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Evaluation of *thrB* and *thrC* gene expression in *Corynebacterium glutamicum* ATCC 13032 during *in vitro* L-threonine production

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Abstract

Background: L-threonine is an essential amino acid required for normal growth and tissue synthesis in humans and animals and is widely used in the food and pharmaceutical industries.

Objectives: The present study aimed to enhance laboratory-scale threonine production by optimizing the culture medium composition and physicochemical conditions of *Corynebacterium glutamicum* as a suitable production host. In parallel, the expression patterns of the key threonine biosynthetic genes, *thrB* and *thrC*, were evaluated using real-time polymerase chain reaction (PCR).

Methods: *C. glutamicum* was initially cultivated in ISP2 medium, after which different temperature ranges, pH values, and alternative carbon sources, including cheesewater, bagasse, molasses, and corn syrup, were assessed to optimize the medium formulation. Threonine production under different sugar sources and pH conditions was quantified using spectrophotometric and chromatographic methods. The optimal physicochemical conditions for threonine biosynthesis were selected, and the expression levels of the key biosynthetic genes *thrB* and *thrC* were analyzed by real-time PCR.

Results: Chromatographic analysis indicated that supplementation with 6 g/L cheesewater and adjustment of the pH to 7.3 provided the most favorable conditions for L-threonine production. The highest threonine concentrations obtained in the tested culture media were 18,263 mg/L for molasses, 16,219.7 mg/L for bagasse, 6,843.2 mg/L for corn syrup, and 39,393.8 mg/L for cheesewater. Among the evaluated substrates, the cheesewater-containing medium yielded the highest threonine concentration. Maximum threonine production was consistently observed at pH 7.3. Real-time PCR analysis demonstrated that the cheesewater-based medium induced a significant upregulation of threonine biosynthetic genes. Specifically, *thrB* expression increased from 1.09-fold to 1.10-fold ($p<0.0001$), while *thrC* expression increased from 1.08-fold to 1.09-fold relative to the standard medium ($p<0.00017$).

Conclusion: These findings indicate that natural substrates such as cheesewater can serve as safe, economical, and effective carbon sources for improving threonine production. Moreover, the results underscore the substantial influence of physicochemical parameters of the culture medium on L-threonine biosynthesis by *C. glutamicum*, as reflected at both the metabolic and transcriptional levels.

Keywords: Threonine, *Corynebacterium glutamicum*, Real-time PCR, *thrB* and *thrC* gene expression.

Introduction

L-threonine is an essential amino acid of major importance for the nutritional requirements of both humans and animals. It plays a fundamental role in the biosynthesis of critical structural tissues, including cardiac muscle, collagen, and elastin.[1] In addition, L-threonine is extensively used in the food and pharmaceutical industries, particularly as a dietary supplement in animal feed to enhance growth performance and immune function. Because humans and other vertebrates are unable to synthesize threonine endogenously, its supply depends entirely on dietary intake.[2] The global market demand for L-threonine, widely applied in food, animal feed, and medical products, exceeds 700,000 tons annually.[3] At present, microbial fermentation represents the principal industrial strategy for L-threonine production.[4] Several approaches have been developed to improve production efficiency, including amplification of key biosynthetic genes and redirection of metabolic fluxes to reduce the formation of unwanted by-products. However, imbalances in intracellular carbon flow and the accumulation of competing amino acids such as L-lysine and L-isoleucine remain major constraints that limit production yield and process efficiency.[5,6]

Microorganisms naturally synthesize only limited amounts of threonine, making direct production economically insufficient to satisfy industrial demands. Therefore, optimization of culture media composition, adjustment of environmental conditions, and selection of suitable microbial strains are critical strategies for enhancing threonine yield. Several bacterial species have been exploited for this purpose, including *Corynebacterium glutamicum,*[7] *Escherichia coli,*[8] *Bacillus subtilis,*[9] *Brevibacterium* spp.,[10] and *Lactobacillus* spp.[11] These microorganisms are frequently subjected to genetic and metabolic engineering to increase threonine output through pathway modification. Each organism offers distinct advantages, such as high production potential, genetic tractability, and efficient metabolite secretion.[8]

Among these candidates, *C. glutamicum* is widely recognized as a particularly suitable host for threonine production because of its unique physiological and metabolic properties. This bacterium naturally exhibits a high capacity for amino acid synthesis, especially glutamic acid, and can be readily engineered to redirect metabolic flux toward threonine biosynthesis. Furthermore, *C. glutamicum* can be genetically modified to enhance threonine production by constructing optimized heterologous biosynthetic pathways.[12] Its ability to tolerate harsh industrial conditions, together with its rapid growth rate and ease of large-scale cultivation, further supports its applicability in industrial fermentation processes. Collectively, these characteristics make *C. glutamicum* an attractive platform for the commercial production of threonine and other amino acids.[13]

Given the nutritional importance of threonine and the substantial costs associated with its industrial production, the present study focused on in vitro threonine biosynthesis using *C. glutamicum*. This strategy offers potential economic advantages while exploiting the organism's intrinsic ability to convert aspartic acid into threonine. By optimizing growth parameters, particularly the nature of the carbon source, this work sought to maximize threonine output. The main objectives of this study were: 1) to determine the most favorable conditions for threonine production by *C. glutamicum*, 2) to optimize physicochemical parameters affecting threonine biosynthesis, and 3) to examine the expression of the *thrB* and *thrC* genes, which are key determinants of threonine biosynthesis.[14,15] The threonine biosynthetic pathway is complex and involves multiple genes, including *lys*, *thrA*, *thrB*, and *thrC*. Among these, *thrB* and *thrC* play central roles in controlling pathway flux. Previous studies have demonstrated a direct relationship between the expression levels of these genes and the amount of threonine produced. In particular, *thrB* is considered a major regulatory point in the pathway and is generally expressed at higher levels than *thrC*, thereby exerting strong control over overall amino acid biosynthesis.[16,17]

The threonine biosynthesis pathway includes several sequential enzymatic steps mediated by *thrA*, *thrB*, and *thrC*. In *C. glutamicum*, the *thrB* gene encodes homoserine kinase, which catalyzes the phosphorylation of homoserine to O-phosphohomoserine.[18] Subsequently, the *thrC* gene encodes threonine synthase, which converts O-phosphohomoserine into L-threonine in the final step of the pathway.[19]

The *thrB* gene thus plays a pivotal regulatory role in threonine biosynthesis by generating O-phosphohomoserine, a critical intermediate in the metabolic sequence.[18] The *thrC* gene encodes threonine synthase, which catalyzes the terminal conversion of this intermediate into threonine. The expression levels of both *thrB* and *thrC* have been reported to correlate positively with total threonine yield, highlighting their importance in pathway control and metabolic efficiency.[20]

Objectives

The present study aimed to: i) evaluate several low-cost carbon sources for L-threonine production by *C. glutamicum*, ii) identify the optimal pH conditions for threonine biosynthesis, and iii) investigate the expression profiles of the key biosynthetic genes *thrB* and *thrC* under optimized culture conditions.

Methods

Cultivation and optimization of *C. glutamicum*

The *Corynebacterium glutamicum* strain ATCC 13032 was obtained from the Microbial Bank of the Iranian Biological Resource Center. A single colony was inoculated into 50 mL of sterile ISP2 liquid medium in a 250 mL Erlenmeyer flask and incubated at 34 °C with shaking at 120 rpm to establish a contaminant-free culture. Bacterial growth was monitored spectrophotometrically at 620 nm, and cultures were harvested for threonine quantification during the logarithmic growth phase (OD620 ≈ 0.8–1.0).

To evaluate low-cost agro-industrial substrates, glucose in the ISP2 medium (per 50 mL: 0.5 g NaCl, 0.5 g malt extract, and 0.2 g yeast extract) was replaced with cheesewater, molasses, bagasse extract, or corn syrup. Each carbon source was supplied at a final concentration of 6 g/L, prepared by dissolving 0.3 g of each substrate in 50 mL of distilled water. Cheesewater and molasses were sterilized through 0.22 μm filters prior to use. Bagasse was processed by autoclaving at 121 °C for 15 min, followed by filtration to obtain a soluble extract. All media were sterilized by autoclaving at 121 °C for 15 min and cooled to room temperature before inoculation.

Each culture flask was inoculated with 1% (v/v) of an overnight seed culture (OD620 ≈ 1.5) and incubated at 34 °C with shaking at 120 rpm. For optimization experiments, the effects of different pH values (7.3, 7.4, and 7.5) and carbon sources on threonine production were assessed. Samples were collected during the logarithmic growth phase for further analysis.

Determination of threonine production by HPLC

Bacterial cultures were grown until the logarithmic phase, representing optimal growth and threonine synthesis. Cultures were centrifuged at 4000 rpm for 20 min to separate the supernatant. For chromatographic analysis, the supernatant was combined with acetic acid, ninhydrin, 0.3% ascorbic acid, and distilled water in defined ratios. The mixture was incubated in a water bath at 85 °C for 40 min and then rapidly cooled under running cold water. Processed samples were analyzed using high-performance liquid chromatography (HPLC) in a specialized laboratory.

Chromatographic separation was performed using a C18 column with a mobile phase consisting of water and acetonitrile. Samples were filtered and diluted prior to injection. Threonine detection was achieved using a UV-VIS detector set at 210 nm, and the column temperature was maintained between 25–30 °C throughout the analysis. Threonine concentrations were quantified using a calibration curve prepared from certified L-threonine standards. Known concentrations were injected into the HPLC system, and peak areas were plotted against concentration to generate a regression equation, which was subsequently applied to determine the threonine content of bacterial samples.

Real-Time Polymerase Chain Reaction (RT-PCR)

RNA was extracted from cultures grown under optimized medium conditions with the highest threonine yield and compared to standard ISP2 medium to evaluate the expression of *thrB* and *thrC*. Culture media were centrifuged at 9000 rpm for 10 min at 4 °C. RNA extraction was performed using the TRIzol method according to the manufacturer's protocol.[21]

To remove residual DNA, DNase treatment was applied, and the enzyme was inactivated by incubation at 75 °C for 5–10 min. RNA purity was assessed using a NanoDrop spectrophotometer by measuring the OD260/280 ratio. Complementary DNA (cDNA) was synthesized using the SinaClon kit based on the extracted RNA concentration. Real-time PCR reactions were performed using Maxima SYBR Green ROX qPCR Master Mix (Fermentas) on a Techne Quantica Real-Time PCR Thermal Cycler. Primers for *thrB* and *thrC* were designed as detailed in Table-1. The *oprF* gene was selected as an internal control after confirming stable expression under all experimental conditions.

Each reaction included an initial denaturation at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 5 s and annealing at 57 °C for 30 s. Primer efficiencies ranged between 90–110%. Gene expression fold changes were calculated using the ΔΔCt method from three biological replicates.

Table-1. Primers used for real-time PCR

Gene	Primer Sequence
thrB-F	ATGGCAATTGAACTGAACGTCG
thrB-R	GGGAGCCATCAAGAGGGA
thrC-F	CGACTATGTACTGCTTAACTGC
thrC-R	AGGTCAACACCCCAATCATC
oprF-F	CTTCGACAAGTCCAAGGTCA
oprF-R	AAGTGGACGGGTACTGCTTC

Statistical analysis

Quantitative gene expression data were analyzed using GenEx software. Threonine production values are presented as mean±standard deviation. Differences between treatment groups were assessed using one-way ANOVA, and statistical significance was defined as $p < 0.05$.

Results

Growth analysis of *C. glutamicum*

The growth behavior of *Corynebacterium glutamicum* was monitored in different nutrient media by measuring optical density at 620 nm over a 15-day period [Figure-1]. During the initial growth phase, a gradual increase in OD was observed over the first 5–7 days, indicating a prolonged adaptation period to the culture conditions. After reaching the peak growth phase, OD values declined from day 15 onward, reflecting the onset of the stationary phase and subsequent decline phase. The extended adaptation period and delayed logarithmic phase are likely attributable to the complexity of the carbon sources and the metabolic adjustments required for their efficient utilization. Average growth values in each medium are summarized in Table 2, illustrating differences in bacterial proliferation across carbon sources and pH conditions.

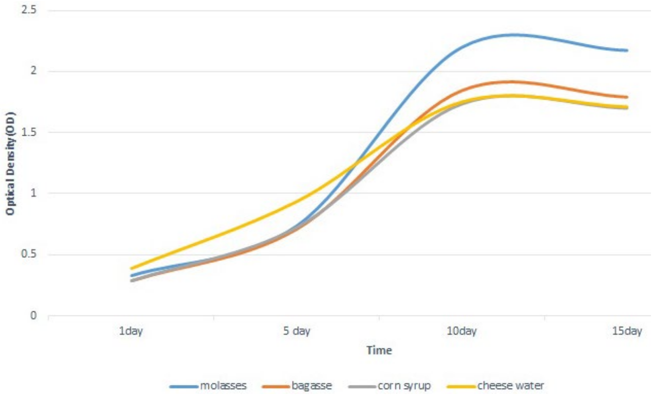

Figure-1. Growth curves of *Corynebacterium glutamicum* cultured in nutrient media supplemented with molasses, bagasse, corn syrup, and cheesewater.

Table-2. Growth of *C. glutamicum* in different culture media at varying pH values (OD620)

Culture	Bagasse			Molasses			Corn syrup			Cheese water		
pH	7.3	7.4	7.5	7.3	7.4	7.5	7.3	7.4	7.5	7.3	7.4	7.5
Day 1	0.263	0.195	0.405	0.358	0.2587	0.293	0.273	0.229	0.349	0.227	0.280	0.656
Day 5	0.802	0.715	0.613	0.651	0.744	0.814	0.653	0.732	0.779	0.997	0.823	0.989
Day 15	2.040	1.645	1.851	2.217	2.225	2.143	1.485	1.910	1.835	1.707	1.877	1.677

HPLC analysis of threonine production

Chromatographic analysis revealed that L-threonine production varied significantly depending on the carbon source [Table-3]. The highest yields under laboratory conditions were observed in cheesewater (39,393.8 mg/L), followed by molasses (18,263 mg/L), bagasse (6,017.7 mg/L), and corn syrup (6,843.2 mg/L). Cheesewater demonstrated the most pronounced effect on threonine synthesis, achieving substantially higher concentrations than other tested substrates. Threonine quantification was performed using standard chromatographic protocols according to the SOP of Atahrdaneh Company. These findings confirm the suitability of cheesewater as an effective carbon source for enhancing threonine production.

Table-3. Threonine production in different culture media measured by HPLC (mg/L)

Row	Amino acid	Carbon source	pH	Threonine concentration	Unit
1	L-threonine	Molasses	7.3	8,617	PPM
2	L-threonine	Molasses	7.4	13,500	PPM
3	L-threonine	Molasses	7.5	18,263	PPM
4	L-threonine	Corn syrup	7.3	5,176	PPM
5	L-threonine	Corn syrup	7.4	6,843.2	PPM
6	L-threonine	Corn syrup	7.5	6,010.5	PPM
7	L-threonine	Bagasse	7.3	16,219	PPM
8	L-threonine	Bagasse	7.4	12,118	PPM
9	L-threonine	Bagasse	7.5	6,017.7	PPM
10	L-threonine	Cheesewater	7.3	39,393.8	PPM
11	L-threonine	Cheesewater	7.4	27,356.9	PPM
12	L-threonine	Cheesewater	7.5	33,063.9	PPM

Real-Time PCR Analysis of *thrB* and *thrC* Expression

The expression levels of the threonine biosynthetic genes *thrB* and *thrC* were assessed by real-time PCR. Cultures grown in cheesewater-supplemented medium exhibited significantly higher gene expression compared to the standard medium. Specifically, *thrB* expression increased from 1.091 ± 0.002 in the control medium to 1.103 ± 0.003 in cheesewater medium ($p < 0.0001$). Similarly, *thrC* expression rose from 1.085 ± 0.003 to 1.094 ± 0.004 under the same conditions ($p < 0.00017$). These results indicate that cheesewater stimulates transcription of both key genes in the threonine biosynthetic pathway, correlating with enhanced L-threonine production [Figure-2].

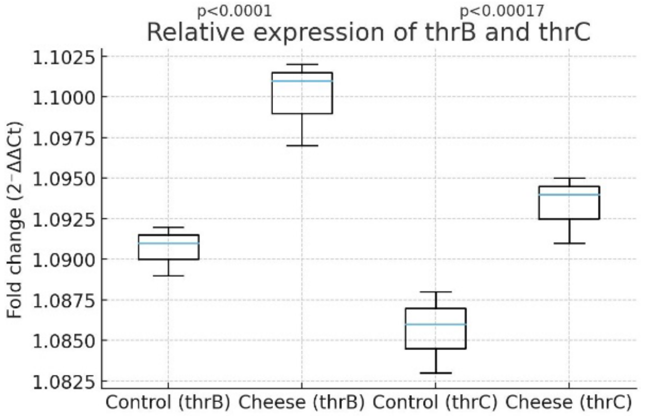

Figure-2. Relative fold change ($2^{-\Delta\Delta Ct}$) of *thrB* and *thrC* gene expression in standard ISP2 medium and cheesewater-supplemented medium. Statistical analysis was performed using a two-tailed t-test. Error bars represent the variability of replicate measurements.

Discussion

L-threonine production by *C. glutamicum* was strongly influenced by the type of carbon source incorporated into the culture medium. In this study, several alternative and low-cost substrates were evaluated, and significant differences in threonine yield were observed among cheesewater, molasses, bagasse, and corn syrup.[22]

Among these substrates, cheesewater yielded the highest L-threonine concentration under laboratory conditions.[23] This superior performance may be attributed to its biochemical composition, which includes lactose as a primary carbon source, as well as vitamins, minerals, free amino acids, and other bioactive compounds.[24] Lactose provides a sustained carbon supply through gradual hydrolysis into glucose and galactose, supporting balanced cellular metabolism.[25] Additionally, growth-promoting components in cheesewater may enhance overall cellular fitness, indirectly favoring metabolic flux toward threonine biosynthesis.

In contrast, although molasses and bagasse are widely employed as economical substrates in amino acid fermentation, their complex composition and potential inhibitory constituents may limit their efficiency under the tested conditions. Chromatographic analysis corroborated these observations, demonstrating that the highest threonine accumulation (39,393.8 ppm) occurred in cheesewater-supplemented medium at pH 7.3. Lower threonine levels were observed in molasses, bagasse, and corn syrup media, highlighting the critical role of carbon source composition in determining threonine yield.[26]

Growth analysis further indicated that *C. glutamicum* maintained stable proliferation in cheesewater-containing medium, particularly during the logarithmic phase, coinciding with enhanced threonine production. This alignment between growth and metabolite accumulation suggests that cheesewater provides a favorable metabolic environment for threonine biosynthesis.

To elucidate the molecular basis of enhanced production, the expression of the key biosynthetic genes *thrB* and *thrC* was examined using real-time PCR.[27] Results revealed a modest but significant increase in gene expression: *thrB* expression rose approximately two-fold, while *thrC* expression showed a smaller increase relative to the standard ISP2 medium. Although these changes are moderate, they likely contribute to elevated threonine levels when combined with improved substrate availability, enzyme efficiency, and potential post-transcriptional regulation.

It is important to recognize that threonine biosynthesis is regulated at multiple levels, and product yield does not necessarily require large fold changes in individual gene expression. A plausible mechanistic explanation for the observed enhancement involves improved intracellular metabolic balance.[28] The additional amino acids and micronutrients present in cheesewater may reduce cellular demand for de novo synthesis, allowing more resources to be directed toward the aspartate pathway from which threonine is synthesized.[29] Moreover, lactose-derived carbon sources may modulate central carbon metabolism differently from simple sugars, potentially increasing precursor availability for threonine biosynthesis.[30]

Overall, these findings suggest that cheesewater represents a promising, low-cost carbon source for augmenting L-threonine production by *C. glutamicum* under laboratory conditions. Nevertheless, further optimization and mechanistic studies are necessary to evaluate its applicability for industrial-scale production.[31]

It should be noted that this study evaluated only two genes within the threonine biosynthetic pathway (*thrB* and *thrC*), while other key regulatory genes, such as *thrA* and *lysC*, were not assessed.[32] Future studies incorporating broader gene expression profiling, enzyme activity measurements, and robust experimental designs are essential to fully elucidate the mechanisms underlying enhanced threonine production.

Conclusions

The present study demonstrates that natural substrates such as cheesewater can serve as safe and cost-effective carbon sources for optimizing threonine production. These findings underscore the significant impact of medium composition and physicochemical conditions on L-threonine biosynthesis by *C. glutamicum*.

Practical points in Biochemistry/Nutrition:

► Cheesewater (dairy effluent) is a superior, low-cost carbon source that significantly enhances microbial L-threonine production and upregulates the key biosynthetic genes *thrB* and *thrC*, offering a practical strategy for optimizing amino acid fermentation.

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Competing interests

The authors declare that they have no competing interests.

Abbreviations

cDNA: Complementary DNA; DNase: Deoxyribonuclease; HPLC: High-Performance Liquid Chromatography; ISP2: International Streptomyces Project Medium 2; OD: Optical Density; PCR: Polymerase Chain Reaction; RT-PCR: Real-Time Polymerase Chain Reaction; SOP: Standard Operating Procedure; UV-VIS: Ultraviolet-Visible (spectroscopy); C. glutamicum: Corynebacterium glutamicum E. coli: Escherichia coli; B. subtilis: Bacillus subtilis.

Authors' contributions

N.D. and N.A. wrote the initial draft of the manuscript. L.R. and A.D. designed the study. A.E., A.SH., P.B., and A.F. contributed to result interpretation. All authors read and approved the final manuscript. All authors take responsibility for the integrity of the data and the accuracy of the data analysis.

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Availability of data and materials

The data used in this study are available from the corresponding author on request.

Ethics approval and consent to participate

This study was conducted following ethical approval from the Ethics Committee of Tabriz University, Tabriz, Iran (Reference number: 961101-114-1851-35-3). Informed consent was not applicable.

Consent for publication

By submitting this document, the authors declare their consent for the final accepted version of the manuscript to be considered for publication.

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