ORGINAL RESEARCH ARTICLE

Identification of the *met* Gene and Kinetic Analysis of Methionine Synthesis in Soil Thermophilic Bacilli

Mona Mohammadi[1], Sarvenaz Falsafi[1], Kumars Amini[2]

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Abstract

Background Methionine, an essential sulfur-containing amino acid, plays a critical role in detoxification via methylation and must be obtained through the diet, as humans cannot synthesize it. This study aimed to identify the met gene and analyze the kinetics of methionine synthesis in soil-derived thermophilic bacteria.

Methods Two hundred soil samples were collected from tree-adjacent areas along Khordin Boulevard, Tehran. After heat treatment and culturing, isolates underwent microscopic, biochemical, and molecular characterization. DNA was extracted using a specialized kit, and the met gene was identified via multiplex PCR and gel electrophoresis. Gene expression was quantified using quantitative real-time PCR (qRT-PCR) under three temperatures (25°C, 35°C, 45°C) with ammonium nitrate (nitrogen source) and glucose (carbon source). Data were analyzed by one-way ANOVA using SPSS v.22 and reported as mean ± SD at a significance level of $p < 0.05$.

Results Bacilli accounted for 30% of isolates from Tehran's urban soil. The met gene was detected in only 6.66% of bacilli. Significant differences in met expression were observed between treated and control groups across all temperatures and nutrient conditions ($p < 0.05$). The most excellent suppression of met expression occurred at 45°C (2.342-fold decrease), while 25°C showed the least reduction (1.649-fold). Glucose and ammonium nitrate synergistically reduced expression (1.914- and 1.834-fold, respectively).

Conclusion Temperature and carbon/nitrogen sources modulate met gene expression, thereby influencing methionine synthesis in soil bacilli. Optimal suppression occurred at 45°C with a combination of glucose/ammonium nitrate, suggesting environmental regulation of this metabolic pathway. In contrast, the lowest suppression of met expression was observed at 25°C.

Keywords Bacillus Thermophilus, Methionine, Gene Expression, Ammonium Nitrate, Soil Microbiology

✉ Mona Mohammadi
monamohammadipyn78@gmail.com

1. Department of Microbiology, Faculty of Basic Sciences, Medical Sciences Branch, Islamic Azad University, Tehran, Iran
2. Department of Microbiology, Faculty of Basic Sciences, Saveh Branch, Islamic Azad University, Saveh, Iran

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1 Introduction

Members of the genus Bacillus exhibit remarkable resistance to environmental stressors, including drought, water scarcity, ultraviolet radiation, and nutrient-deficient conditions, owing to their unique physiological traits. The phylum Firmicutes comprises several families, such as Bacillaceae and Paenibacillaceae. Bacillus species and related genera are ubiquitous, thriving in diverse natural and anthropogenic environments.[1]

The global demand for amino acid production is steadily increasing. To develop a cost-effective microbial process for methionine production, high-yielding strains with efficient biosynthetic capabilities must be identified. Although Bacillus species display diverse and industrially valuable properties, bacterial isolates from distinct geographic regions may share close relationships. Thermophilic Bacillus species have been isolated not only from hot springs but also from compost and fruit juices. Many Bacillus strains produce industrially significant enzymes in substantial quantities, such as α-amylase (for starch hydrolysis) and subtilisin protease (used in detergents).[2]

Several essential amino acids, including lysine, isoleucine, valine, and methionine, are currently produced industrially through microbial fermentation. Given the cost-effectiveness of fermentation for amino acid production, there is considerable interest in developing microbial processes for commercial methionine synthesis. However, methionine fermentation differs significantly from conventional methods used for ethanol, lactic acid, or citric acid production.[3]

Methionine biosynthesis is an energy-intensive and ATP-dependent process, leading microorganisms to produce only the minimal amounts required for growth. Microorganisms with simpler regulatory mechanisms are preferable candidates for methionine production. Bacillus subtilis, a prominent species within this genus, can synthesize and secrete lipopeptides, particularly surfactins and mycosubtilins. Strains of B. subtilis isolated from marine sponges (e.g., WS1A and YBS29) have demonstrated the ability to produce multiple antimicrobial peptides. Generally, Bacillus species exhibit limited divergence in their 16S rRNA gene sequences, and this minimal genetic variation shows weak correlation with phenotypic traits.[4]

L-amino acids are widely used in food and feed biotechnology, serving as key intermediates in the chemical industry. Essential amino acids are critical for parenteral nutrition in humans, while L-glutamate and its salts act as flavor enhancers in food products. The expanding market for L-amino acids—driven by global population growth and rising demand for animal-derived products—necessitates strain optimization and process intensification to enhance production efficiency.[5]

Currently, the amino acid fermentation industry produces over five million tons annually, with production volumes steadily increasing. Methionine, alongside cysteine, is one of the two sulfur-containing essential amino acids required for protein synthesis. Like humans, animals rely on dietary methionine. Microbial methionine production has been reported in 16 species, including Bacillus, Pseudomonas, Arthrobacter, and Micrococcus. Since plant-based proteins are often deficient in methionine, vegetarian diets may fail to meet nutritional requirements. Methionine deficiency is linked to disorders such as toxemia, rheumatic fever, muscle paralysis, hair loss, depression, schizophrenia, liver degeneration, Parkinson's disease, and growth impairment.[6]

Sustainable, cost-effective microbial production of L-methionine from renewable resources has gained prominence. However, research on optimizing critical parameters (temperature, pH, incubation time, and agitation rate) remains limited. Fine-tuning these parameters could significantly boost methionine yields in soil-derived bacteria.[7]

Methionine supplementation in animal feed enhances reproductive performance, meat/egg quality, and immune function while mitigating immunological stress. It aids detoxification by methylating toxins or drugs and is the first limiting amino acid in commercial feed. Additionally, methionine acts as a fat-metabolizing agent by regulating protein balance, supporting methyl group transfer, and participating in the synthesis of choline, betaine, and folate/vitamin B12 metabolism.[8, 9]

Soil microbes, especially Bacillus species, are primary methionine producers. These spore-forming, rod-shaped bacteria thrive in soil environments.[10] Among bacteria, soil-derived Bacillus strains are top candidates for L-methionine production. Metabolic engineering—using modified strains with streamlined pathways—has shown promise. This study focuses on identifying the met gene and analyzing the kinetics of methionine biosynthesis in Bacillus isolates. Globally, major producers are transitioning from DL-methionine (mixed form) to bacterial L-methionine, reflecting rapid market growth. This study aimed to identify the met gene and analyze the kinetics of methionine synthesis in soil-derived thermophilic bacteria.

2 Methods

Sample Collection

A total of 200 soil samples were collected from the rhizosphere of oak trees in Tehran, Iran, at depths of 0–10 cm. To select for spore-forming bacteria, samples were subjected to thermal pretreatment: 10 g of soil was suspended in 100 mL of distilled water and heated at 80°C for 10 min. Serial dilutions (10^{-1} to 10^{-6}) were prepared in sterile distilled water, and 1 mL aliquots were

plated on nutrient agar. Plates were incubated at 37°C for 24 hours, and colonies were purified via streak plating.

Identification of Suspected Bacillus Isolates

Out of the 200 soil samples collected (0–10 cm depth), biochemical assays were conducted to identify Bacillus species. These included catalase, oxidase, Simmons' citrate, Gram staining, nitrate reduction, starch hydrolysis, and bile salt tolerance tests.

DNA Extraction

Genomic DNA was extracted using the Sinagene DNA Extraction Kit (Iran), following the manufacturer's protocol. DNA quality was verified via spectrophotometry (A_{260}/A_{280} ratio).

16S rRNA Gene Amplification and Sequencing

To confirm the presence of Bacillus, PCR amplification was performed using standard primers targeting the 16S rRNA gene. The PCR products were visualized on a 1.5% agarose gel via electrophoresis. For sequencing the 16S rRNA gene in soil-isolated Bacillus strains, isolates harboring the gene were selected and their sequences amplified individually in 50 µL reaction volumes. Three microliters of each PCR product were loaded on a 1% agarose gel to verify the presence of specific bands and rule out false positives. Samples were sent to Takapo Zist Co. (Iran) with dry ice for sequencing. Primer tubes were sealed with parafilm to prevent evaporation. Upon receiving the sequences, genetic similarity with sequences in the NCBI database was assessed, and a phylogenetic tree was constructed. The 16S rRNA gene sequences were aligned using the ClustalW tool on the NCBI website.

Detection of the met Gene

- PCR primers: Custom primers targeting the met gene (see Table 1) were designed based on conserved regions in Bacillus spp.
- Amplification: Conditions matched 16S rRNA PCR, with an annealing temperature optimized for met-specific primers.
- Confirmation: Amplicons were electrophoresed (1% agarose) and sequenced for validation.

Table 1 Primer Sequences for 16S rRNA and met Genes [1]

Reference	PCR Product Size	Sequence	Primer	Target Gene
[1]	1400 bp	AGAGTTTGATCCTGGCTCAG / GACGGGCGGTGTGTACAA	Forward / Reverse	16S rRNA
Designed	1042 bp	ATGAGCATCACCCAGAAC / TCATTTTICCAAICTTCCAT	Forward / Reverse	*met*

Gel Electrophoresis

PCR products were separated on 1.5% agarose gels in 1× TBE buffer at 90 V for 1 hour. Gels were stained with ethidium bromide (0.5 µg/mL) for 10 minutes, destained in deionized water, and imaged using a gel documentation system.

Expression Analysis of the met Gene Under Various Conditions Using Real-Time PCR

Effect of Temperature on Gene Expression

Bacterial pre-cultures were prepared at a half McFarland turbidity standard and used in experiments examining the effects of temperature, nitrogen source, and carbon source. For temperature effects, cultures were incubated at 25°C, 35°C, and 45°C for 16 hours. Fresh cultures were used for RNA extraction.

Effect of Nitrogen Source (Ammonium Nitrate)

Pre-cultures were inoculated into media containing 1% ammonium nitrate and incubated for 16 hours. Cultures were used for RNA extraction.

Effect of Carbon Source (Glucose)

Pre-cultures were inoculated into media containing 1% glucose and incubated for 16 hours. Cultures were used for RNA extraction.

RNA Extraction

RNX reagent (Sinagene, Iran) was used for cell lysis. Samples were incubated at room temperature for 5 minutes to ensure complete cell lysis, after which they were further processed.

Real-Time PCR Protocol

Real-time PCR was conducted in 20 µL reaction volumes using the SMOBIO kit (South Korea; LOT: IQ11002108901), with specific reaction components and thermal cycling conditions detailed in Tables 2 and Table 3.

Table 2 Reaction Components for Real-Time PCR

Component	Volume
SYBR Green Master Mix	10 µL
Forward Primer	1 µL
Reverse Primer	1 µL
cDNA	1 µL
RNase-Free Water	7 µL

Data Analysis

Data were analyzed using the ΔΔCT method. Mean ± standard deviation values were reported, and statistical analysis was performed using one-way ANOVA in SPSS version 22.

$$\Delta CT_{treat} = CT_{gene-treat} - CT_{housekeeping-treat}$$
$$\Delta CT_{non-treat} = CT_{gene-non-treat} - CT_{housekeeping-non-treat}$$
$$\Delta\Delta CT = \Delta CT_{treat} - \Delta CT_{non-treat}$$
$$Fold\ Change = 2^{-\Delta\Delta CT}$$

Table 3 Real-Time PCR Thermal Profile

Step	Temperature	Duration
Initial Denaturation	95°C	3 min
Denaturation	95°C	30 sec
Annealing	59°C	30 sec
Extension	72°C	30 sec
Final Extension	72°C	5 min
Number of Cycles	40–45	

3 Results

Isolation and Identification of Bacillus Strains

After heat treatment and serial dilution culture, 200 collected samples underwent biochemical testing to identify Bacillus species. Samples displaying the characteristics outlined in Table 4 were identified and isolated as *Bacillus* spp.

Table 4 Differential biochemical tests used for Bacillus identification

No.	Test	Bacillus Reaction
1	Gram Staining	Positive
2	Methyl Red	Positive
3	Voges-Proskauer	Positive
4	Simmons Citrate	Positive
5	Triple Sugar Iron Agar	Positive
6	Catalase	Positive
7	Starch Hydrolysis	Positive
8	Oxidase	Negative
9	Nutrient Agar	Grayish-white colonies

Of the samples examined, 60 isolates (30%) were identified as Bacillus. The distribution of these isolates is illustrated in Figure 1.

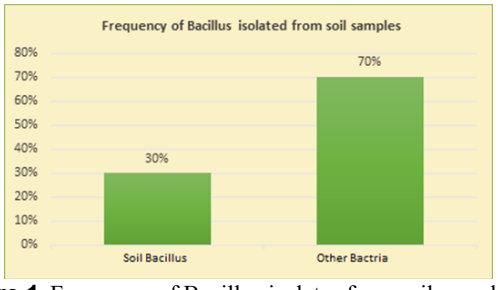

Figure 1 Frequency of Bacillus isolates from soil samples

Molecular Identification of Bacillus Strains

All 60 confirmed samples were subjected to PCR analysis using the 16S rRNA gene and were molecularly confirmed as belonging to the genus Bacillus. The electrophoresis gel results for selected positive samples are shown in Figure 2.

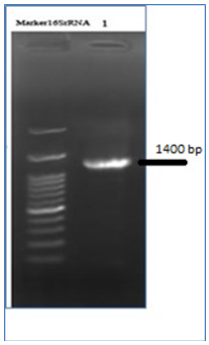

Figure 2 Molecular identification of soil Bacillus strains using 16S rRNA primers; lane 1: 1400 bp band corresponding to Bacillus

Detection of the Methionine Gene

Bacillus isolates were analyzed for the methionine amino acid gene using PCR, and the amplified products were evaluated through gel electrophoresis. The results are presented in Figure 3 and Figure 4.

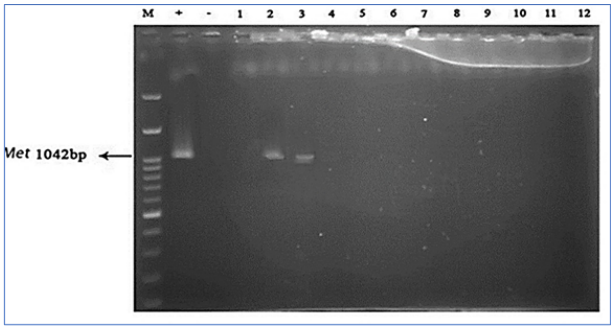

Figure 3 Electrophoresis of Bacillus isolates for the met gene (samples 1–12). Lane M: marker (100–2000 bp); lanes + and −: positive and negative controls; lanes 1–12: test samples. Samples 2 and 3 were positive.

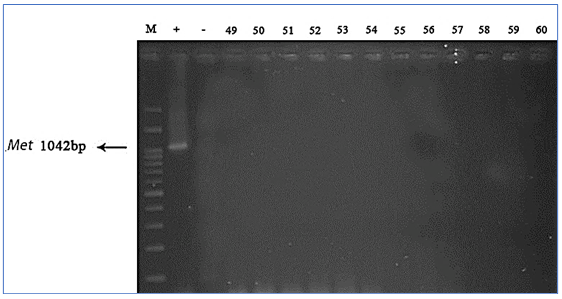

Figure 4 Electrophoresis of Bacillus isolates for the met gene (samples 49–60). No amplification of the met gene was observed in these samples.

Based on electrophoresis results, the frequency of the met gene among tested Bacillus isolates was 66.6%, as shown in Table 5.

Table 5 Frequency of the methionine gene in Bacillus isolates

Gene	Total Samples	Positive Samples	Frequency (%)
met	60	4	66.6

Sequencing Results of the 16S rRNA PCR Product

The PCR products of the methionine gene were sent to Bioneer for sequencing. The sequencing results are displayed in Figure 5.

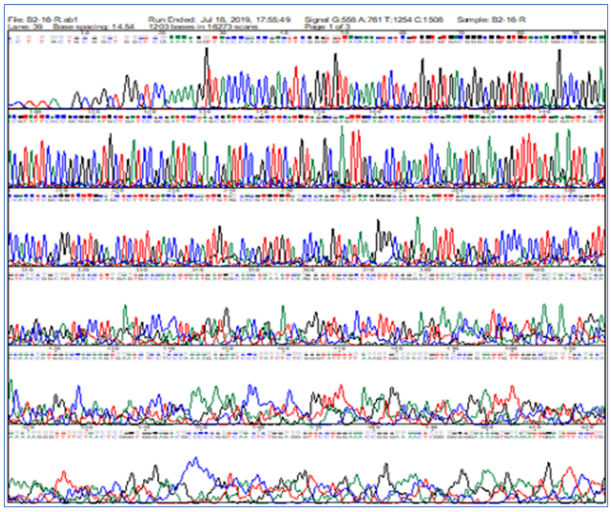

Figure 5 Sequencing results of the met gene PCR product

Phylogenetic Tree Construction

Phylogenetic analysis was conducted using ClustalX and MEGA5 software with the Neighbor-Joining method. A phylogenetic tree was constructed based on 16S rRNA sequences to analyze evolutionary relationships. As shown in Figure 6, Bacillus subtilis and Bacillus cereus clustered together with 100% similarity, indicating a close phylogenetic relationship.

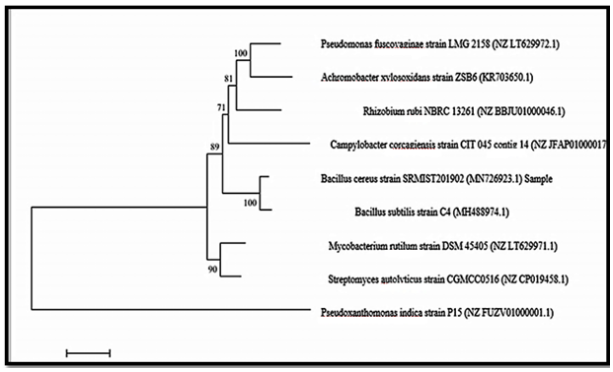

Figure 6 Phylogenetic tree based on 16S rRNA gene sequences

Gene Expression Analysis of the Methionine Gene via Real-Time PCR

RNA Extraction Validation

RNA integrity and quality were assessed using gel electrophoresis. The corresponding electrophoresis image is shown in Figure 7.

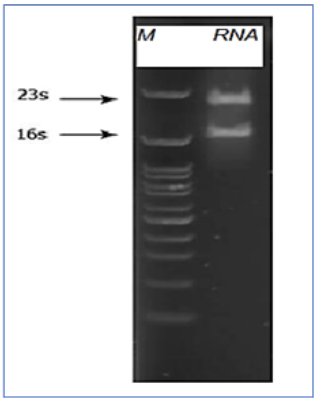

Figure 7 RNA validation from the tested samples

Melting Curve Analysis

Each gene produces a unique melting curve, visualized using SYBR Green dye during Real-Time PCR. The target met gene was confirmed by the uniform melting peaks across all samples at 85.37°C, indicating specific amplification and absence of nonspecific products (Figure 8).

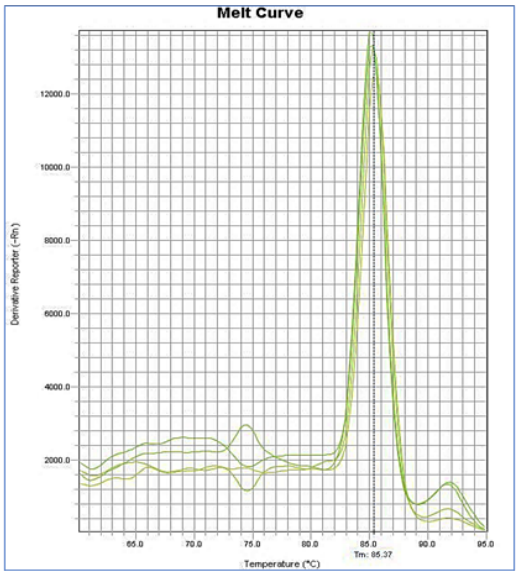

Figure 8 Melting curve of met gene PCR products

Effect of 25°C on Gene Expression

Amplification Curve

Amplification curves were analyzed based on the CT (threshold cycle) value. A lower CT indicates higher gene expression. Amplification curves are shown in Figure 9.

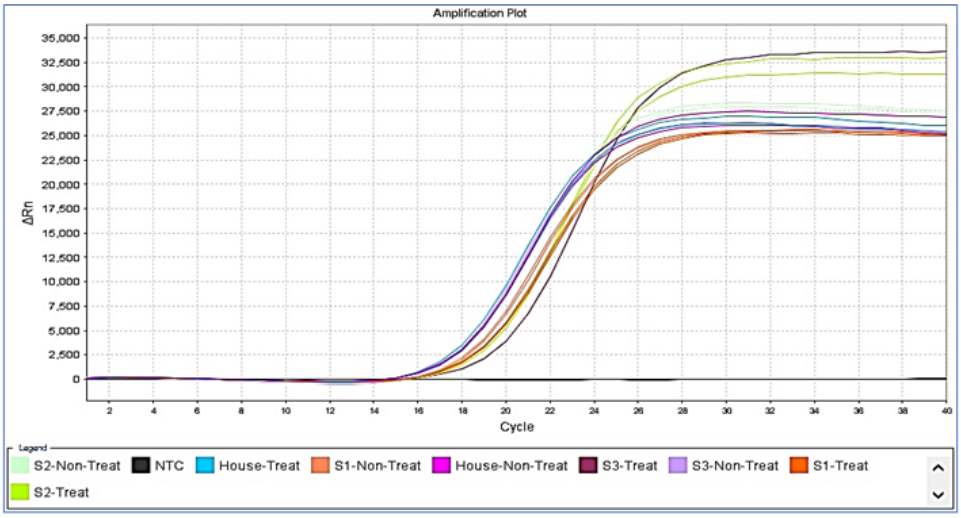

Figure 9 Amplification of met gene in treated and untreated Bacillus samples at 25°C

Expression levels were ordered from highest to lowest as follows:

House-Treat > House-Non-Treat > S3-Non-Treat > S2-Non-Treat > S1-Non-Treat > S1-Treat > S2-Treat > S3-Treat

Detailed CT and fold change results are provided in Tables 6, Table 7 and Table 8.

Table 6 Threshold Cycle (CT) Results for Internal Control Sample

Housekeeping Gene				
Average (CT)	**Sample Name**	**CT**	**CT**	**Average (CT)**
18.43	Treat	18.45	18.41	18.43
18.435	Non-Treat	18.41	18.46	18.435

Table 7 Threshold Cycle (CT) Results for Treated and Untreated Bacillus Samples (Gene: Met)

Gene (Met)			
Sample Name	CT	CT	Average (CT)
Sample 1 Treat	20.11	20.18	20.145
Sample 2 Treat	20.23	20.22	20.225
Sample 3 Treat	20.58	20.51	20.545
Sample 1 Non-Treat	19.48	19.41	19.445
Sample 2 Non-Treat	19.32	19.27	19.295
Sample 3 Non-Treat	19.15	19.19	19.17

Based on Figure 10, expression of the met gene decreased 2-fold in samples treated at 25°C compared to untreated controls, confirming the suppressive effect of thermal treatment.

Table 8 Fold Change Calculations (ΔΔCT Method)

ΔΔCT Method			
Sample Name	**ΔCT**	**ΔΔCT**	**Fold Change**
Sample 1 Treat	1.715	0.705	0.613442489
Sample 2 Treat	1.795	0.935	0.52304247
Sample 3 Treat	2.115	1.38	0.384218795
Sample 1 Non-Treat	1.01	0.14166667	0.720298431
Sample 2 Non-Treat	0.86	-0.0083333	1.065600502
Sample 3 Non-Treat	1.84	-0.1333333	1.302846093
Average Fold Change			0.5069013

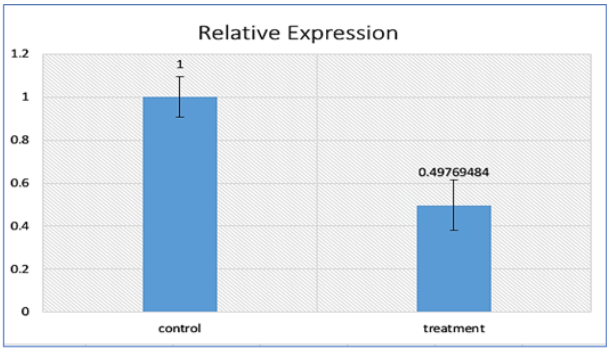

Figure 10 Relative met gene expression in Bacillus treated at 25°C vs. untreated

PCR reaction efficiency and slope were calculated using serial dilution and the Pfaffl method.

The slope and PCR efficiency for the met gene were determined using serial dilution method. Based on the standard curve calculations using the Pfalz method, the slope was -3.6671 and the PCR efficiency for met gene expression was 87.36784% (Figure 11).

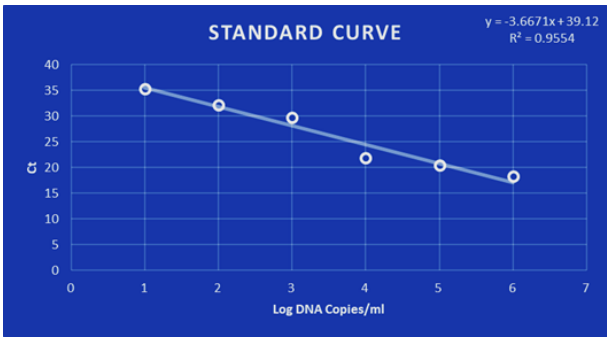

Figure 11 Determination of PCR Efficiency and Slope for the met Gene Using Serial Dilution Method

4 Discussion

Bacillus species are widely used in biotechnology for the production of various enzymes and as probiotics in animal feed. The collected samples were evaluated for Bacillus identification using biochemical tests after heat treatment and sequential culturing.[12] Among the analyzed samples, 60 (30%) were identified as Bacillus, while the remaining belonged to other genera. The prevalence observed in this study, as well as in previous research, suggests that variations may be due to factors such as regional differences, sampling periods, collection sites, and methods used for isolation and identification.

Genes involved in methionine biosynthesis and transport are regulated through different mechanisms across microbial species. In *Bacillales* and *Clostridia*, the S-box system, and in methionine-specific *Lactobacilli*, the T-box system, are RNA-dependent regulatory mechanisms controlling transcription termination via distinct pathways. The S-box RNA structure is stabilized by direct binding of S-adenosylmethionine (SAM), while the T-box senses amino acid deficiency through uncharged tRNAs.[13] In Gram-negative Enterobacteria such as *Escherichia coli (E. coli)*, regulation is DNA-dependent, involving the activator MetR and the repressor MetJ, which bind operator sites separate from the MET-box. [14] Increasing bacterial treatment temperature reduces enzyme kinetic activity and substrate affinity.

Anakwenze et al. (2021) optimized fermentation conditions for *Bacillus thuringiensis EC1* to enhance methionine production. They found that Zn^{2+} stimulated methionine production at all concentrations, while Mg^{2+} (0.1 µg/ml) and Ba^{2+} (10 µg/ml) improved yield. Submerged culture optimization resulted in a methionine yield of 3.18 mg/ml.[15]

Kharayat et al. (2022) increased L-methioninase production by optimizing *Pseudomonas stutzeri* culture conditions using artificial neural networks, achieving 285.63 U/L activity. Growth kinetics indicated non-growth-associated production, accompanied by an enhanced enzyme output.[16] Similarly, increased bacterial treatment temperature reduces enzyme activity and substrate affinity.

Yocum et al. (1996), in a study titled "Cloning and characterization of the metE gene encoding S-adenosylmethionine synthetase from Bacillus subtilis," found that the cloned gene complemented the *metE1* mutation and integrated at or near the chromosomal *metE1* locus. Expression of S-adenosylmethionine synthetase two was suppressed by exogenous methionine. Overexpression from a strong constitutive promoter led to methionine excretion in *Bacillus subtilis*, indicating that S-adenosylmethionine acts as a key regulatory hub for methionine biosynthesis in *B. subtilis*, as previously demonstrated in *Escherichia coli*.[17]

Alyousif et al. (2022) isolated and screened *Bacillus*-related species from Iraqi soil samples. PCR-based identification of 43 isolates revealed the presence of *Bacillus, Cytobacillus, Priestia, and Peribacillus*, with *B. subtilis* and *B. megaterium* being the most prevalent. [18] Their reported Bacillus prevalence was higher than in this study, indicating regional distribution differences. Again, higher bacterial treatment temperature reduces enzyme activity and substrate binding.

Environmental factors, such as temperature, soil pH, pesticide residues, and organic matter, influence the abundance and diversity of *Bacillus*. Sediment type, climate, and microbial interactions also affect colonization.[19]

Auta et al. [20] and Ayandiran et al. [21] used similar methodologies but reported differing Bacillus prevalence rates (67.1% vs. 8.9%). In another study, only 15% of 400 forest soil samples contained *Bacillus*. Temperature has a critical effect on cellular processes, necessitating bacterial thermal sensing mechanisms, including methyl-accepting proteins and heat shock responses.[22] *E. coli* grows optimally near 40°C, with growth rates increasing with temperature within the 20–37°C range, resembling a chemical reaction.[23]

5 Conclusion

The current lack of a comprehensive understanding of methionine biosynthesis and its feedback regulation has hindered the development of commercial production processes. This study evaluated the impact of different incubation temperatures (25°C, 35°C, and 45°C) on the expression of the met gene in Bacillus. The results revealed a temperature-dependent suppression of met gene expression, with reductions of 1.914-fold at 25°C, 2-fold at 35°C, and 2.342-fold at 45°C relative to untreated controls. These findings demonstrate that elevated temperatures significantly downregulate the met gene expression. While prior research has predominantly examined the effects of temperature on bacterial virulence

factors, this study provides evidence that increased temperatures also diminish enzymatic kinetic activity and substrate binding affinity.

Phylogenetic analysis placed Bacillus subtilis and Bacillus cereus within the same clade with 100% bootstrap support, confirming their close evolutionary relationship. Significant correlations were observed between met gene expression levels in treated and untreated samples across all temperature conditions. The study further identified glucose (a carbon source) and ammonium nitrate (a nitrogen source) as additional regulatory factors. Among the tested temperatures, 45°C induced the most substantial reduction in both met gene expression and enzymatic activity, while 25°C exhibited the least pronounced effect.

Declarations

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Authors' Contributions
All authors contributed to the initial conceptualization, study design, data collection, and drafting of the manuscript. All authors have read and approved the final version of the manuscript and declare no conflicts regarding any part of the study.

Availability of Data and Materials
The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Conflict of Interest
The authors declare that there is no conflict of interest.

Consent for Publication
All authors have read and approved the final manuscript and have provided their consent for publication.

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Ethical Considerations
Ethical principles were adhered to in accordance with institutional guidelines. This study was approved by the Ethics Committee of the Islamic Azad University, Tehran Medical Sciences Branch, under the code IR.IAU.PS.REC.1403.159.

Artificial Intelligence Disclosure
The authors declare that this manuscript was prepared without the use of AI tools.

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