

# Amelioration of L-methionine production by *Alcaligenes faecalis* ATCXT3624: Empirical optimization of culture conditions

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## ABSTRACT

**Background:** L-methionine production at the industrial scale suffers from various drawbacks, including the production and separation of the L-enantiomer of the amino acid methionine by chemical production methods. This strain exhibiting resilience to feedback inhibition by L-methionine provided a promising avenue for enhanced production. The present study has tried to uplift the production rate of the amino acid by a developed L-methionine-resistant strain *Alcaligenes faecalis* ATCXT 3624 through surpassing feedback inhibition. **Methods:** Empirical optimization of all fermentation conditions was studied by observing the effects of initial pH, temperature, inoculum age, medium volume, cell density, and different micronutrients, which enhanced L-methionine production. In addition, efforts were made to find suitable nitrogen and carbon sources. **Results:** Eventually,  $23.8 \pm 0.22$  mg/mL of L-methionine was obtained under optimized fermentation conditions, which is significantly ( $p < 0.05$ ) higher than that produced before optimized fermentation conditions. **Conclusion:** Eventually, it can be assumed that the strain effectively produced the amino acid at an enhanced rate under optimized conditions.

**Keywords:** Fermentation, L-methionine, *Alcaligenes faecalis* ATCXT 3624, Optimization.

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## INTRODUCTION

L-methionine is a crucial sulfur-containing amino acid necessary in the diets of both humans and mammals to support normal growth and the proper functioning of the body's metabolism.<sup>1</sup>

The demand for L-methionine (L-Met) has surged in recent years, driven by the global expansion of the feed additive market, which is being propelled by the increasing worldwide consumption of meat and dairy products as primary sources of protein and essential nutrients.<sup>2</sup>

Methionine is typically manufactured using chemical and enzymatic processes, both of which are costly. The chemical method involves using hazardous substances like acrolein, methyl mercaptan, and hydrocyanic acid, while the enzymatic approach necessitates expensive enzymes. However, the commercial production of L-methionine has not been realized due to feedback inhibition and repression<sup>3</sup> issues.

The identification of glutamic acid-producing bacteria ultimately paved the way for fermentation techniques in the production of a wide range of other amino acids,<sup>4</sup> including lysine, threonine, isoleucine, and histidine, which have effectively been synthesized by fermentation processes.<sup>5-7</sup> Hence, the exploration of cost-effective and environmentally sustainable production of pure L-methionine using microorganisms and naturally renewable resources is gradually drawing the scientific community's attention at a significant perspective.<sup>2</sup>

Development of a resistant strain of *Alcaligenes faecalis* ATCXT3624 overcoming feedback inhibition by L-methionine has already been ventured.<sup>8</sup> Moreover, there is a scarcity of research focused on optimizing key parameters (such as pH, temperature, age of inoculum, incubation time, cell

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density, carbon concentration, nitrogen concentration,  $\text{KH}_2\text{PO}_4$ ,  $\text{K}_2\text{HPO}_4$ ,  $\text{CaCO}_3$ , and  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ) for L-methionine production.

This study aimed to develop and optimize the production of L-methionine by a developed resistant strain of *A. faecalis* ATCXT3624 strain derived from freshly obtained soil samples.

## MATERIALS AND METHODS

### Sample Preparation

L-methionine over producer strain *A. faecalis* ATCXT3624 was developed from an *A. faecalis* strain isolated from soil samples collected from Sambalpur district near Hirakud dam (Odisha), India (Latitude: 21° 31' 12.00 N and Longitude: 83° 52' 12.00" E).<sup>8</sup>

### Effects of Physical Parameters

#### Effects of initial pH

The impact of initial pH on the strain was assessed using 3.8 to 6.67 mg/mL of biomass of yeast extract peptone dextrose

(YEPD) broth solution. The temperature was held constant at 30°C. The experiment encompassed a pH range of 6 to 8 and was monitored with a digital pH meter (CL110-chemLine). Adjustments to pH were made with 0.1 M NaOH and 0.1 M HCl solutions.

#### *Effect of temperature*

The effect of temperature on L-methionine production was assessed within the 25 to 33°C temperature range using a BOD incubator (Model Number: ENT-B-01 with a temperature range of 5–50°C). The biomass dosage employed was 3.26 to 6.8 mg/mL, dissolved in yeast extract peptone dextrose broth.

#### *Effect of inoculum age*

The strain was incubated for various durations, ranging from 12 to 72 hours. The biomass dosage employed ranged from 3.43 to 6.65 mg/mL of media in the study.

#### *Effect of medium volume*

The strain was incubated with various volumes of Yeast Extract Peptone Dextrose (YEPD) media, ranging from 20 to 26 mL. The study used a Biomass dosage from the 4.27 to 6.67 mg/mL range.

#### *Effect of cell density*

The strain was cultured with varying cell densities (ranging from  $2 \times 10^6$ – $12 \times 10^6$  cells/mL) in 100 mL of yeast extract peptone dextrose (YEPD) media. The biomass dosage employed in the study ranged from 3.92 to 6.7 mg/mL of media.

### **Selection of Carbon/Nitrogen Source**

The selection of appropriate carbon and nitrogen sources influencing enhanced L-methionine production has been carried out. Various carbon sources, including glucose, fructose, xylose, arabinose, ribose starch, and dextrin, were examined along with various nitrogen sources such as ammonium sulfate, urea, and ammonium nitrate. The basal medium for the study consisted of  $K_2HPO_4$  (0.1–0.4%),  $KH_2PO_4$  (0–0.4%),  $CaCO_3$  (0–0.2%) and  $MgSO_4 \cdot 7H_2O$  (0.02–0.03%). Various carbon and nitrogen sources were introduced to this basal medium. The medium was then inoculated and incubated under shaking conditions using a Remi RS-12 R shaker at 200 rpm and a temperature of 30°C for a duration of 48 hours. Following the incubation period, cell separation was achieved through centrifugation using a Remi c24BL centrifuge at 10,000 rpm for 10 minutes, and L-methionine levels were determined in the supernatant using a UV Spectrophotometer (Shimadzu UV-1280).

### **Effects of Micronutrients**

#### *Effect of $K_2HPO_4$*

The strain was cultured with varying concentrations of  $K_2HPO_4$  (ranging from 0.1–0.4%) in 100 mL of yeast extract peptone dextrose (YEPD) media. The biomass dosage employed in the study ranged from 6.89 to 7.83 mg/mL in 100 mL of media.

#### *Effect of $KH_2PO_4$*

The strain was exposed to various concentrations of  $KH_2PO_4$  (ranging from 0.0–0.4%) in 100 mL of YEPD media. The biomass dosage employed in the study ranged from 7.55 to 8.21 mg/mL in 100 mL of media.

#### *Effect of $CaCO_3$*

The strain was exposed to varying concentrations of  $CaCO_3$  (ranging from 0.0–0.2%) in 100 mL of YEPD media. The biomass dosage employed in the study ranged from 6.92 to 8.59 mg/mL in 100 mL of media.

#### *Effect of $MgSO_4 \cdot 7H_2O$*

The strain was exposed to varying concentrations of  $MgSO_4 \cdot 7H_2O$  (ranging from 0.02–0.03%) in 100 mL of YEPD media. The biomass dosage employed in the study ranged from 7.29 to 9.12 mg/mL in 100 mL of media.

### **Statistical Analysis**

All the experimental data was represented as Mean  $\pm$  SEM, with a sample size (n) of 6. A confidence level of 95% was considered in the present study. Hence, the factors having *p-values* less than 0.5 were considered significant in the present study. Student's t-test was conducted to determine the significance of the increase in L-methionine production after optimization of all criteria affecting L-methionine production by the strain.

## **RESULTS**

### **Effects of Physical Parameters**

#### *Effect of initial pH*

An experimental study spanning pH values from 6 to 8 investigated the impact of varying medium pH. The results revealed that the maximum L-methionine yield, reaching  $20.12 \pm 0.41$  mg/mL with a biomass dosage of  $6.67 \pm 0.43$  mg/mL, was achieved at a pH of 7.5 as demonstrated in Figure 1(A).

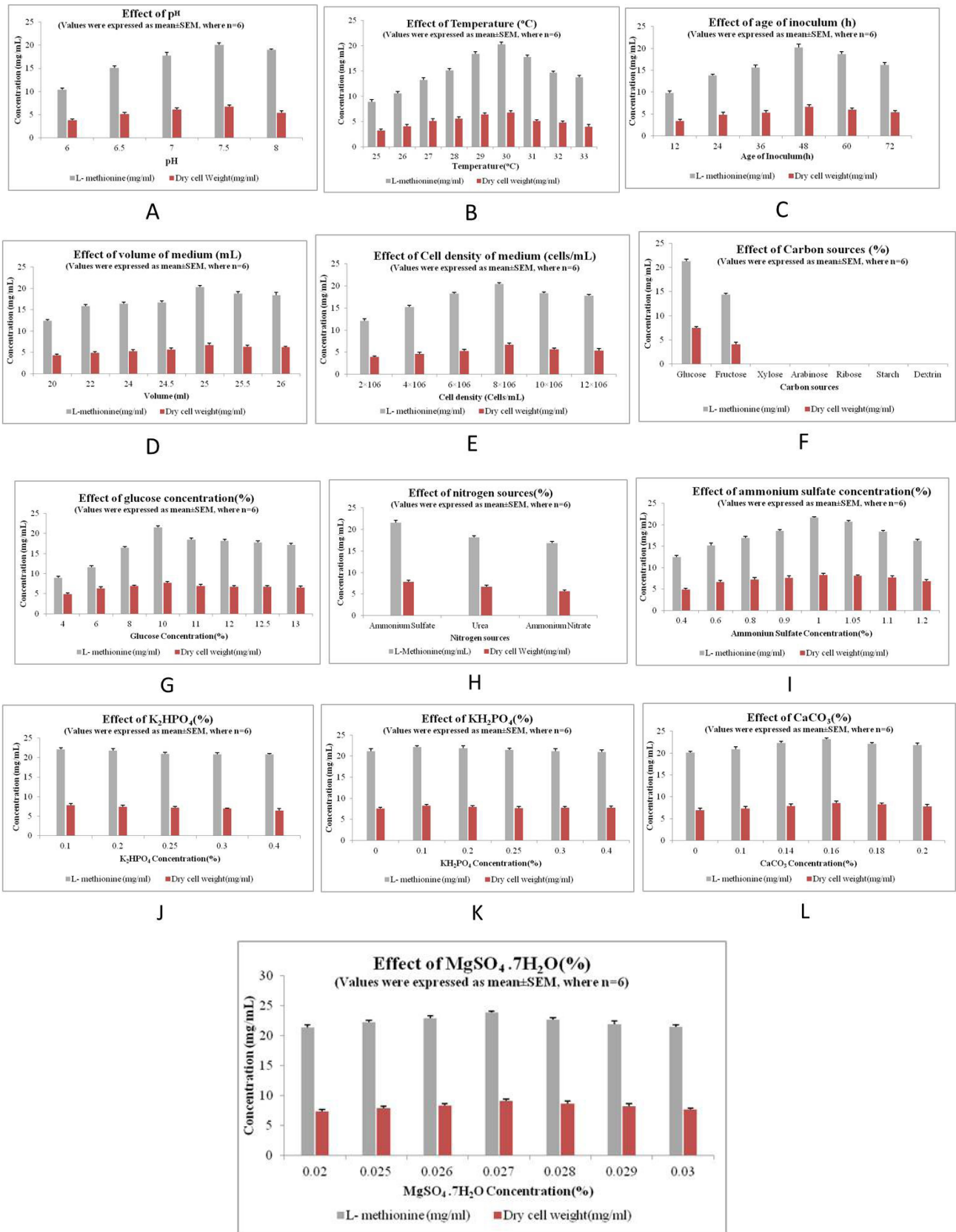
#### *Effect of temperature*

We examined the influence of temperature on L-methionine production by varying temperatures within the range of 2 to 33°C while maintaining all other variables constant. The highest yield of L-methionine, reaching  $20.26 \pm 0.46$  mg/mL with a biomass dosage of  $6.8 \pm 0.36$  mg/mL, was achieved at 30°C, as demonstrated in Figure 1(B).

#### *Effect of age of inoculum*

We examined the impact of incubation time on L-methionine production by varying the duration of fermentation from 12 to 72 hours, while holding all other variables constant. Intriguingly, the highest L-methionine yield, reaching  $20.27 \pm 0.76$  mg/mL with a biomass dosage of  $6.65 \pm 0.44$  mg/mL, was achieved after 48 hours of fermentation, as illustrated in Figure 1(C).

## Optimization of fermentation conditions for L-methionine production



**Figure 1:** Effect of (A) pH, (B) temperature (°C), (C) age of inoculum (h), (D) volume of medium (mL), (E) cell density (cells/mL), (F) carbon sources (%), (G) glucose concentration (%), (H) nitrogen sources (%), (I) ammonium sulfate (%), (J) K<sub>2</sub>HPO<sub>4</sub> (%), (K) KH<sub>2</sub>PO<sub>4</sub> (%), (L) CaCO<sub>3</sub> (%) and (M) MgSO<sub>4</sub>·7H<sub>2</sub>O (%) on L-methionine production by *A. faecalis* ATCXT 3624

### Effect of volume of medium

We examined the impact of the volume of the medium on L-methionine production by varying the volume of the medium from 20 to 26 mL while holding all other variables constant. Intriguingly, the highest L-methionine yield, reaching  $20.3 \pm 0.34$  mg/mL with a biomass dosage of  $6.67 \pm 0.5$  mg/mL, was achieved at 25 mL of medium after fermentation, as illustrated in Figure 1(D).

### Effect of cell density

We examined the impact of cell density on L-methionine production with varying cell densities ranging from  $2 \times 10^6$ - $12 \times 10^6$  cells/mL while holding all other variables constant. Intriguingly, the highest L-methionine yield reaching  $20.45 \pm 0.24$  mg/mL with a biomass dosage of  $6.71 \pm 0.41$  mg/mL was achieved at  $8 \times 10^6$  cells/mL of medium after fermentation, as illustrated in Figure 1(E).

### Selection of Carbon and Nitrogen Source

Selecting proper carbon and nitrogen sources plays a pivotal role in media composition. It is the most important step in governing microbial growth and its L-methionine production ability. Efficient microbial growth and metabolism depend on optimizing media components, focusing primarily on the carbon and nitrogen sources. Among all the carbon sources tested in our study, the strain showed optimized L-methionine production  $21.22 \pm 0.43$  mg/mL in glucose (10%) at a biomass dosage of  $7.44 \pm 0.28$  mg/mL Figure 1 (F), (G). The effectiveness of other carbon sources of L-methionine production in the following order:

L-methionine production by the strain was thereafter studied under various glucose concentrations from (4–13%). It was observed that maximum L-methionine production of  $21.51 \pm 0.38$  mg/mL occurred at 10% glucose concentrations, and above this concentration, the production decreased a little bit Figure 1 (G). Among the various nitrogen sources selected for the present study, ammonium sulfate (1%) showed maximum L-methionine production of  $21.63 \pm 0.48$  mg/mL under the biomass dosage of  $7.83 \pm 0.35$  mg/mL (Figure 1(H)). During the study of the efficacy of various ammonium sulfate concentrations (0.4–1.2%) of ammonium sulfate showed maximum L-methionine production of  $21.7 \pm 0.19$  mg/mL at a biomass dosage of  $8.29 \pm 0.37$  mg/mL Figure 1(I).

### Effects of Micronutrients

$K_2HPO_4$  and  $KH_2PO_4$  have important effects on microbial growth and L-methionine production. It has been observed that at 0.1%  $K_2HPO_4$  and  $KH_2PO_4$  concentrations, L-methionine production increased at  $22.07 \pm 0.47$  and  $22.13 \pm 0.31$  mg/mL at biomass dosages of  $7.83 \pm 0.38$  mg/mL and  $8.21 \pm 0.32$  mg/mL, respectively. Figure 1(J), (K). At 0.16%,  $CaCO_3$  L-methionine production increased further to  $23.13 \pm 0.35$  mg/mL at a biomass dosage of  $8.59 \pm 0.45$  mg/mL Figure 1(L).  $MgSO_4 \cdot 7H_2O$  (0.027%) has also influenced the L-methionine production further of  $23.8 \pm 0.22$  mg/mL at a biomass dosage of  $9.12 \pm 0.28$  mg/mL Figure 1(M).

## DISCUSSION

The microbial production of metabolites is greatly influenced by their growth pattern, which is governed by various physicochemical and nutritional conditions in the surrounding environment. The microbial growth phase or exponential phase constitutes a rapid growth phase named trophophase, followed by the production phase called idiophase.<sup>9</sup> The factors that have the most intriguing effect on microbial growth are pH, temperature, volume of media, and carbon and nitrogen supplementation in the media. Solution pH plays a vital role in governing the metabolic activity of any organism. *A. faecalis* is an alkali-tolerant bacterium that survives in an alkaline environment consisting of ammonia liberated from nitrogenous sources such as urea. Despite this capability, it still retains its cytosolic pH towards neutrality and protects its indispensable macromolecules driving its metabolic reactions.<sup>10</sup> Our finding in this regard is pH  $\sim 7.5$ , which supports the maximal production of L-methionine by the developed resistant strain. In the study by <sup>11</sup>, which documented a peak methionine yield of 2.13 g/L at pH 7, it was observed that many microorganisms thrive within the range of neutral pH values (6.5–7.5). However, certain microorganisms generate acids during their growth. The impact of varying pH levels on methionine production by *C. glutamicum* was investigated. To maintain the desired pH values, HCl and NaOH were used in conjunction with a pH digital meter. The strain was then inoculated into shake flasks at various pH levels and subsequently incubated on an orbital shaker for 48 hours. The results, as depicted in the aforementioned figure, clearly indicate that L-methionine production is influenced by pH. *A. faecalis* is basically a mesophilic bacterium, and the temperature (30°C) in which it showed maximal production of the amino acid also falls within the range. Metabolic activity increases with increasing temperature, but temperature optimization is needed. Interestingly, the data showed an increasing trend in L-methionine production as temperature increased, but it reached its maximum at 35°C. Beyond this point, higher temperatures led to a decline in L-methionine production due to their adverse effects on microorganisms. At elevated temperatures, enzymes operate at an accelerated pace, leading to denaturation. The ideal temperature of 35°C, as evidenced by our findings, aligns with the work of,<sup>12</sup> which also identified it as the optimal temperature for maximum L-methionine production. This contrasts with the findings of,<sup>11</sup> who reported a maximum of 2.16 g/L at 30°C. The age of culture also has a keen effect on the metabolite production capability of an organism under fermenting conditions. This result notably aligns with findings from previous studies conducted<sup>11,13</sup> who reported 4.55 mg/mL and 4.6 g/L, respectively, after the same 96-hour fermentation period. The correlation between methionine production and sugar consumption closely mirrors results previously documented.<sup>13,14</sup> According to <sup>15</sup>, the decline in L-methionine production observed in *Bacillus cereus* after four days can be

attributed to several factors, including the bacterial culture's age, sugar content depletion, and reduced availability of nitrogen in the fermentation medium. It's worth noting that microbial production of metabolites typically initiates after a lag phase of one day, with peak production occurring during the onset of the stationary phase or later in the fermentation process. A higher amount of media aggravates high microbial growth and activity in batch conditions where supplementation of fresh media doesn't occur. A higher media volume suffers from adequate oxygen supply, inhibiting microbial growth.<sup>11</sup> So, optimization of the volume of the culture media is mandatory. In general, studies show that L-methionine production increases with increasing volume of media.<sup>11</sup> Maximal L-methionine production has been reported to have a 30% volume of medium.<sup>11</sup> Similarly, inoculum size is also a crucial effector of microbial growth. Our study has shown 25% of the volume of media showed optimal L-methionine production by the developed resistant strain of *A. faecalis* ATCXT3624. Inoculum size also has a keen impact on microbial growth and metabolite production. The growth period gets enhanced with a small inoculum size, and a larger inoculum size reduces DO in the media with increased competition for nutrients.<sup>11</sup> The strain showed maximal L-methionine production ( $20.45 \pm 0.24$  mg/mL) at the inoculum size of  $8 \times 10^6$  cells/mL.

Proper nutrient composition is a quintessential component of any culture media supporting optimal microbial growth and activity. Proper selection of carbon and nitrogen sources is a quintessential step in optimizing microbial growth and its L-methionine production capability. The impact of micronutrients has already been evaluated. However, efficient microbial growth and its production capability depend on optimizing media components, especially on the carbon and nitrogen source.<sup>16</sup> Among all the carbon sources tested in our study, the strain showed maximal L-methionine production of  $21.22 \pm 0.43$  mg/mL in glucose (10%) at a biomass dosage of  $7.44 \pm 0.28$  mg/mL.

Among the various nitrogen sources selected for the present study, ammonium sulfate of 1% showed maximum L-methionine production ( $21.63 \pm 0.48$  mg/mL) under the biomass dosage of  $7.83 \pm 0.35$  mg/mL.

Apart from the carbon and nitrogen sources, the micronutrient composition in the medium also has quintessential effects on microbial growth and metabolism.  $K_2HPO_4$  and  $KH_2PO_4$  have important effects on microbial physiology. These two compounds act as buffers, helping maintain the medium's pH and playing a pivotal role in maintaining microbial viability and growth.<sup>17</sup> It has been observed that at 0.1%  $K_2HPO_4$  and  $KH_2PO_4$  concentrations, L-methionine production increases at a maximum of  $22.07 \pm 0.47$  and  $22.13 \pm 0.31$  mg/mL at biomass dosages of  $7.83 \pm 0.38$  and  $8.21 \pm 0.32$  mg/mL, respectively.  $CaCO_3$  at a concentration of 0.16% has aggravated L-methionine production of  $23.13 \pm 0.35$  mg/mL at a biomass dosage of  $8.59 \pm 0.45$  mg/mL. Eventually, adding 0.027%  $MgSO_4 \cdot 7H_2O$  also influenced the L-methionine

production with a maximum of  $23.8 \pm 0.22$  mg/mL at a biomass dosage of  $9.12 \pm 0.28$  mg/mL.  $Mg^{2+}$  acts as cofactors of various vital enzymes controlling cellular metabolism and viability.<sup>18</sup>

By using student's t-test for testing the significance of the increase in L-methionine production after the optimization process compared to the unoptimized one,<sup>8</sup> it can be shown that a significant increase ( $p < 0.05$ ) of L-methionine production has occurred under the optimized fermentation conditions.

The fermentation conditions and nutritional parameters for microbial production of L-methionine by *A. faecalis* ATCXT3624 using various carbon and nitrogen sources along with vital micronutrients were optimized in the current study. Among the carbon and nitrogen sources, glucose and ammonium sulfate were selected for maximum L-methionine production, and among the micronutrients,  $CaCO_3$  and  $MgSO_4 \cdot 7H_2O$  had maximum impacts on the fermentation process. Optimum values of the physical parameters of the fermentation conditions for L-methionine production were temperature 30°C, pH 7.5, volume ratio of medium 25 mL, cell density  $8 \times 10^6$  cells/mL, and age of inoculum 48 hours. At these optimum values, biomass and methionine production were  $9.12 \pm 0.28$  and  $23.8 \pm 0.22$  mg/L after 48 hours of fermentation. Thus, it can be concluded that the most effective L-methionine production ( $23.8 \pm 0.22$  mg/mL) will be achieved by maintaining all the optimum conditions and can be used effectively for fermentation on a large scale.

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## PEER-REVIEWED CERTIFICATION

During the review of this manuscript, a double-blind peer-review policy has been followed. The author(s) of this manuscript received review comments from a minimum of two peer-reviewers. Author(s) submitted revised manuscript as per the comments of the assigned reviewers. On the basis of revision(s) done by the author(s) and compliance to the Reviewers' comments on the manuscript, Editor(s) has approved the revised manuscript for final publication.