Eurasian Journal of Physics and Functional Materials

Volume 9 | Number 3

Article 6

Pages: 1 - 1

2025

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## Recommended Citation

Bekbayev, Kairat S.; Melissova, Aknur A.; Kuterbekov, Kairat A.; Bekmyrza, Kenzhebatyr Zh.; and Kabyshev, Asset M. (2025) "OPTIMIZATION OF BIOHYDROGEN SYNTHESIS CONDITIONS FROM SUGAR PRODUCTION WASTE," *Eurasian Journal of Physics and Functional Materials*: Vol. 9: No. 3, Article 6.
DOI: https://doi.org/10.69912/2616-8537.1256

**REVIEW**

# Optimization of Biohydrogen Synthesis Conditions From Sugar Production Waste

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**Abstract**

**This study aims to optimize the conditions for biohydrogen synthesis from beet molasses using the dark fermentation method. To achieve this, a Central Composite Design and Response Surface Methodology were applied, which allow for identifying the relationship between different process parameters and their influence on synthesis efficiency. The experiment investigated key factors such as beet molasses concentration, sulfuric acid ($H_2SO_4$) concentration, and fermentation duration. Response Surface Methodology enabled the determination of optimal conditions for achieving maximum biohydrogen yield, based on indicators such as pH and the redox potential of the substrate. The results showed that the highest biohydrogen yield could be achieved with a beet molasses concentration of 7 %, an $H_2SO_4$ concentration of 0,75 %, and pH adjustment using $K_2PO_4$. These parameters ensure the most efficient and prolonged biohydrogen synthesis process. The study concludes that careful control of these factors could significantly improve biohydrogen yields, open up new prospects for more efficient use of sugar beet molasses as a feedstock for clean energy production, and promote the development of sustainable energy technologies.**

***Keywords:*** Biohydrogen synthesis, Optimization, Central composite design, Response surface methodology

## 1. Introduction

In recent decades, renewable energy sources have been attracting increasing attention due to global challenges related to climate change and the depletion of fossil resources. One promising direction is biohydrogen - a clean fuel derived from organic materials [1,2]. Molasses, a by-product of the sugar industry, represents a rich source of carbon that can be effectively used for biohydrogen synthesis. The results of this work highlight the potential of molasses as a raw material for sustainable biohydrogen production and can serve as a basis for further research in this field.

Optimization of molasses fermentation can increase its industrial value and reduce negative environmental impact [3].

Modern biotechnological productions require highly efficient processes that allow for maximum yield of target products with minimal costs [4,5]. The growing interest in renewable energy sources and biochemical processes makes the task of efficient use of raw materials extremely relevant [6].

The application of Response Surface Methodology (RSM) and Central Composite Design (CCD) for molasses fermentation represents an innovative approach compared to traditional experimental planning methods. This allows not only to reduce the number of experiments but also to obtain more accurate results, making the research particularly relevant in conditions of limited resources and the need to increase productivity.

Many studies have looked at the molasses fermentation process using surface response methodology

Received 19 August 2025; accepted 25 August 2025.
Available online 7 October 2025

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*https://doi.org/10.69912/2616-8537.1256*

(RSM), but with different feedstocks and different objective functions. Hawaz et al. [5] applied RSM to optimize the conditions for bioethanol production using Meyerozyma caribbica strain [5]. CCD helped to identify important variables such as molasses concentration, pH, and fermentation time. Beigbeder et al. [6] investigated the optimal yeast, sugar, and nutrient concentrations to increase the ethanol production rate using beet molasses using CCD and RSM to analyze the relationships between variables [6]. Abdel-Rahman et al. [7] used RSM to optimize lactic acid production from beet molasses using Enterococcus hirae, showing the importance of sugar and nutrient content to maximize the product yield [7]. Application of the methodology of Altõnõşõk et al. [8] response surface modeling (RSM) and central composite planning improved fermentation conditions, resulting in high ethanol yields [8]. The work considered various combinations of variables such as free sugar concentration and pH of the medium.

The study by Cruz et al. [9] investigated the fermentation process under very high gravity conditions using a mixture of cane juice and molasses [9]. Response surface methodology (RSM) and central composite design (CCD) were used to optimize cell concentration and other variables, resulting in increased ethanol yield. It was shown that the combination of cane juice and molasses under high gravity conditions effectively improved the overall fermentation productivity. Papizadeh et al. [10] investigated experimental design approaches to increase the cell density of the probiotic strain Lactobacillus plantarum in cane molasses-based medium [10]. Using response surface methodology (RSM) and central composite design (CCD), the fermentation process was optimized and the yield of lactic acid and cellular biomass was significantly increased. The study by Saavedra et al. [11] focused on the optimization of lactic acid production using Lactobacillus plantarum strain in cane molasses-based [11]. The authors used central composite design (CCD) to study the effect of sugar concentration, medium pH, and fermentation time on lactic acid yield. It was shown that the use of RSM was effective in improving fermentation. The study by Farimani et al. [12] focused on the optimization of bioethanol production from sweet sorghum bagasse using RSM [12]. Surface response methodology and central composite design (CCD) helped in identifying the optimal fermentation conditions to enhance the yield of reducing sugars and hence bioethanol. The study by Amiri et al. [13] investigated the optimization of bacterial cellulose production in kombucha culture using cane molasses [13]. The application of the central composite design (CCD) and response surface methodology (RSM) allowed the identification of the optimal conditions for maximum pulp production and reduction of residual sugars.

All the studies reviewed highlight the importance of using response surface methodology (RSM) and central composite design (CCD) to optimize fermentation processes to increase the yield of target products from various types of carbohydrate-containing feedstocks. Optimization of key parameters such as sugar concentration, pH, and fermentation duration can significantly improve the overall productivity of biotechnological processes.

The aim of this study is to optimize the dark fermentation conditions of molasses for biohydrogen production using the response surface method (RSM). The main focus was on the influence of key factors such as molasses concentration, $H_2SO_4$ concentration and fermentation time on pH, oxidation-reduction potential (ORP) and optical density (OD) of the substrate to study the optimal conditions for biohydrogen synthesis.

Objectives of the study: to study the effect of molasses concentration on oxidation-reduction potential; to study the effect of selected factors on pH; to study the effect of selected factors on optical density (OD); to use the response surface method (RSM) to analyze nonlinear effects of factors and interactions between them, as well as to identify optimal fermentation conditions.

Coding levels were set for each factor:

| Factor | -α (Star points) | −1 (Low level) | 0 (Central level) | +1 (High level) | +α (Star points) |
|---|---|---|---|---|---|
| Molasses concentration (%) | 2 % | 4 % | 7 % | 10 % | 12 % |
| Concentration of $H_2SO_4$ (%) | 0.5 % | 0.75 % | 1.125 % | 1.5 % | 1.75 % |
| pH adjustment method | KOH | KOH | – | $K_2HPO_4$ | $K_2HPO_4$ |
| Duration (hours) | 0 | 24 | 48 | 72 | 96 |

Optimization of conditions for the synthesis of biohydrogen from industrial waste contributes to the development of sustainable economic principles, supporting global trends towards the use of renewable resources and innovative technologies.

## 2. Experimental part

### 2.1. Materials and methods

Raw materials: beet molasses (hereinafter referred to as molasses) used in this study was obtained from Taraz Sugar Plant LLP (RK, Taraz). The amount of dry matter in beet molasses was 65.11 %, of which 57 % were carbohydrates.

Beet molasses was subjected to acid–hydrothermal pretreatment in the presence of diluted sulfuric acid using an autoclave at 121 °C (1.1 MPa) for 20 min. After treatment, the hydrolysate was clarified by sequential filtration and centrifugation at 4000 rpm for 15 min to remove suspended solids and impurities.

The carbohydrate composition of beet molasses and its hydrolysates was determined by high-performance liquid chromatography (HPLC, Agilent 1200 Series) equipped with a refractive index detector. Organic acids were quantified using capillary electrophoresis.

Prior to dark fermentation, the pH of the medium was adjusted to 7.5 with KOH and $K_2HPO_4$. pH calibration and monitoring during fermentation were performed with a HI 3220 pH meter (Hanna Instruments, Portugal) equipped with a pH-selective electrode.

For inoculum preparation, wild-type Escherichia coli BW25113 was cultivated under anaerobic conditions in peptone medium (20 g/L peptone, 2 g/L $K_2HPO_4$, 5 g/L NaCl) at 37 °C for 18–20 h. The genotype of the strain was lacl q rrnB T14 Δ lacZ W116 hsdR514 ΔaraBAD AH33 Δ rhaBAD LD78.

Pretreated substrates were inoculated with the prepared bacterial cultures. Dark fermentation was conducted in sealed 500 mL flasks at 37 °C.

The overall hydrogen yield was quantified in a laboratory-scale setup employing 500 mL glass reactors under continuous stirring.

Design of experiments: A central composite design (CCD) was used to study the influence of three factors on ORP: (A) molasses concentration with levels from 4 % to 10 %; (B) concentration of the hydrolyzing agent - $H_2SO_4$ (0.75 %–1.5 %); (C): fermentation duration from 0 to 96 hours.

The factors were coded for analysis, where the coded value of 0 corresponded to a molasses concentration of 7 %, 0.75 % $H_2SO_4$, and a fermentation time of 24 hours. The experiment was conducted with five replications at the central points to ensure statistical accuracy.

Response Surface Methodology (RSM): The response variable was the concentration of reducing sugars (mg/ml), measured using HPLC. The data were analyzed using RSM to construct a second-order quadratic model, which was used to predict optimal conditions for sugar yield. In the experiment, a central composite design (CCD) was used to optimize conditions for biohydrogen synthesis. The following variables were selected to study the influence of factors: (1) Molasses concentration (A) - from 4 % to 10 %; (2) $H_2SO_4$ concentration (B) - from 0.75 % to 1.5 %; (3) pH adjustment method (C) – KOH or K2HPO4; (4) Fermentation duration (D) - from 0 to 96 hours.

## 3. Experimental design matrix

The design matrix includes coded values for each of the factors, which allows for easy variation of the levels of each factor in the experiments:

| A (Molasses concentration) | B (Concentration of $H_2SO_4$) | C (pH adjustment) | D (Duration, hours) | Encoded ORP value (MB) | pH | OD |
|---|---|---|---|---|---|---|
| −1 | −1 | −1 | −1 | −502 | 5.25 | 0.439 |
| −1 | −1 | −1 | 0 | −511 | 5.21 | 0.456 |
| −1 | −1 | −1 | +1 | −520 | 5.17 | 0.478 |
| −1 | +1 | +1 | −1 | −326 | 7.50 | 0.0584 |
| −1 | +1 | +1 | 0 | −334 | 7.40 | −0.0265 |
| +1 | −1 | −1 | −1 | −89 | 5.46 | 0.602 |
| +1 | −1 | −1 | +1 | −120 | 5.38 | 0.623 |
| +1 | +1 | +1 | +1 | −609 | 6.75 | 0.7304 |
| 0 | 0 | 0 | 0 | −530 | 5.10 | 0.512 |
| +α | -α | +α | +α | −575 | 6.74 | 0.8091 |

Biohydrogen production was assessed by monitoring the oxidation–reduction potential (ORP), with hydrogen formation confirmed at ORP values $\leq -400$ mV. Measurements were performed using a HI 2216 pH/ORP meter (Hanna Instruments, Portugal).

## 4. Research results and discussion

As a result of dark fermentation of the molasses substrate, optimal conditions were identified based on the measurement of the oxidation-reduction potential of the substrate, as shown in Fig. 1.

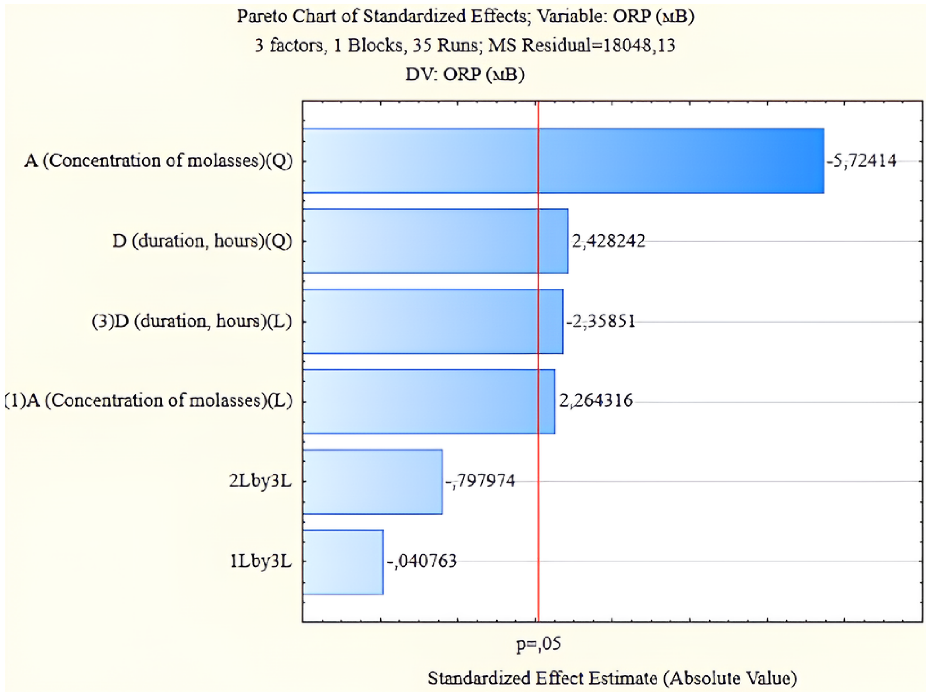


*Fig. 1. Pareto chart for standardized effects of variables.*

The graph (Fig. 1) depicts a Pareto chart for the standardized effects of variables influencing the oxidation-reduction potential (ORP) in the molasses fermentation experiment. The chart shows the absolute values of standardized effects for various factors. The quadratic effect of this factor has the greatest influence on ORP with a negative value (−5.72414), indicating a significant impact of high molasses concentration on reducing ORP. The quadratic effect of duration also has a significant influence on ORP (2.428242), showing that fermentation time strongly affects ORP changes. The value (−2.35851) also shows that increasing fermentation time linearly affects ORP. The linear effect of molasses concentration has a positive influence on ORP (2.264316), indicating a certain correlation between molasses concentration and ORP. The interaction between factors proved to be less significant compared to the main factors, with their influence on ORP being small. The red line on the chart denotes the significance level of $p = 0.05$. Variables crossing this line are considered statistically significant for ORP changes during the experiment.

The presented graph (Fig. 2) shows the response surface for the ORP (Oxidation-Reduction Potential) variable. The graph demonstrates how molasses concentration and duration influence ORP, with different ORP levels highlighted by a color scale:

- **Red** color corresponds to positive ORP values (above 0 mV), indicating more oxidative conditions.
- **Yellow and green** colors indicate a decrease in ORP (between −100 and −500 mV), showing a gradual transition to more reducing conditions.
- **Dark green and blue** colors correspond to the lowest ORP values (down to −700 mV), indicating strong reducing conditions.

From the graph (Fig. 2), it can be seen that at low molasses concentrations and short durations, the ORP is higher, and as the molasses concentration and time increase, a significant decrease in ORP is observed, reaching minimum values at the bottom of the graph (red zones transition to green and blue).

This confirms that to maximize ORP reduction (achieving reducing conditions), it is important to use higher molasses concentrations and increased fermentation time.

Thus, the concentration of molasses had a strong influence on all key parameters: ORP, pH, and OD. As shown in the Pareto chart (Fig. 1) and on the response surface (Fig. 2), increasing the molasses concentration up to 10 % led to a significant decrease in ORP, reaching minimum values (−609 mV). For pH, molasses concentration had the greatest impact, where the linear effect of concentration was 21.05005. The optimal OD level was achieved at a molasses concentration of about 10 % (Fig. 3). The optimal molasses concentration for the fermentation process varies within the range of 7–10 %. Increasing the molasses concentration above 10 % reduces the fermentation

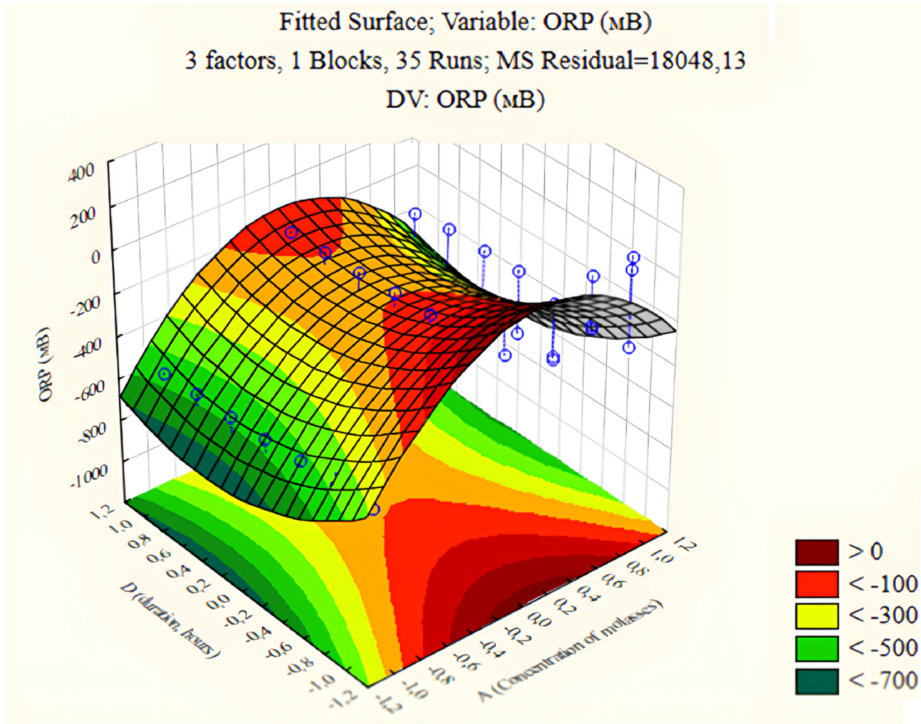


*Fig. 2. Response Surface for the ORP (Oxidation-Reduction Potential) variable as a function of two factors: A (Molasses Concentration, %) - X-axis, D (Experiment Duration, hours) - Y-axis.*

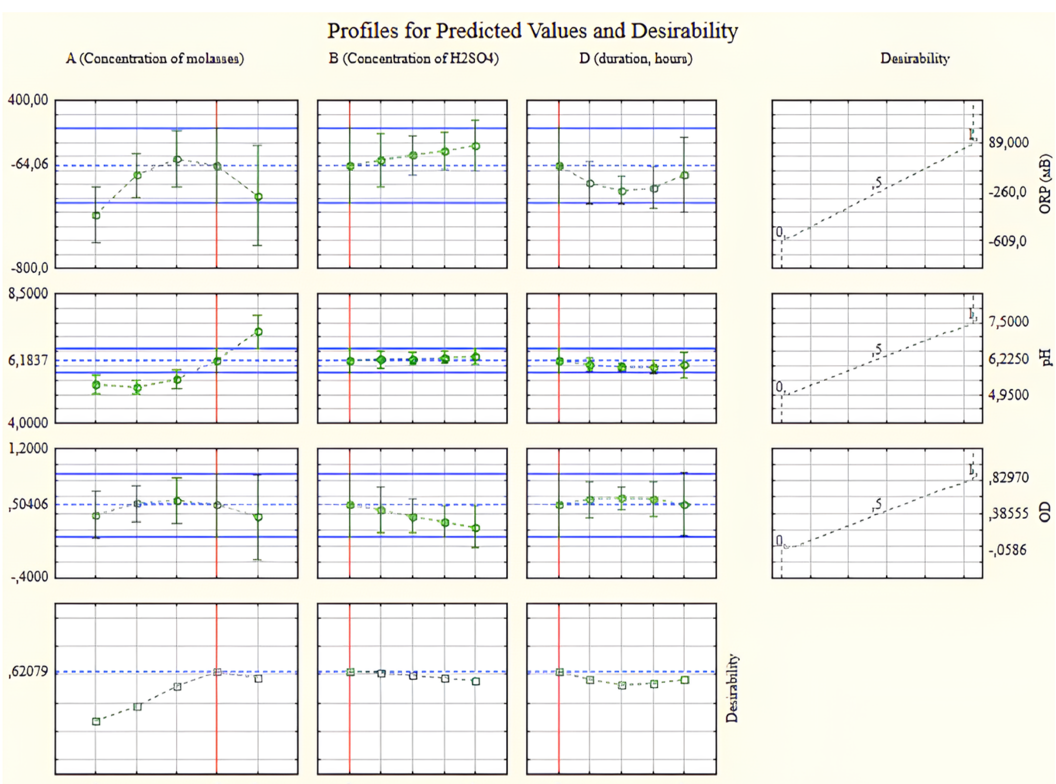


*Fig. 3. Profiles for predicted values and desirability.*

efficiency, which is associated with inhibitory substances in the substrate affecting microbial activity. This is confirmed both in the Pareto chart and on the response surfaces.

The optimal fermentation time for maximum hydrogen yield is 48–72 hours. Further increase in fermentation time does not contribute to an increase in hydrogen synthesis, which is associated with the depletion of resources for microorganisms. This is confirmed by the Pareto diagram data for all variables.

The graph (Fig. 3) shows the profiles of predicted values and desirability, which reflect the influence of three factors on the key parameters of the experiment: ORP (oxidation-reduction potential), pH, OD (optical density), as well as the overall desirability of the process:

- A (Molasses Concentration): −1 (Low) to +1 (High)
- B ($H_2SO_4$ Concentration): −1 (Low) to +1 (High)
- D (Experiment Duration): −1 (Start of Experiment) to +1 (Max Time)

The profile graph shows that the ORP value has the greatest decrease (to −609 mV) at high molasses concentrations and over a long period of time. The $H_2SO_4$ concentration also has an effect, but to a lesser extent. Increasing the molasses concentration increases the pH. The $H_2SO_4$ concentration has a smaller effect on the pH change, and the duration of the experiment has a minimal effect. The optical density increases with increasing molasses concentration and has maximum values at long time intervals.

Thus, the best results for ORP, pH and OD are achieved with high molasses concentrations and experiment duration. The $H_2SO_4$ concentration has less effect on desirability than molasses and time. The maximum desirability (Desirability) exceeds 0.6 under optimal conditions for each parameter.

## 5. Conclusion

In the study of molasses fermentation for biohydrogen synthesis, the key process parameters were optimized using the surface response method (RSM). Molasses concentration, $H_2SO_4$ concentration, and fermentation time were selected as the main factors influencing biohydrogen yield and related parameters (ORP, pH, and OD). The following key findings were obtained from the data analysis:

1. **Molasses concentration** had a significant effect on all parameters studied. Both linear and quadratic effects of molasses concentration demonstrated that increasing this factor to a certain level (about 10 %) improved ORP, pH and OD. However, with further increase in concentration, an inhibitory effect was observed, leading to a decrease in optical density and less pronounced hydrogen synthesis.
2. **Fermentation duration** also had a significant effect, especially on optical density (OD) and pH. A linear increase in time had a positive effect on OD, reaching a maximum at long fermentation intervals (more than 48 hours). However, too long a time led to a decrease in the efficiency of the process, as confirmed by the quadratic effects of time in the models.
3. **The concentration of $H_2SO_4$** had a less pronounced effect compared to the concentration of molasses and the fermentation time. Its optimal value varied within the range of 0.75 %–1.5 %, while higher concentrations reduced the efficiency of the process.
4. **Parameter optimization:** The surface response method showed that the most effective conditions for biohydrogen synthesis were molasses concentration of about 7–10 %, $H_2SO_4$ concentration of 0.75 %, and fermentation duration of about 48 hours. These parameters provided the most favorable values for all the parameters studied, from oxidation-reduction potential to pH and optical density.
5. **Pareto diagrams and response surfaces** confirmed the importance of nonlinear effects and interactions of factors. The quadratic effects of molasses concentration and time were key to achieving optimal results, indicating the importance of carefully tuning each factor to achieve maximum biohydrogen yield.

## 6. Recommendations

To improve the efficiency of the biohydrogen synthesis process, it is recommended to focus on fine-tuning the molasses concentration and fermentation time, avoiding both too high and too low values. The results of this study can be used to further optimize biotechnological processes and develop more efficient fermentation strategies to increase biohydrogen yield.

The results of this study open up new perspectives for further research in the field of using molasses as a feedstock for the production of sustainable biohydrogen and can also be applied to the development of more efficient technologies in the field of biotechnology and clean energy sources. Future research can focus on optimizing additional parameters and exploring different fermentation methods to improve biohydrogen yield.

## Funding information

This research was funded by the Committee of Science of the Ministry of Science and Higher Education of the Republic of Kazakhstan Grant No. BR21882359.

## Conflict of interest

The authors declare that there is no conflict of interest.

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