A cyanobacterial biomass productivity of 0.15 g/L/d was attained in both media after five days of cultivation (Fig. 3a). In the initial three days, nitrate assimilation in both media was fairly consistent, at 311 mg/L in CM and 272 mg/L in MM (Fig. 3b). However, given the lower starting nitrate concentration in MM, it was depleted after this period. The phosphate assimilation showed no significant difference between the two media, with levels recorded at 22 mg/L in CM and 20 mg/L in MM, respectively (Fig. 3c). The quantum yield of the cyanobacterial culture, indicative of photosynthetic efficiency, was assessed, revealing no significant variations in both media, with CM and MM ranging from 0.18 to 0.32 and

**Table 5**
Nitrate and phosphate adsorption capacities using adsorbents derived from drinking water treatment residuals (DWTR).

| Adsorbent | Adsorption capacity (mg/g) | |
|---|---|---|
| | Nitrate | Phosphate |
| DWTR-pristine | 1.16 ± 0.03 | 8.93 ± 0.03 |
| DWTR-nitrogen | 3.62 ± 0.05 | 9.76 ± 0.05 |
| DWTR-CH (0.25 wt%) | 4.44 ± 0.11 | 6.08 ± 0.21 |
| DWTR-CH (1.25 wt%) | 2.32 ± 0.03 | 4.22 ± 0.1 |
| DWTR-CH (2.5 wt%) | 1.81 ± 0.03 | 3.73 ± 0.07 |

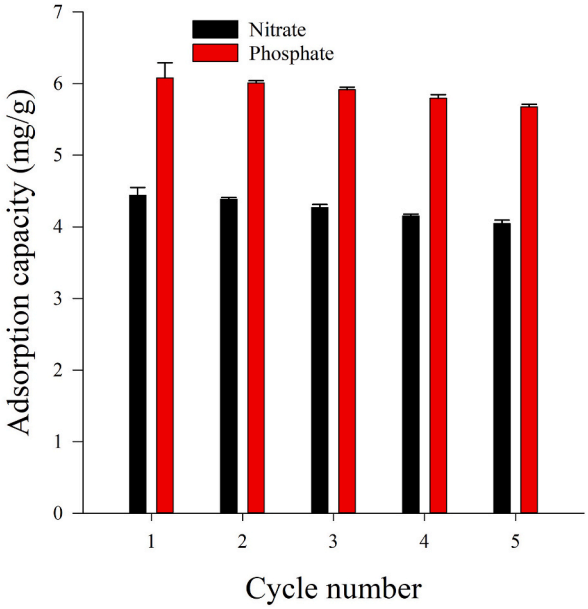


**Fig. 2.** The recyclability of DWTR-CH at a concentration of 0.25 wt% for the adsorption of nitrate and phosphate.

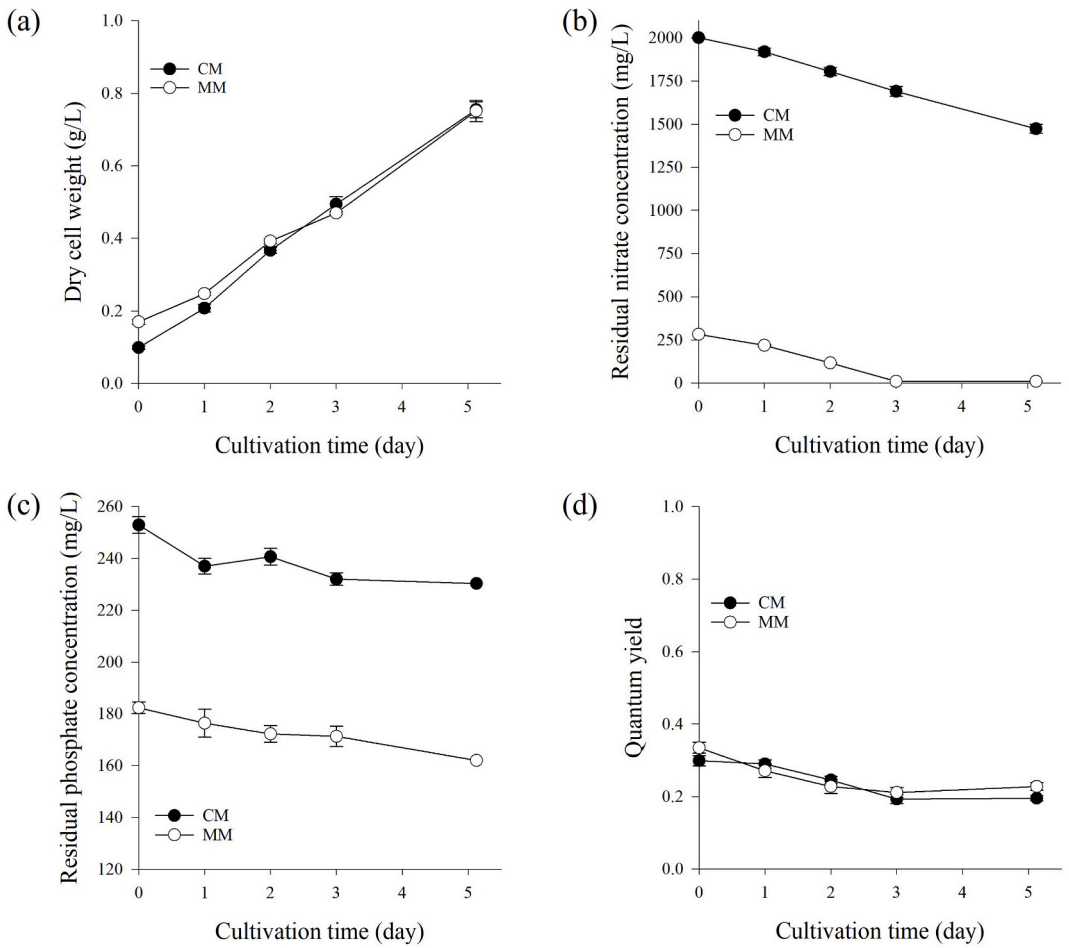


**Fig. 3.** Growth curve (a), nitrate removal (b), phosphate removal (c), and quantum yield (d) of *S. platensis* cultivated in both MM and CM.

0.18 to 0.35, respectively (Fig. 3d). These observations imply that employing MM, which is formulated with nitrate and phosphate from wastewater (WW), does not detrimentally impact the photosynthetic activity of the cyanobacterial culture.

### 3.3. Potential of *S. platensis* biomass from modified medium (MM) as animal feed

We evaluated in vitro toxicity using a hot water extract of *S. platensis* cultivated in MM. The viability of cells from the *S. platensis* extracts cultured in MM paralleled those from CM, with no observed cytotoxicity at any treatment concentrations, as depicted in Fig. 4. In vivo toxicity was subsequently investigated via a limit test on *S. platensis* grown in MM, involving both male and female rats. Following oral administration, neither physical nor behavioral changes, nor mortality, were observed (Table 6). While significant weight loss typically signals declining animal health [41,42], the rats exhibited normal weight gain over a 14-day period, showing no signs of health deterioration (as detailed in Table 7). An examination of the internal organs revealed no abnormalities, suggesting an absence of toxicity (refer to Table 8). A single-dose oral administration of 2000 mg-biomass/kg-body weight resulted in no acute toxicity, classifying *S. platensis* biomass as either unclassified or category 5 under the globally harmonized system of classification and labeling of chemicals (GHS), in accordance with OECD Guideline 33 [43]. This suggests that *S. platensis* biomass, cultivated using nutrients recovered from WW, exhibits minimal acute toxicity and doesn't manifest any specific organ or systemic toxicity.

In addition to ensuring safe cyanobacterial biomass production, the cost-effectiveness of the produced biomass is crucial for the successful scale-up and real-world application of our approach. As detailed in Table 9, we have itemized the costs associated with producing DWTR-CH, taking into account electricity and materials. The cost stands at $21.89 per kg, with chitosan being the primary expense, followed by electricity and chemicals. When reused (maintaining 95 % efficiency for five cycles), the cost drops to $4.61 per kg. Thus, reusability is essential for enhancing affordability. We also computed the cost of MM, as shown in Table 9. Comparing the cost of CM ($14.82 per 3.4L) with that of MM reveals an approximate medium cost reduction of 18 %. In the CM (Zarrouk medium), nitrate constitutes 16 %, and phosphate 10 %, of the overall medium cost. Notably, nitrate and phosphate rank among the priciest components in various cultivation media. For example, in the WC, CHU, and BG-11 media, the cost percentages for nitrate are 33.1 %, 22.1 %, and 83.3 %, respectively. Meanwhile, phosphate's cost in CHU is 31 % [44]. This indicates that the potential for cost savings through our strategy fluctuates based on the specific culture medium in use. Typically, media costs account for between 20 % and 50 % of the total biomass production costs, contingent upon the target species and medium types [44,45]. Given that our strategy can potentially trim medium costs by 18 %, this can lead to an overall production cost savings of 4–9 %. Although our approach shows promise, it does pose challenges, particularly when juxtaposed against the direct use of wastewater for cultivating microalgae or cyanobacteria, wherein the medium cost is virtually nil. One avenue to heighten the cost-effectiveness of our method lies in identifying adsorbents adept at ensnaring a wider variety of nutrients, such as carbonate (which converts to bicarbonate upon merging with carbon dioxide) and calcium. This is paramount since bicarbonate and calcium together contribute to roughly 60 % of the Zarrouk medium's culture cost and 25 % in the WC medium [44]. Moreover, strategies like employing $CO_2$ and slaked lime for pH regulation or replenishing used-up nutrients to reuse the culture medium can further augment the cost-effectiveness and scalability of our methodology.

As shown in Table 10, the carbohydrate content of *S. platensis* grown in both MM and CM was fairly similar, registering at 6.9 % and 7.3 %, respectively. The lipid content of *S. platensis* cultivated in MM was double that found in CM, suggesting a potential nitrogen

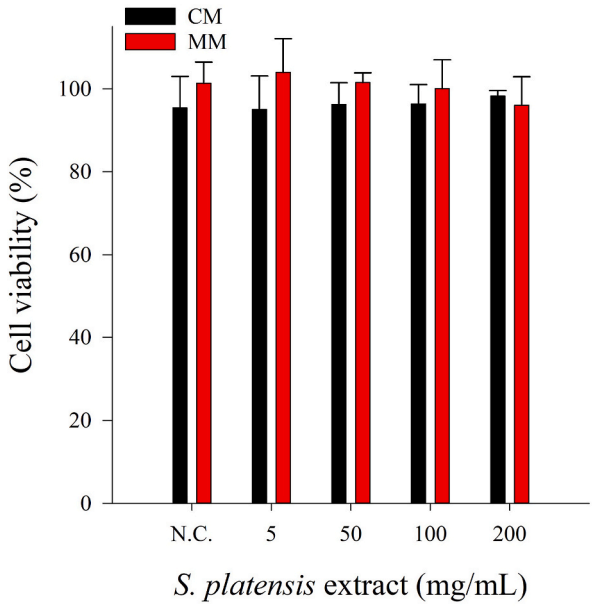


**Fig. 4.** Cell viability of the hot water extract of *S. platensis* cultivated in both MM and CM.

**Table 6**
The clinical observations of the test animals.

| General symptom observations | Limit test (2000 mg/kg) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Male | | | | | Female | | | | |
| | Rat 1 | Rat 2 | Rat 3 | Rat 4 | Rat 5 | Rat 1 | Rat 2 | Rat 3 | Rat 4 | Rat 5 |
| Changes in skin and fur | Absent | Absent | Absent | Absent | Absent | Absent | Absent | Absent | Absent | Absent |
| Changes in eyes and mucous membrane | Absent | Absent | Absent | Absent | Absent | Absent | Absent | Absent | Absent | Absent |
| Changes in behaviour pattern | Absent | Absent | Absent | Absent | Absent | Absent | Absent | Absent | Absent | Absent |
| Tremors and convulsions | Absent | Absent | Absent | Absent | Absent | Absent | Absent | Absent | Absent | Absent |
| Salivation | Absent | Absent | Absent | Absent | Absent | Absent | Absent | Absent | Absent | Absent |
| Diarrhoea | Absent | Absent | Absent | Absent | Absent | Absent | Absent | Absent | Absent | Absent |
| Death | No | No | No | No | No | No | No | No | No | No |

deficiency [46]. After three days of cultivation in MM, the nitrogen concentration was almost exhausted, primarily because the initial nitrate concentration in MM was eight times lower than in CM (refer to Table 4). This issue could be potentially addressed by either augmenting the initial concentration in CM using wastewater with higher nitrogen content or by diminishing the volume of the desorption solution. The protein content in MM and CM was 50 % and 57 %, respectively. Notably, despite the nitrogen depletion, the protein content in MM was on par with or even surpassed figures reported in prior studies using food processing wastewater [47,48].

As mentioned before, many parts of microalgae and cyanobacteria can be useful ingredients for animal food. In our study, we focused on making protein because our chosen example, *S. platensis*, is rich in it. Based on the amino acid composition (Table 11), the overall free amino acid content in MM-grown *S. platensis* biomass was about 17 % lower than that in CM, primarily due to nitrogen depletion in MM. However, despite the different nitrogen sources—wastewater for MM and reagent for CM—the amino acid pattern remained consistent between MM and CM [49]. Exceptions included glutamic acid, alanine, methionine, and tryptophan, which did exhibit changes. The concentrations of glutamic acid, alanine, and methionine declined by more than 5 percentage points, while the concentration of tryptophan rose by over 5 % in the *S. platensis* biomass grown in MM. These changes could be attributed to metabolic alterations related to nitrogen depletion. In the case of *S. obliquus* BR003, ornithine decreased and tryptophan increased when exposed to nitrogen-depleted conditions for 36 h of cultivation [50]. The contents of threonine and tryptophan decreased, while the glycine content increased during nitrogen limitation in *P. tricornutum*, possibly due to changes in catabolic pathways [51].

Feedstocks abundant in methionine and lysine are known to augment the dimensions of chicken breast and thigh muscles, enhancing both the quality and quantity of the meat [2]. Concurrently, histidine plays a vital role in aquaculture, affecting both the growth of fish and the structural integrity of their gills [27,52]. Al-Dhabi and Arasu [53] documented a variance in total free amino acid content, ranging between 11 and 56 mg/100 g across 37 commercial *Spirulina* products, with the essential amino acid content fluctuating between 2 and 32 mg/100 g. Remarkably, despite its potentially lower protein productivity, the *S. platensis* biomass cultivated in MM mirrored the essential amino acid content found in mainstream protein sources like eggs, milk, and soy proteins, with the exception of cysteine [54]. This suggests that the *S. platensis* biomass harvested from MM, which contains 560 mg/100 g (in free form) of all ten essential amino acids, holds promise for utilization in both the food and feed sectors.

## 4. Conclusion

Unlike prior studies that used raw Wastewater (WW), our research stands out with a distinctive strategy: we extracted and concentrated specific nutrients, notably nitrate and phosphate, from WW. This allowed for a controlled and safer cultivation of *Spirulina platensis*. The effectiveness of our method is underscored by nutrient assimilation rates similar to those achieved with conventional commercial sources. A key highlight of our work is the quality of the *S. platensis* biomass derived from these reclaimed nutrients. Our results indicate this biomass is not only free from toxins but also protein-rich, boasting an essential amino acid profile. This makes it a strong contender for animal feed, suitable for diverse livestock and fish species. Our study goes beyond merely addressing concerns over using WW in biomass production. It underscores the broader potential of repurposing waste materials. However, our work is not without its challenges. Future refinement is needed in areas like developing improved adsorbents and refining our extraction methods. By optimizing these aspects, we aim to make our approach both cost-effective and scalable, ensuring it transitions from a scientific concept to a widely-adopted practice.

## Author contribution statement

Seung Won Nam: Software, Resources. Seong-Hoon Park: Writing – review & editing, Funding acquisition. Seng-Min Back: Resources, Formal analysis. Byung-Gon Ryu: Resources, Formal analysis. Je-Hein Kim: Resources, Formal analysis. Won Noh: Methodology, Investigation, Formal analysis. Yeong Jin Kim: Validation, Resources, Formal analysis. Seonghwan Park: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. Sang-Jun Lee: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. Jungmin Kim: Writing – original draft, Supervision, Methodology, Investigation, Conceptualization

**Table 7**
The body weights of the test animals.

| Changes in body weights | Limit test (2000 mg/kg) | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Male | | | | | | | Female | | | | | | |
| | Rat 1 | Rat 2 | Rat 3 | Rat 4 | Rat 5 | Mean | SD | Rat 1 | Rat 2 | Rat 3 | Rat 4 | Rat 5 | Mean | SD |
| day 0 (g) | 233.3 | 217.1 | 232.1 | 221.2 | 223.7 | 225.5 | 7.0 | 168.9 | 160.7 | 162.6 | 160.2 | 152.8 | 161.0 | 5.8 |
| day 1 (g) | 258.5 | 242.2 | 258.6 | 248.1 | 245.8 | 250.6 | 7.5 | 184.1 | 178 | 181.1 | 186.6 | 173.5 | 180.7 | 5.1 |
| day 7 (g) | 308.3 | 287 | 313 | 298.8 | 289.4 | 299.3 | 11.4 | 208.1 | 198.5 | 204.5 | 201.1 | 197.8 | 202.0 | 4.3 |
| day 14 (g) | 354.1 | 318.2 | 347.3 | 344.2 | 334.8 | 339.7 | 13.9 | 230.6 | 218.2 | 216.6 | 215.5 | 215.1 | 219.2 | 6.5 |
| Weight gain (g) | 120.8 | 101.1 | 115.2 | 123 | 111.1 | 114.2 | 8.7 | 61.7 | 57.5 | 54 | 55.3 | 62.3 | 58.2 | 3.7 |
| Percentage of weight gain (%) | 51.8 | 46.6 | 49.6 | 55.6 | 49.7 | 50.7 | 3.3 | 36.5 | 35.8 | 33.2 | 34.5 | 40.8 | 36.2 | 2.9 |

**Table 8**
The gross pathological findings of the test animals.

| Gross pathological findings | Limit test (2000 mg/kg) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Male | | | | | Female | | | | |
| | Rat 1 | Rat 2 | Rat 3 | Rat 4 | Rat 5 | Rat 1 | Rat 2 | Rat 3 | Rat 4 | Rat 5 |
| Abnormality of appearance | No | No | No | No | No | No | No | No | No | No |
| Abnormality of abdominal cavity | No | No | No | No | No | No | No | No | No | No |
| Abnormality of chest cavity | No | No | No | No | No | No | No | No | No | No |
| Abnormality of cranial cavity | No | No | No | No | No | No | No | No | No | No |

**Table 9**
Cost analysis for DWTR-CH production and modified medium preparation.

| Unit cost | Item | | Cost | Note |
|---|---|---|---|---|
| | Electricity | | 0.10 USD/kWh | South Korea, Business in Dec. 2022 |
| | Chitosan | | 15.00 USD/kg | Standard quality |
| | Acetic acid | | 0.47 USD/kg | Northeast Asia in Aug. 2023 |
| | NaOH | | 0.57 USD/kg | 570 USD/ton in May 2023 |
| | HCl | | 0.02 USD/kg | Northeast Asia in Aug. 2023 |
| Production of DWTR-CH bead (1 kg) | Item | Amount | Cost | Note |
| | Furnace | 40.00 kWh | 3.96 USD | 4 h |
| | Mixing | 0.68 kWh | 0.07 USD | 2 h for acetic acid and 2 h for NaOH |
| | Vacuum oven | 15.31 kWh | 1.52 USD | 12 h |
| | DW | 33.55 L | 0.19 USD | |
| | Chitosan | 0.91 kg | 13.64 USD | |
| | Acetic acid | 1.82 kg | 0.85 USD | |
| | NaOH | 2.93 kg | 1.67 USD | |
| | DWTR | 0.09 kg | 0 USD | From wastewater facility |
| | Total | | 21.89 USD/kg·DWTR-CH | |
| | | | **4.61** USD/kg·DWTR-CH | Assuming 5 times reuse with 95 % efficiency |
| Modified medium (MM) (3.4 L) | Item | Amount | Cost | |
| | WW | 100 L | 0 USD | From wastewater facility |
| | DWTR-CH | 0.25 kg | 1.15 USD | Assuming 5 times reuse with 95 % efficiency |
| | HCl | 0.01 kg | 0.0002 USD | |
| | NaOH | 0.004 kg | 0.002 USD | |
| | DW | 3.51 L | 0.02 USD | |
| | Mixing | 0.68 kWh | 0.07 USD | 2 h for adsorption, 2 h for desorption |
| | Other nutrients | | 10.89 USD | Except for nitrate and phosphate |
| | Total | | **12.13** USD/3.4L·MM | |

**Table 10**
Composition of *S. platensis* biomass grown in modified medium (MM) and control medium (CM).

| Medium | Biomass composition (% of dry cell weight) | | |
|---|---|---|---|
| | Carbohydrate | Protein | Lipid |
| MM | 6.86 ± 0.42 | 50.44 ± 1.13 | 17.29 ± 0.65 |
| CM | 7.27 ± 0.68 | 57.16 ± 0.76 | 8.53 ± 0.49 |

## Ethics statement

This study was approved by Korea Institute of Toxicology's IACUC (Institutional Animal Care and Use Committee), with the approval number: AEC-20121211-0002, and the Animal Care and Use Protocol, with the approval number: 1807-0004.

## Data availability statement

Data will be made available on request.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

**Table 11**
Free amino acid profile of *S. platensis* cultivated in modified medium (MM) and control medium (CM).

| Amino acid | Amino acid content (mg/100 g of dry cell weight) | | | |
|---|---|---|---|---|
| | MM | | CM | |
| Phospho-L-serine | 166.58 ± 33.11 | (13.24) | 182.00 ± 10.56 | (12.00) |
| Taurine | 1.15 ± 1.07 | (0.09) | 0.81 ± 1.40 | (0.05) |
| Phosphoethanolamine | 21.21 ± 1.84 | (1.69) | 23.63 ± 2.47 | (1.56) |
| Urea | 17.42 ± 30.17 | (1.38) | 0.00 | (0.00) |
| Aspartic Acid | 19.49 ± 0.08 | (1.55) | 21.92 ± 0.53 | (1.45) |
| Hydroxy proline | n.d. | (0.00) | n.d. | (0.00) |
| Threonine[a] | 47.12 ± 4.50 | (3.75) | 46.42 ± 3.22 | (3.06) |
| Serine | 44.04 ± 0.69 | (3.50) | 53.42 ± 0.34 | (3.52) |
| Asparagine | 35.44 ± 3.08 | (2.82) | 15.94 ± 1.72 | (1.05) |
| Glutamic acid | 60.85 ± 0.66 | (4.84) | 148.77 ± 0.58 | (9.81) |
| L-Glutamine | n.d. | (0.00) | n.d. | (0.00) |
| Theanine | n.d. | (0.00) | n.d. | (0.00) |
| α-Aminoadipic Acid | n.d. | (0.00) | 1.28 ± 2.21 | (0.08) |
| Proline | 28.36 ± 2.84 | (2.25) | 33.19 ± 0.56 | (2.19) |
| Glycine | 23.60 ± 0.26 | (1.88) | 31.81 ± 0.44 | (2.10) |
| Alanine | 177.35 ± 0.82 | (14.10) | 288.32 ± 2.98 | (19.02) |
| Citrulline | n.d. | (0.00) | n.d. | (0.00) |
| α -Aminobutyyrid acid | n.d. | (0.00) | n.d. | (0.00) |
| Valine[a] | 52.72 ± 2.00 | (4.19) | 47.35 ± 1.95 | (3.12) |
| Cystine | n.d. | (0.00) | n.d. | (0.00) |
| Methionine[a] | 38.95 ± 2.76 | (3.10) | 135.16 ± 117.06 | (8.91) |
| lsoleucine[a] | 44.35 ± 0.38 | (3.53) | 37.87 ± 0.32 | (2.50) |
| Leucine[a] | 116.53 ± 0.23 | (9.26) | 103.49 ± 0.97 | (6.83) |
| Tyrosin | 60.88 ± 0.62 | (4.84) | 43.55 ± 1.19 | (2.87) |
| Phenyalanine[a] | 64.70 ± 1.21 | (5.14) | 63.77 ± 0.48 | (4.21) |
| β-Alanine | n.d. | (0.00) | n.d. | (0.00) |
| β-Aminoisobutyric acid | n.d. | (0.00) | n.d. | (0.00) |
| γ-Aminobutyric acid | 10.27 ± 0.23 | (0.82) | 10.68 ± 1.13 | (0.70) |
| Histidine[a] | 16.78 ± 0.18 | (1.33) | 11.23 ± 0.61 | (0.74) |
| 3-Methylhistid | n.d. | (0.00) | 0.29 ± 0.51 | (0.02) |
| 1-Methylhistid | 1.61 ± 2.80 | (0.13) | 17.49 ± 2.76 | (1.15) |
| Tryptophan[a] | 75.15 ± 28.80 | (5.97) | 13.69 ± 6.31 | (0.90) |
| Ornithine | 5.42 ± 0.40 | (0.43) | 16.79 ± 0.31 | (1.11) |
| Lysine[a] | 40.32 ± 0.12 | (3.20) | 59.10 ± 0.51 | (3.90) |
| Ammonia | 23.22 ± 1.40 | (1.85) | 38.27 ± 1.49 | (2.52) |
| Arginine[a] | 64.44 ± 0.82 | (5.12) | 70.05 ± 0.97 | (4.62) |
| Total | 1257.95 ± 74.77 | (100.00) | 1516.27 ± 95.99 | (100.00) |

n.d., not detected.
Data in parentheses indicate the percentage of each amino acid relative to the total amount.
[a] Essential amino acids.

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