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Obtaining of osmoresistant mutants in L-histidine-producing coryneform bacterium to improve efficiency of target amino acid biosynthesis

Susanna Keleshyan[1], Zhaneta Karapetyan[1], Anna Toplaghaltsyan[1,*], Lusine Melkonyan[1,2], Vahe Ghochikyan[1], Luiza Manukyan[1], Gevorg Tsarukyan[1], Sofya Martirosyan[1], Tamara Davidyan[1], Nelli Avetisyan[1] and Gayane Avetisova[1,2]

[1] SPC "Armbiotechnology" NAS RA, 14 Gyurjyan, 0056, Yerevan, Armenia; [2] Yerevan State University, 1 Alex Manoogian, 0025, Yerevan, Armenia

[*]**Corresponding Author:** Anna Toplaghaltsyan, MD, PhD student, Junior Researcher, "SPC Armbiotechnology" NAS RA, 14 Gyurjyan, Yerevan, 0056, Armenia

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ABSTRACT

Background: L-histidine, an essential amino acid, plays an important role in the regulation of vital metabolic processes in living organisms. Global L-histidine production was valued at $0.24 billion in 2024 and is expected to double by 2033. This amino acid is utilized extensively in the pharmaceutical, food, and livestock industries. 75% of the world's L-histidine is produced by microbial means, with coryneform bacteria being the predominant organism. Coryneform bacteria are a valuable model for studying the response of bacteria to osmotic stress. Increased osmotic pressure in the fermentation process is observed due to the changing composition of the nutrient medium during the synthesis of the target amino acid, which has a negative effect on bacterial cells. To increase survival under such conditions without altering metabolic functions, microorganisms synthesize substances called osmolytes, one of which is the amino acid L-histidine.

Objective: The aim of this study is to obtain osmotolerant coryneform L-histidine producer mutants and evaluate their synthesizing activity in increased osmotic pressure.

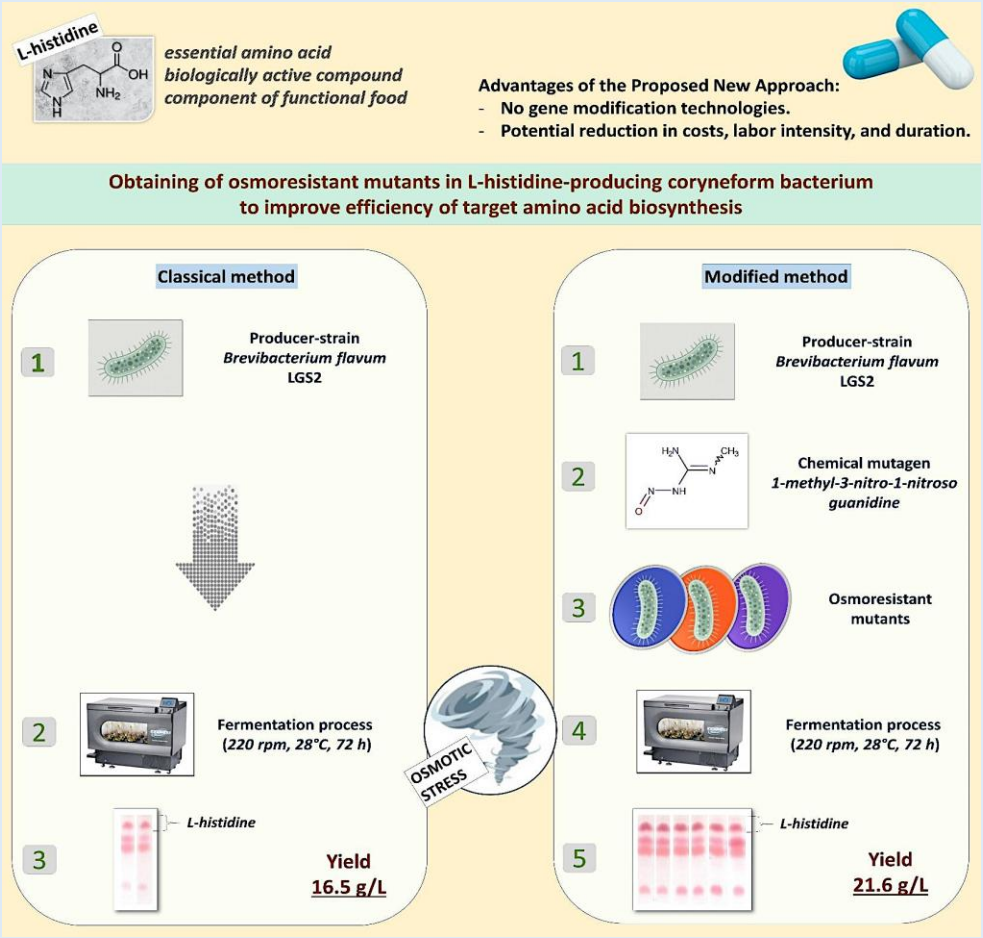
Methods: Osmotolerant mutants were obtained from the L-histidine producer *Brevibacterium flavum* LGS2 by chemical mutagenesis using 1-methyl-3-nitro-1-nitrosoguanidine. The impact of elevated osmotic pressure on the synthesis of the

target amino acid was determined in submerged fermentation within a shaker incubator at 220 rpm, 30°C for 72 hours. The amount of histidine was determined by thin-layer chromatography.

Results: Chemical mutagenesis, excluding gene-modifying technologies, was employed to obtain 16 osmoresistant mutants of *B. flavum* LGS2 capable of growing in mediums containing concentrations greater than or equal to 0.5 M NaCl. The findings indicated that three mutants demonstrated the highest yield, with an average production of 21.6 g/L L-histidine, in comparison to the 16.5 g/L produced by the control strain.

Conclusion: In the study, the obtained osmoresistant mutants exhibited an increased level of L-histidine synthesis, exceeding the productivity of the initial strain by an average of 13.2%. The scientific innovation of this research lies in its practical application of mutations that increase osmotic pressure resistance, addressing real-world issues. This shows the potential for improving the productivity of industrial strains that produce L-histidine.

Keywords: biologically active compound, L-histidine, strain-producer, chemical mutagenesis, osmoresistant mutant



Graphical Abstracts: Obtaining of osmoresistant mutants in L-histidine-producing coryneform bacterium to improve efficiency of target amino acid biosynthesis

INTRODUCTION

L-histidine is an essential amino acid that is one of the few proteinogenic amino acids. It serves as a precursor for the synthesis of histamine, glutamine, and other biologically important compounds. Histidine, in combination with other amino acids, performs a significant function in the nutrition and metabolism of living organisms, serving as one of the most critical precursors for the synthesis of a diverse range of vital compounds. Amino acids have been demonstrated to regulate key metabolic pathways and processes that are crucial for the body's vital functions, growth, development, reproduction, and homeostasis [1-3].

Due to its indispensable role in living organisms, L-histidine is a component of many functional products. The latter are defined as natural foods and/or their components that supply the live organism with biologically active compounds necessary for normal growth and development [4-6].

Essential amino acids, found in muscle proteins, can serve as an energy source during prolonged physical activity. Since skeletal muscles contain high concentrations of L-histidine, products containing this amino acid are commonly used in the food industry to nourish athletes and vegetarians and are used in the production of feed for farm animals [7-10].

Furthermore, L-histidine has a broad range of applications in the medical field. It is a component of numerous pharmaceutical preparations and is also employed as a supplement to fundamental drug treatment regimens, with the objective of promoting the healing process. The prevalence of histidine usage can be attributed to its role as a constituent of oxygen delivery; in hemoglobin and myoglobin. A deficiency of histidine has been demonstrated to result in alterations to blood composition, hearing loss, delayed recovery of damaged tissues, and impaired mental development and intelligence. The amino acid has also been utilized in the treatment of allergies, arthritis, hyperacidity, anemia, peptic ulcer disease, cardiovascular disease, and diabetes mellitus [11-13].

A close examination of the global L-histidine market reveals that it is undergoing a period of substantial growth. At present, the annual production capacity is 3,000 tons, with an annual growth rate of over 20%. This phenomenon can be attributed to an increase in demand from the aforementioned key industries, including food, feed, and pharmaceuticals. From a financial perspective, the global histidine market was valued at US$0.24 billion in 2024. According to forecasts, this figure will reach US$0.41 billion by 2033, with an average annual growth rate of 6.0% between 2025 and 2033 [14-15].

Microorganisms play a key role in producing functional foods. They can be used to produce various biological additives through microbiological synthesis or to deliver functional substances [16].

The predominant method of synthesizing L-histidine is through microbiological means, employing producer strains, predominantly coryneform bacteria that are GRAS (Generally Regarded as Safe) qualified [17-18]. During the amino acid fermentation process, the cells of the producer strains undergo various changes in the culture medium, including changes in pH, nutrient concentration, and the accumulation of the target product. These alterations significantly modify the osmotic pressure of the fermentation medium, which ultimately leads to the inactivation of the producer strain cells, suppressing their growth and, consequently, the synthesis of the target product. Maintaining the osmotic pressure of the nutrient medium within acceptable limits is a prerequisite for normal growth and division of bacterial cells and for the maximum expression of the synthetic abilities of the producer strains [11, 19-20]. The use of corynebacteria as a subject for studying the response of microorganisms to osmotic stress is particularly convenient, as it is known that this bacterium is capable of effectively adapting to changes in osmotic pressure.

In order to counteract the high osmotic pressure of the external environment and thereby increase their chances of survival in these conditions, organisms use special organic substances called osmolytes. These osmolytes are synthesized in cells or enter the organism from the environment [21-22]. The presence of these substances enables cells to restore osmotic pressure balance without altering their metabolic functions. Such substances include amino acids such as proline, histidine, glycine, and alanine, etc. [20, 23].

As stated in the relevant literature, elevated osmotic pressure demands additional energy, leading to microbial cells sustaining their metabolism through an increase in ATP synthesis [24]. The first step in histidine biosynthesis in coryneform bacteria is N-5-phosphoribosyl-ATP, which is formed by the condensation of ATP with 5-phosphoribosyl-L-pyrophosphate. Thereafter, an imidazole ring is formed, and glutamate transfers the alpha-amino group to N-5-phosphoribulosylformimino-5-aminoimidazole-4-carboxamide. In the final stage, an amine is formed, which is oxidized to histidine. Consequently, the substrates histidine-5-phosphoribosyl-L-pyrophosphate and ATP play a key role in intermediate and energy metabolism [25].

It can be hypothesized that culturing an osmoresistant histidine producer in a fermentation medium with high osmotic pressure will result in an increase in the ATP pool, which will, in turn, lead to the activation of the target product synthesis process. The activation of synthesis is also facilitated by the fact that the amino acids glycine and glutamine, which participate in the synthesis of histidine, are osmolytes that accumulate in cells under conditions of high osmotic pressure, thereby restoring intracellular pressure. The accumulation of these amino acids can also ultimately lead to an increase in the level of histidine synthesis [26].

The aim of this work is to obtain osmoresistant mutants based on the coryneform L-histidine producer *B. flavum* LGS2 and to study the effect of increased osmotic pressure on the growth and biosynthesis activity of these mutants.

MATERIALS AND METHODS

Strains: The influence of the osmotic pressure of the medium on the growth and histidine synthesizing activity was studied on the L-histidine-producing strain *B. flavum* LGS2, previously obtained at SPC "Armbiotechnology" NAS RA. The strain is resistant to the L-histidine analog 3-(4-thiazolyl)-L-alanine and synthesizes up to 16.5 g/L histidine. The histidine auxotrophic mutant *B. flavum* T3 (*his*$^-$) was used as a test culture for the semi-quantitative evaluation of L-histidine hypersynthesis.

Growth medium: Soyabean Casein Digest (Caso) Medium (HiMedia Laboratories; Thane, India) was used as the enriched medium. Glover's medium was utilized as a minimal medium, with the following composition (g/L): glucose - 8.0, NH_4Cl - 5.0, NH_4NO_3 - 1.0, Na_2SO_4 - 2.0, K_2HPO_4 - 3.0, KH_2PO_4 - 1.0, $MgSO_4 \cdot 7H_2O$ - 0.5, $FeSO_4 \cdot 7H_2O$ - 0.01, $MnSO_4 \cdot 5H_2O$ - 1.0, biotin - 0.0001, thiamine - 0.0001, agar - 15.0, pH 7.5. The following composition (g/L) of fermentation medium was used for the L-histidine synthesis: sucrose - 150.0, $(NH_4)_2SO_4$ - 40.0, yeast extract - 30.0, urea - 2.0, KH_2PO_4 - 1.5, K_2HPO_4 - 0.5, Na_2HPO_4 - 0.5, $MgSO_4 \cdot 7H_2O$ - 1.0, $CaCO_3$ - 40.0, pH 7.5.

Obtaining of osmoresistant mutants: In order to obtain osmoresistant mutants of *B. flavum* LGS2, 1-methyl-3-nitro-1-nitrosoguanidine (NG) (Merk; Darmstadt, Germany) was used by means of a chemical mutagenesis process [27-28]. The strain was cultured overnight on a shaking incubator (Faithful FS-70B, Huanghua, China) at 180 rpm and 28°C to obtain a bacterial suspension at a titer of 10^9 cells per millilitre

(ColonyStar counter, Funke Gerber, Berlin, Germany). The overnight culture was subsequently centrifuged (Biobase BKC-MF5B, Jinan, China) at speed 12,000×g for 5 minutes at room temperature (15-25°C). The pellet was resuspended in acetate buffer with a pH of 5.6, containing NG at a concentration of 300 µg/mL. The mutagenesis process was carried out for a duration of 30 minutes. The mutagen-treated culture was subsequently plated on medium containing the minimum inhibitory concentration of NaCl (0.5M).

Evaluation of the influence of different concentrations of NaCl on the growth of osmoresistant mutants: The impact of the high osmotic pressure of the medium on the growth of the histidine-producing strain and its osmoresistant mutants was assessed during the growth process of the cultures on a shaking incubator at 180 rpm and 28°C for 30 hours. To construct the growth curve, samples were collected at five-hour intervals, and the optical density was measured using a UV-VIS spectrophotometer (Peak Instruments C-7200S, Shanghai, China) at a wavelength of 540 nm.

A semi-quantitative assessment of the histidine-synthesizing activity of osmoresistant mutants: In the first stage, the histidine synthesizing activity of the obtained strains was evaluated using a semi-quantitative method. The histidine auxotrophic mutant *his*$^-$ was used as a test culture. A suspension of the test culture with a final concentration of 10^9 cells per millilitre was added to Glover agar. Subsequently, a total of ten holes, each with a diameter of 0.2 cm, were made in the agar. Then, 50 µL of an overnight culture of the mutant (with a concentration of 10^8-10^9 cells per millilitre) was added to each hole. A pure Caso-broth served as the negative control, while the initial strain *B. flavum* LGS2 was used as the positive control [27, 29]. The size and density of the growth zone formed by the test culture surrounding the holes were used as a criterion for the intensity of histidine synthesis.

A quantitative assessment of the histidine-synthesizing activity of osmoresistant mutants: In order to quantitatively determine the synthesis activity of histidine, submerged fermentation of mutants was performed [27]. The fermentation medium (15 ml) was transferred to a 500 ml Erlenmeyer flask, and 5% of the inoculum pre-cultured in Caso broth with aeration for 18 h was added. The fermentation process was then carried out on an incubator-shaker (Innova 43 Shaker, New Brunswick Scientific, Edison, USA) at 220 rpm and 28°C for a period of 72 hours. The quantification of histidine concentration in the culture broth was performed by thin layer chromatography [30]. The ammonia-butanol-ethanol-acetone-water system in a ratio of 2.5:2:2:4:1 was used for separation. The ninhydrin dye (Merck; Darmstadt, Germany) was used to visualize amino acids [31].

Statistical Analysis: Experiments were performed in triplicate and data analysis was performed by Analysis of variance (ANOVA) with Dunnett's test using Minitab 17.1 statistical program (Pennsylvania, USA), $p<0.05$.

RESULTS AND DISCUSSION

In industrial settings, the osmotic pressure of the medium tends to increase during the fermentation process due to the build-up of the final product. Consequently, the disorder of growth and inactivation of various bioprocesses in the cells of the producer strain occur [11, 20]. One of the strategies that microbes employ to counteract high osmotic pressure is the synthesis of intracellular substances. The regulation of the concentration of them enhances the ability of cells to adapt to changes in the osmolality of the environment.

The valuable model for studying the response of bacteria to osmotic stress are coryneform bacteria due to their ability to adapt to changes in osmotic pressure [25]. However, there is limited knowledge regarding the survival of these bacteria under conditions of high

osmotic pressure, which underscores the importance of further research in this area [32]. It has been demonstrated that the osmotic resistance of corynebacteria increases in response to the incorporation of compounds such as glutamate, proline, arginine, histidine, and trehalose, collectively referred to as osmolytes, into the culture medium [11]. The presumed function of these substances is to maintain the tonicity of the cytoplasm at a level higher than that of the medium, thereby ensuring the appropriate turgor pressure within the cells.

Within the domain of microbiology, coryneform bacteria have attracted particular interest due to their prevalence as producers of amino acids. These bacteria include *B. flavum*, which has been used to obtain numerous active strains that produce amino acids, including histidine [33-35].

Obtaining of osmoresistant mutants: The chemical mutagenesis method was employed to obtain osmoresistant mutants based on the existing strain *B. flavum* LGS2. NG was used as the mutagen, and NaCl was employed as a selective agent. Initially, the minimum inhibitory concentration of NaCl was established through the cultivation of bacteria in minimal Glover's liquid medium, which contained varying concentrations of NaCl ranging from 0.3M to 0.7M. Consequently, the minimum inhibitory concentration of NaCl was found to be 0.5M (Fig. 1).

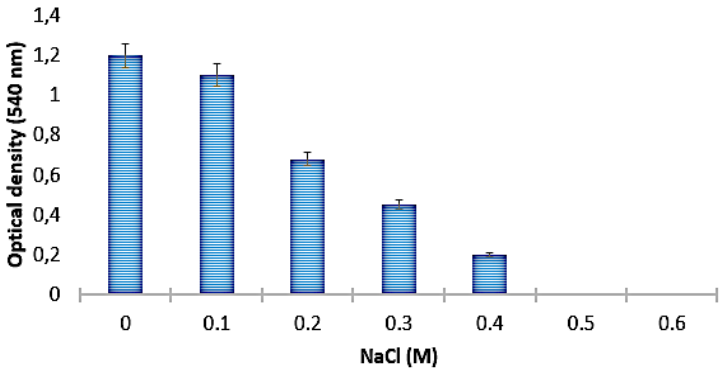


Figure 1. Determination of the minimum inhibitory concentration of NaCl for *B. flavum* LGS2.

Then, the mutagen-treated culture, which exhibited a survival rate of 50% to 40%, was plated on minimal Glover's medium containing the minimum inhibitory concentration of NaCl. Consequently, a total of 16 osmotic-resistant mutants were isolated. The relatively low frequency of mutants indicates the high sensitivity of the producer strain to osmotic pressure in the medium and confirms the advisability of increasing its osmotic resistance.

Evaluation of the influence of different concentrations of NaCl on the growth of osmoresistant mutants: It is imperative to assess the impact of elevated osmotic pressure in the medium on their viability. Consequently, a series of experiments was conducted to investigate the growth of 16 mutants in a medium with increased NaCl content. As predicted, the growth of the initial producer strain *B. flavum* LGS2 was significantly suppressed at a concentration of 0.5M NaCl in the medium, while all selected mutants demonstrated a clear resistance to high osmotic pressure (Fig. 2). It is notable that eight mutants with designated numbers M2, M4, M5, M8, M10, M14, M15, and M16 demonstrated growth rates that exceeded that of the original culture by 10–15% in both the absence and presence of osmotic stress.

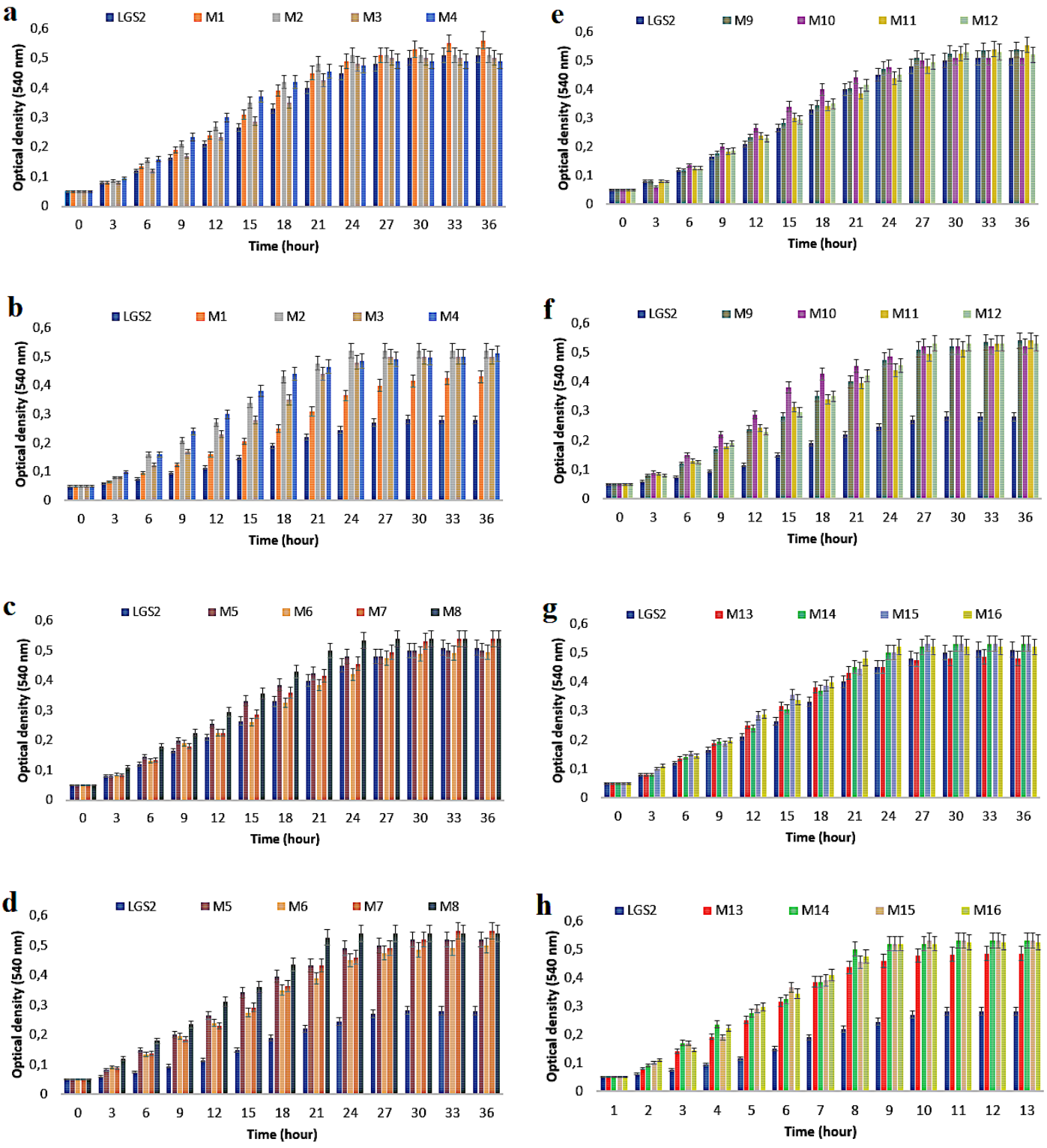


Figure 2. Growth curves of *B. flavum* LGS2 and its mutants. a, b, c, d – absence of osmotic stress; e, f, g, h – presence of osmotic stress

A semi-quantitative assessment of the histidine-synthesizing activity of osmoresistant mutants: To evaluate the synthesis of histidine, a semi-quantitative test was performed on all 16 mutants. L-histidine over synthesis was assessed using a test culture *his*$^-$ that was auxotrophic for this amino acid. The results indicated that 90% of the mutants exhibited a significant stimulatory effect on the growth of the test culture, in

comparison with the initial culture. Notably, eight mutants (with designated numbers M2, M4, M5, M8, M10, M14, M15, and M16) exhibited growth rates 10–15% higher than the original culture, in both the absence and presence of osmotic stress (Fig. 3). This ability, acquired by the mutants, will significantly reduce the duration of the fermentation process, which is especially important under industrial conditions.

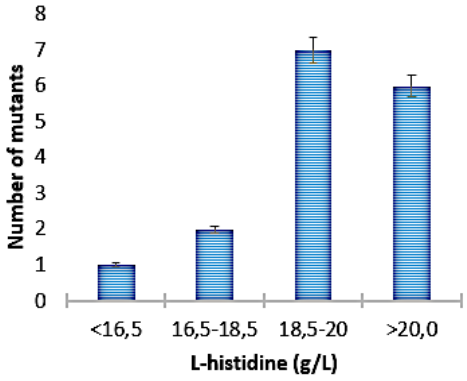


Figure 3. A semi-quantitative assessment of the histidine-producing activity of osmoresistant mutants.

A quantitative assessment of the histidine-synthesizing activity of osmoresistant mutants: In order to confirm the reliability of the positive effect of mutations leading to osmotic resistance, as well as the stability of the results obtained, the levels of histidine synthesis were checked in six selected mutants under conditions of submerged fermentation. As demonstrated in Figure 4, the mutants designated M2, M5, and M15 exhibited the highest levels of L-histidine synthesis (21.4–21.8 g/L).

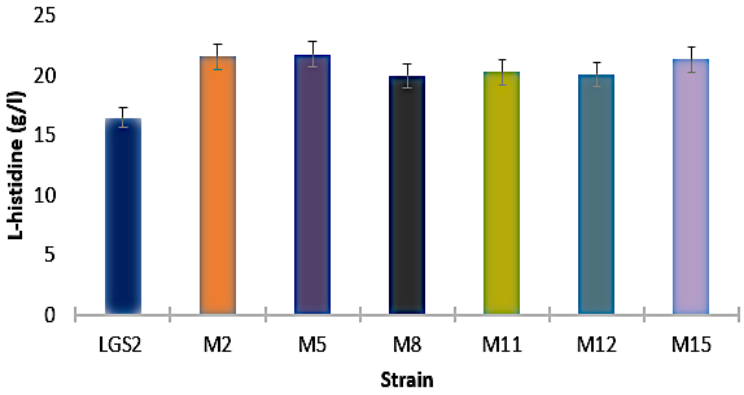


Figure 4. A quantitative assessment of the histidine-synthesizing activity of osmoresistant mutants.

Consequently, three osmoresistant mutant producers were obtained, exhibiting an average histidine yield of 21.6 g/L, which is significantly higher than the yield of the original strain, which was 16.5 g/L. In addition to elevated synthesis, all three mutants exhibited 14–15% accelerated growth relative to the original strain *B. flavum* LGS2 (Fig. 2).

The results obtained demonstrated the possibility of increasing the viability of osmotic mutant producers of L-histidine by increasing the synthesis of the target amino acid, which is an osmolyte. Yılmaz et al. (2024) and Duveau et al. (2023) also confirmed the feasibility of this approach when they investigated the effect of environmental stress factors on enhancing the

synthesis of certain amino acid derivatives [11, 20]. Furthermore, these findings suggest the potential for cost, labor intensity, and duration reduction in industrial processes for the production of L-histidine through the implementation of the presented mutants.

Thus, the scientific innovation of this research lies in identifying new approaches to using non-standard methods in biosynthesis. These methods increase the productivity of producers of certain secondary metabolites, such as amino acids, while reducing production costs.

CONCLUSION

In the work, obtained osmoresistant mutants exhibited an increased level of L-histidine synthesis, exceeding the productivity of the initial strain by an average of 13.2%. These findings validate the practicability of leveraging mutations that result in elevated osmotic pressure resistance to address applied issues, thereby demonstrating the potential to enhance the productivity of industrial strains capable of producing L-histidine. It is also important to note that the proposed method does not involve the use of gene-modifying technologies, which is undesirable in the food and pharmaceutical industries. The results of this study may provide the foundation for the development of novel strategies for the creation of new foods and medicines with the desired composition of bioactive L-amino acids.

List of Abbreviation: GRAS: Generally Regarded as Safe, *his*$^-$: *B. flavum* T3, NG: 1-methyl-3-nitro-1-nitrosoguanidine, Caso medium: Soyabean Casein Digest Medium, ANOVA: Analysis of variance.

Competing interests: 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.

Authors' contributions: SK: supervision, methodology, writing – original draft preparation, data curation. ZhK: investigation, methodology, writing – review and editing, data curation. AT: investigation, methodology, writing – original draft preparation, writing – review and editing. LMe: data curation, writing – review and editing. VG: supervision, data curation. LMa: methodology, investigation. GT: investigation, data curation. SM: investigation, methodology. TD: investigation, methodology. NA: investigation, methodology. GA: supervision, writing – review and editing.

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