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Research Article

Investigation of Cysteine Production Capacities in Bacillus Species Isolated from Turkish Soil

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ABSTRACT

This study aimed to investigate the cysteine production capacities of microbial strains isolated from the environment (mainly soil). In total, 262 bacterial colonies were isolated from 47 soil samples collected from 7 different locations. Isolated bacterial colonies were first grown in tryptic soy broth to determine their cysteine production capacity. The amounts of cysteine produced by isolated bacteria varied from 0.3 to 2.0 g.L-1. Five out of 262 isolates produced a significant amount of cysteine. Based on 16S rRNA gene sequence analysis using the BLAST (basic local alignment search tool) database., the highest cysteine-producing strain was identified as Bacillus sp. strain CG1. Cysteine production potential was evaluated by adjusting the growth medium composition as well as incubations conditions. Sixteen different chemical compositions of growth media were evaluated. The incubation parameters adjusted to produce cysteine were temperature, pH, time, and agitation speed. The highest cysteine yield was 4.24 ± 0.47 g.L-1 under growth conditions of pH 7.5, 175 rpm, and 40 °C at 84 h. The recovery efficiency was found to be around 65% using an ion-exchange resin adsorbent.

1. Introduction

The demand for proteins and amino acids has been increasing due to the continuous increase of the world population [1]. Cysteine is one of the twenty natural amino acids and contains a sulfur atom in its chemical structure. Cysteine is a biologically important amino acid, crucial both for biological functions and in commercial applications. It is an EU-approved food additive (E920) and has been utilized in the food, pharmaceutical, and cosmetics industries [2, 3]. In the food industry, it has been used as a flavor enhancer and as a flour treatment agent to improve baking functionality [1, 3].

Reports indicate that cysteine can be produced through acid or alkali hydrolysis of human hair or animal byproducts, including hair, skin, feathers, or nails [4, 5]. This type of production of cysteine may cause some inconveniences for consumers having religious and/or special diet preferences. If animal-derived ingredients are used and originate from non-halal or otherwise non-permitted sources under Halal or Kosher standards [6], the final product may be considered doubtful or not permissible for consumption. [7, 8].

Although extraction from protein hydrolysates can be used for the industrial production of cysteine [9], it can also be produced by fermentation [1]. The microbial production of cysteine has the potential to lower production costs

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and reduce environmental burdens, particularly those related to wastewater treatment. Microbial cysteine may also be used in food products to fulfill the requirements of the vegetarian, vegan, and religious dietary practices. Microorganisms, medium composition, and process conditions such as pH, temperature, time, and agitation are the main parameters for microbial cysteine production. Previous studies have reported the production of cysteine by bacterial isolates in various fermentation media [1, 10, 11, 12, 13]. A few studies report genetically modified and engineered microorganisms to be used for the production of cysteine [3, 11, 14, 15, 16]. However, there is still a lack of information about an efficient fermentative production method of cysteine in the literature.

This study aimed to evaluate the cysteine production capacities of bacteria isolates collected from soil samples from different locations to identify the strain with the highest cysteine-production. Furthermore, optimization of growth conditions to obtain increased cysteine yield was studied. Unlike previous studies that largely focus on genetically engineered strains or laboratory collections, the present work investigates naturally occurring *Bacillus* isolates from ecologically diverse Turkish soils as potential non-GMO sources for sustainable cysteine production. This approach aligns with the increasing demand for halal- and vegan-compliant bio-based amino acids.

2. Material And Methods

2.1. Materials

All chemicals and reagents used in this study were of analytical grade. Tryptic soy broth (TSB), nutrient agar (NA), plate count agar (PCA), violet red bile glucose agar (VRBGA), and other media components were sourced from Merck KGaA (Darmstadt, Germany). Ninhydrin reagent, glacial acetic acid, and cysteine standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ion-exchange resin (Dowex 50Wx8-400, fine mesh spherical) were supplied by Dow Chemical Company (Midland, MI, USA). Taq DNA polymerase was purchased from a local provider of Fermentas (St. Leon-Rot, Germany). All solutions were prepared using distilled and deionized water. Glassware and instruments were sterilized as required prior to use.

2.2. Sample Collection

The colonies of bacteria were isolated from soil samples received from seven different regions of Türkiye. The soil samples were gathered from random locations in the cities of Bursa, İzmir, Antalya, Konya, Sinop, Erzurum, and Kahramanmaraş (Fig. 1).

2.3. Methods

2.3.1. Isolation of Bacteria

Two g of soil sample was taken into a sterile 5 mL tube and 2 mL of isotonic solution (1.5% NaCl) was added prior to vortex mixing. Next, 0.1 mL was pipetted and spread onto the surface of agar media (NA, TSA, NA enriched with 5 $g.L^{-1}$ peptone, PCA and VRBGA) in Petri plates by using a sterile bent glass spreader. Incubation was performed at 37 ºC for 24 h. Three streak-platings on the appropriate agar were used to purify each bacterial isolate. After incubation, a single colony was selected and transferred into vials containing glycerol and kept in frozen storage (-70 ºC) for further analysis [17].



Figure 1. A map showing the collection points of soil samples

2.3.2. Identification of Bacterial Strains

The best cysteine-producing strain among the isolated colonies was identified using nucleotide sequencing of 16S rRNA (ribotyping). All molecular identification procedures described in this section (gen omic DNA extraction, PCR amplification, cloning, and Sanger sequencing) were outsourced to Biogen İlaç Tic. Ltd. Şti. (Türkiye), with the methodological details provided by the service provider. Genomic DNA was isolated for molecular identification as explained by Tilahun et al. [18]. Following the directions, the isolates' DNA was extracted and purified using an Invisorb Spin DNA Extraction kit.

The PCR amplification procedure was carried out in accordance with [19] and [20]. Using 16S rRNA primers (RS-1; 5′-AGA GTT TGA TCC TGG CT C AG-3′ and RS-3; 5′-AAG GAG GTG ATC CAG CCG CA-3′), fragments of the bacterial isolates' 16S rRNA genes were amplified by PCR [21]. This was accomplished by creating PCR reaction mixtures (50 µL) using 1 µL of the extracted DNA, 5 µL of dNTPs, 1 µL of each of the primers RS-1 and RS-3, 1 µL of Taq DNA polymerase, and 5 µL of PCR buffer. The cycle of reactions began when 50 µL of filtered water was added to this combination. Initially, the denaturation temperature program was set at 95 °C for 60 seconds. The primer was then denaturated 35 times at 94 °C for 60 seconds, annealed at 51 °C for 30 seconds, extended at 72 °C for 60 seconds, and finally extended at 72 °C for 60 seconds. The amplicons of the bacterial isolates were then separated by gel electrophoresis using a 3% agarose gel after 1 µL dye and 5 µL PCR products were added and stained with ethidium bromide.

Before being inoculated onto a medium containing 100 μg/mL ampicillin for selection, the genomic region of interest from each bacterial isolate was ligated into a vector (pGEM®-T easy) and converted into XL1-blue cells. The medium was vigorously shaken and incubated for 16 hours at 37 °C. After the plasmids were separated, an automated DNA sequencer (ABI Prism 377, Applied Biosystems, USA) was used to perform the sequencing.

In order to find the most homologous sequence based on average nucleotide identity (ANI) values, the amplified and sequenced 16S rRNA gene sequences of bacterial isolates were uploaded to the National Center for Biotechnology Information (NCBI) website (https://blast.ncbi.nlm.nih.gov/Blast.cgi) using the Basic Local Alignment Search Tool (BLAST) [22, 23]. The sequence outputs received from the service provider were used for BLAST-based identification. The gene sequence that was deposited has accession number M W237712.

2.3.3. Production of Cysteine

Each of the 262 bacterial colonies isolated from soil samples were inoculated into 100 mL of TSB to find the best cysteine generating candidates. For 48 hours, the flasks were incubated at 37 °C at 125 rpm in an orbital shaker (Daihan-IS20R, South Korea). Ten milliliters of fermented broth were obtained after incubation, centrifuged for seven minutes at 4100 rpm. For separation of the cells, the liquid part was filtered using a membrane filter with a hole size of 0.45 µm [1, 10]. It was then stored in a refrigerator at 4 °C for additional analysis.

2.3.4. Quantitative Determination of Cysteine

The content of cysteine was measured using the acidic ninhydrin method, which was described by Ali [1]. To do this, 550 µL of ninhydrin reagent was added to a 5 mL screw-capped Pyrex tube containing 50 µL of supernatant. The tubes were stored in a water bath at 100 °C for an hour. After that, the tubes were cooled at room temperature, and 1.6 mL of acetic acid (glacial) was added. Utilizing a UV spectrophotometer (Metas UV-5100, China), absorbances were measured at 365 nm. Standard cysteine solutions with known concentrations (0.25, 0.5, 1.0, and 2.5 $g.L^{-1}$) were prepared, and a calibration curve was constructed using the same procedure.

2.3.5. Optimization of Cysteine Production Conditions

Different temperature, pH, duration, and agitation speed parameters were used to screen for cysteine synthesis in TSB in order to optimize the fermentation conditions. Thirty-one runs were performed in all (Table 1). To maximize the yields of cysteine production, incubation methods were conducted under various growing circumstances. The growth parameters (pH, time, temperature, and rpm) were established using the Plackett–Burman design [24].

The effects of growth conditions and the effects of the composition of growth medium (Carbon-sources, amino acids, nitrogenous compounds, and minerals) on cysteine production were evaluated. To do this, TSB was mixed with various amino acids, carbon sources, nitrogen sources, and minerals to create sixteen distinct growth media compositions (Table 2) to screen based on cysteine production at 40°C, 175 rpm, 84 h, and pH 7.5. The effects of different amino acids (serine and methionine) on the production of cysteine were studied.

These amino acids were added to the formulation of the growth medium at a level of 2 $g.L^{-1}$. The impact of several carbon sources, including lactose, fructose, glucose, and starch, on cysteine

synthesis was investigated. To achieve this, the fermentation medium was supplemented with 2.5 $g.L^{-1}$ of each carbon source. In 250 mL conical flasks, the components of various growth media formulations were combined, and the flasks were incubated for 48 hours at 37 °C at 125 rpm in an orbital shaker [10]. Following each run, the spectrophotometer based on the acidic ninhydrin technique was used to measure the cysteine concentration. The center point was repeated three times during the experimental design method's 31 runs to assess the repeatability of the test results. Cysteine concentration was the response variable assessed for statistical analysis.

Table 1. Incubation Parameters used (Plackett–Burman design)

Run	pH	Time (h)	Shaking speed (rpm)	Incubation Temperature (°C)	Run	pH	Time (h)	Shaking speed (rpm)	Incubation Temperature (°C)
1	7.5	48	75	37.5	17	7.5	24	125	37.5
2	7.5	48	100	37.5	18	7.5	36	125	37.5
3	7.5	48	125	37.5	19	7.5	48	125	37.5
4	7.5	48	150	37.5	20	7.5	60	125	37.5
5	7.5	48	175	37.5	21	7.5	72	125	37.5
6	7.5	48	200	37.5	22	7.5	84	125	37.5
7	7.5	48	225	37.5	23	7.5	96	125	37.5
8	7.5	48	250	37.5	24	7.5	108	125	37.5
9	7.5	48	125	10	25	5.5	48	125	37.5
10	7.5	48	125	15	26	6	48	125	37.5
11	7.5	48	125	20	27	6.5	48	125	37.5
12	7.5	48	125	25	28	7	48	125	37.5
13	7.5	48	125	30	29	7.5	48	125	37.5
14	7.5	48	125	35	30	8	48	125	37.5
15	7.5	48	125	40	31	8.5	48	125	37.5
16	7.5	48	125	45					

2.3.6. Extraction of Cysteine

Numerous contaminants, including bacterial cells, macromolecules, colors, inorganic materials, and organic materials, were present in the fermented broth. In order to purify cysteine, contaminants have to be eliminated using filtration, centrifugation, and ion exchange resin. Following purification procedures, the eluent was cooled and the pH was adjusted to the isoelectric point (pH 5.0) to precipitate cysteine. Cysteine was extracted using Dowex fine mesh spherical ion-exchange resin (Dowex 50Wx8-400). After removing contaminants by soaking the resin in 95% ethanol, they were rinsed with distilled water and then subjected to three cycles of alternating 2 $mol.L^{-1}$ HCl and 2 $mol.L^{-1}$ NaOH. After that, distilled water was used to wash the resin.

To enable the resin to absorb cysteine molecules, 10 g of cleaned resin was introduced to 1 L of fermenting medium in a flask. As a result, the flask was shaken in a water bath (Daihan, S. Korea) for two hours at 42 °C and 120 rpm. Using the acidic ninhydrin method, a UV spectrophotometer was used to measure the amount of cysteine present in liquid media. Filtration was used to separate the resin loaded with cysteine. After that, the resin was added to a 1 L flask with 1 L of HCl solution (1.0 $mol.L^{-1}$) and agitated at 200 rpm for two hours at 30 °C to desorb the cysteine from the resin.

The acidic ninhydrin technique was used to measure the amount of cysteine present in the liquid phase. By adding 1 N HCl solution, the liquid phase containing cysteine was acidified to pH 5.0, which is the isoelectric point of cysteine. Cysteine crystals were obtained by resting the solution at 20°C for 48 hours. The dry solid crystals were produced by evaporating the eluent [25, 26].

2.3.7. Chemical Analysis of Cysteine

The purity of solid crystal forms of cysteine was evaluated by using liquid chromatography with tandem mass spectrometry (LC-MS/MS) (AGILENT-Infinity 1260), HPLC (AGILENT-6460 Triple Quad) [27], FTIR spectrometer (BRUKER- Verter 70), and RAMAN spectrometer (WİTEC-Alpha 300R) [28]. Cysteine standard with 99% purity was used as a control for confirmation.

Table 2. Composition of production medium used for the optimization of the cysteine production of Bacillus sp. strain CG1

Ingredients (g.L^{-1})	**M1**	**M2**	**M3**	**M4**	**M5**	**M6**	**M7**	**M8**	**M9**	**M10**	**M11**	**M12**	**M13**	**M14**	**M15**	**M16**	**M17**
D-Glucose	2.5	2.5	2.5	2.5	2.5	2.5	2.5	5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Peptone (Caseine)	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16
Soy Peptone	2.5	2.5	2.5	5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
NaCl	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
K_2HPO_4	2.6	2.6	2.6	2.6	2.6	2.6	2.6	2.6	2.6	2.6	2.6	2.6	2.6	2.6	2.6	2.6	5.2
Lactose									2.5								
Fructose										2.5							
Urea							8										
Peptone					3												
Beef Extract											1.2						
Malt Extract												1.2					
Yeast Extract													1.2				
KH_2PO_4														0.5			
$FeSO_4$ $7H_2O$															0.26		
$MgSO_4$ $7H_2O$																0.26	
Methionine		2.1															
Serine			2.1														

2.4. Statistical Analysis

The analyses were run in triplicate. The mean values and standard deviations were determined. Tukey's tests and one-way ANOVA were used to examine significant differences with a 95% confidence interval ($p<0.05$). The Minitab 17 package program (Minitab Ltd, Coventry, UK) was used to conduct the studies.

3. Results and Discussion

3.1. Isolation, Selection and Identification of Top Five Cysteine-Producing Bacteria

The yields of cysteine produced by bacterial isolates were varied from 0.3 to 2.0 g.L^{-1} after 48 h of incubation. The cysteine production capacities of the top five out of 262 isolates

are given in Table 3. The rest of the data is not provided in the table. Cysteine production tests were repeated five times for the top five cysteine-producing isolates among 262 isolates. It was noted from Table 3 that TI.5.8.1 coded isolate produced the highest amount of cysteine (1.87 ± 0.37 $g.L^{-1}$) when compared to others. Accordingly, the highest cysteine-producing isolate (TI.5.8.1, later identified as *Bacillus* sp. strain CG1) was sourced from the Central Anatolia sampling stations, while the other high-performing isolates (TK.3.1.1 and the TM-coded isolates) were obtained from the Black Sea and Marmara regions, respectively.

Based on 16S rRNA gene sequence similarity analysis using BLAST, TI.5.8.1 showed high sequence similarity (99%) to members of the *Bacillus subtilis* group. Therefore, the best producing specie was noted as *Bacillus* sp. strain CG1. The strain CG1 exhibited typical *Bacillus* morphology with endospore formation and positive catalase and oxidase reactions. The presence of cysteine biosynthetic genes (cysE and cysK) was confirmed through BLAST-based annotation, supporting its metabolic potential for cysteine production. Different wild and mutant strains of *Bacillus* species as cysteine producers (e.g., *B. subtilis*) were also reported in the literature [29, 30]. Ali [30] studied the cysteine production of environmental isolates, and they reported from 0.045 to around 0.2 $g.L^{-1}$ cysteine yield from different isolates, indicating much lower values than the data obtained in this study.

Table 3. Amount of cysteine produced by top five cysteine producing isolates ($g.L^{-1}$)

Isolate Code	Run 1	Run 2	Run 3	Run 4	Run 5	($g.L^{-1}$)
TI.5.8.1	1.38	1.68	2.05	2.35	1.90	1.87 ± 0.37^{A}
TK.3.1.1	1.68	1.74	1.15	1.36	1.23	1.43 ± 0.13^{AB}
TM.4.6.1	1.21	1.61	1.50	1.04	1.41	1.36 ± 0.09^{B}
TM.4.5.1	1.34	1.35	1.22	1.52	1.09	1.31 ± 0.03^{B}
TM.3.7.1	1.23	1.70	1.47	1.60	1.56	1.51 ± 0.18^{AB}

TI: Central Anatolia region, TK: Black Sea region, TM: Marmara region

The reason could be attributed to the type of strain used and differences in the growth conditions. They also report that using different fermentation media and isolates' mutations using UV rays and nitrous oxide improved the cysteine yield up to 7.8 $g.L^{-1}$. According to Takumi et al. [11]'s findings, the cysteine production of *Pantoea ananatis* was approximately 2.2 $g.L^{-1}$ as the highest yield. Similarly, Yamamoto et al. [31] report 2.3 $g.L^{-1}$ cysteine yield from *E. coli*. They obtained significantly higher yields when compared to the data derived from this study. This result could be attributed to the variation of the production capacities of different species.

3.2. Effects of Incubation Conditions on Cysteine Production

Cysteine production was affected by the incubation parameters (Fig. 2). While an increase in incubation time improved cysteine production, increasing pH (especially over 8.0) decreased the cysteine yield (Fig. 1A). Similar behavior was observed for the interaction between temperature and pH (Fig. 1C). The data obtained in this study revealed better cysteine production ability of *Bacillus* sp. strain CG1 when agitation speed and pH ranged from 175 to 225 rpm and from 7.0 to 8.0, respectively (Fig. 1B). Similarly, a better L-lysine yield was reported at an agitation speed of 200 rpm and a pH value of 7.0 [32]. It can be stated that the cysteine production was affected by incubation temperature (Fig. 1C, 1D, and 1F). Better cysteine production was observed when the temperature value was around 35-40 °C.

However, according to [32], 30 °C was the most favored temperature for L-lysine fermentation by *C. glutamicum*, and a further increase in temperature resulted in a decrease in production. This outcome might be linked to the temperature sensitivity of the bacterial strain. In this study, increasing the temperature and incubation time resulted in an increased yield of cysteine (Fig. 1D, 1E, and 1F). Based on the data obtained, the optimum incubation conditions for better cysteine production were observed at around 84 h, pH 7.5, 175 rpm, and 40°C.

Application of optimum conditions resulted in the cysteine yield of 3.48 $g.L^{-1}$ using TSB as

a growth medium. Shakoori et al. [10] studied the optimization of fermentation conditions to improve amino acids production by environmental bacterial isolates and reported similar optimum growth conditions, which resulted in 11.4 $g.L^{-1}$ cysteine production from *B. cereus.* According to reports, 30 °C and pH 7.0 are the ideal growth conditions for *Corynebacterium glutamicum*, a Gram-positive soil bacterium that is extensively used in the amino acid synthesis business, in order to create an enhanced output of amino acids [9]. L-glutamic acid and other amino acids have been shown to be more productive at high temperatures (up to 41 °C) in another study [33]. Similarly, 37 °C and a pH of 7 were shown to be the ideal growth conditions for *E. coli* [34]. It should be mentioned that the majority of these results concur with those of this investigation. [35] examined *Bacillus* sp.'s ability to produce amino acids and found that, depending on the fermentation conditions, the yield ranged from 2.8 to 4.5 $g.L^{-1}$. This yield is consistent with the yield found in the current work (around 3.5 $g.L^{-1}$).

While the optimization strategy employed classical statistical design, its outcomes demonstrated that cysteine yields could be substantially enhanced using cost-effective media formulations, such as TSB supplemented with animal peptone and phosphate salts. Future work may involve employing response surface methodology (RSM) or fed-batch strategies using biowaste substrates to further improve process efficiency and economic viability.

3.3. Effects of the Chemical Composition of Growth Media

The primary carbon source that is preferred by bacteria is glucose. In addition, they can also utilize other sugars such as sucrose, ribose, mannose, maltose, xylose, arabinose, galactose, and fructose [36–38]. According to the data obtained from this study, the highest cysteine production was observed when glucose was added into the medium as a carbon source (around 30% increase). Other carbon sources reduced the concentration of cysteine (fructose reduced by 3% and lactose reduced by 3%) when compared to glucose (Fig. 2B). Similar data were reported by Shiio et al. [39].

They obtained lower L-Phenylalanine produced by *Brevibacterium flavum* with fructose as the carbon source than with glucose. Therefore, the difference in cysteine production yield between fructose, lactose, and glucose seems to be due to their metabolic difference in the biosynthesis of cysteine precursors. In another study, L-lysine production by *Corynebacterium glutamicum* was affected by sugar concentration, ammonium sulfate concentration, and incubation period [32]. A maximum yield of L-lysine was reported as 8.2 $g.L^{-1}$. The addition of amino acids to the fermentation medium caused a decrease in cysteine production (around 38% decrease when serine added and around 8% decrease when methionine added) compared to TSB (Fig.. 2A).

Different concentrations of monopotassium phosphate, dipotassium phosphate, iron sulfate, and magnesium sulfate were added into the fermentation medium to evaluate their effects on cysteine production.

The addition of 0.5 $g.L^{-1}$ monopotassium phosphate to the medium as a mineral source resulted in 18% increase in cysteine production. The addition of 2.5 $g.L^{-1}$ dipotassium phosphate into the medium resulted in 14% increase in cysteine production. The addition of 0.25 $g.L^{-1}$ iron sulfate to the medium resulted in 25% increase in cysteine production while adding 0.25 $g.L^{-1}$ magnesium sulfate resulted in 36% reduction in cysteine production (Fig. 2C).

Furthermore, the study assessed the impact of several nitrogen sources, including urea, casein peptone, soybean peptone, animal peptone, yeast extract, beef extract, and malt extract, on the yield of cysteine production (Fig. 3). Yeast extract, beef extract, and malt extract were added to the fermentation medium at a concentration of 1 $g.L^{-1}$, along with casein peptone, soybean peptone, and animal peptone. The generation of cysteine was reduced by 30% when 8 $g.L^{-1}$ urea was added to the medium as a nitrogen source (Fig. 2D). When compared to TSB, the cysteine output was higher from other nitrogen sources (yeast extract, beef extract, and malt extract, respectively) by 8%, 12%, and 17%.

The most significant increase in cysteine production (around 20% increase) was observed when animal peptone was added to the growth medium as a nitrogen source. Furthermore, casein peptone showed a slight increase in cysteine yield (2% increases) compared to animal peptone. However, the addition of soybean peptone resulted in 26% reduction in cysteine production (Fig. 2D).

The yield of 4.24 ± 0.47 $g.L^{-1}$ cysteine could be produced using optimized growth media

composition and growth conditions. Ali et al. [1] achieved 8.76 $g.L^{-1}$ cysteine production due to the medium and process optimization from the environmental isolates (isolated from soil, water, honey, milk, and waste), exhibits almost two-fold of the data obtained from this study. Shakoori et al. [10] reported a maximum of 13.2 $g.L^{-1}$ cysteine production under optimized conditions, revealing a much higher yield when compared to the findings of this study. Takumi et al. [11] obtained 2.2 $g.L^{-1}$ cysteine as a result of the incubation of *Pantoea ananatis* in optimized glucose and thiosulfate-based nutrient medium. 5.1 $g.L^{-1}$ cysteine yield under optimized conditions was reported by Liu et al. [15]. The differences in the cysteine yields reported might be attributed to the variations in growth media composition, growth conditions, and the strains used.

3.4. Separation, Purification and Characterization

The separation of cysteine from the fermentation broth using an efficient technique is essential to improve the yield and reduce production costs. In this study, fine mesh resin spheres were employed to adsorb cysteine molecules from fermentation media. Time-dependent cysteine-resin interaction profiles in the fermentation medium are shown in Fig 3. Maximum cysteine

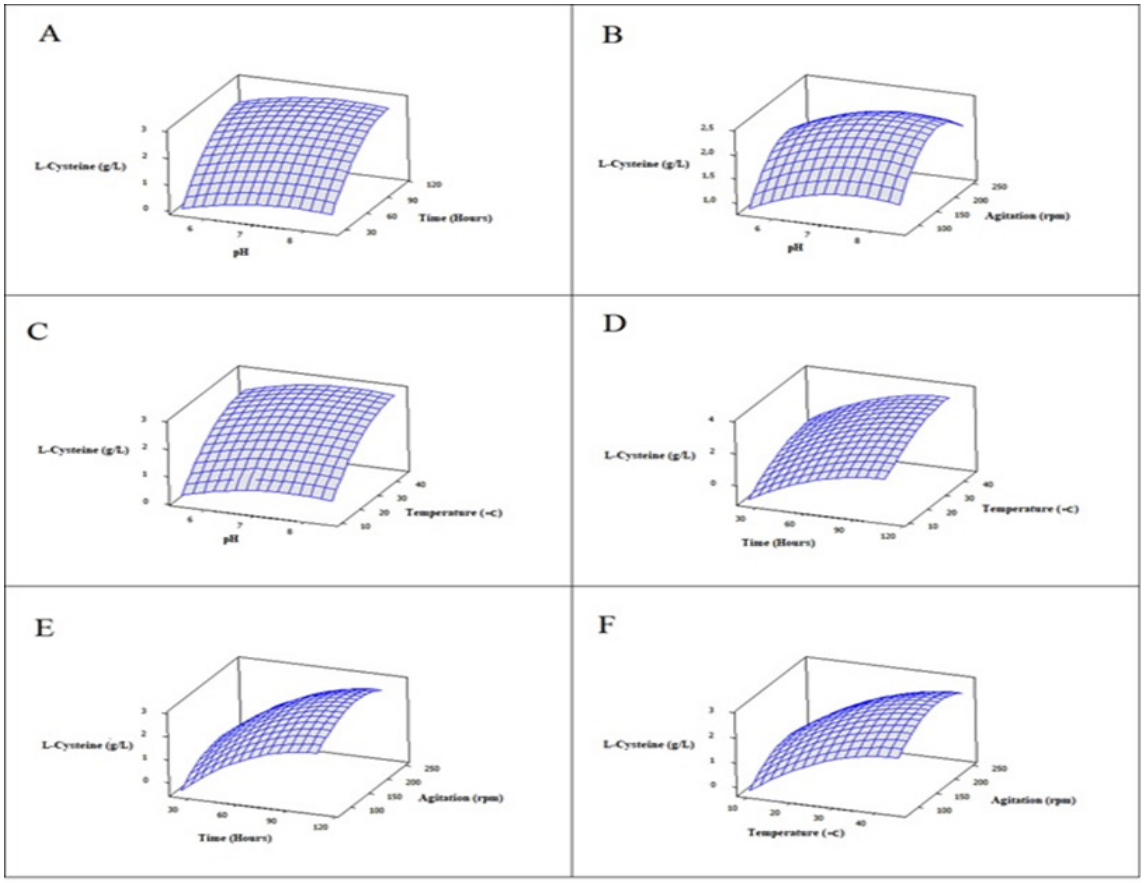

Figure 2. 3D plots of the combined effects of different variables on cysteine production. A- the combined effects of pH and time, B- the combined effects of pH and agitation speed, C- the combined effects of pH and temperature, D- the combined effects of time and temperature, E- the combined effects of time and agitation speed, 1F- the combined effects of temperature and agitation speed.

resin interaction was observed in the 150th minute. The recovery efficiency was obtained as 64.26 ± 4.08% at this point (Fig. 4), which seems lower when compared to the findings of [32]. They reported around 85% L-lysine recovery efficiency by Seralite SRC120 resin. This difference could be attributed to the type of resin used, the type of amino acid to be adsorbed, and the differences in purification protocols.

The purity of the extracted product was confirmed by using FT-IR and Raman spectrometers in comparison with the cysteine standard. The FT-IR and Raman spectra showed similarities between extracted cysteine and standard cysteine (Fig. 5 and Fig. 6).

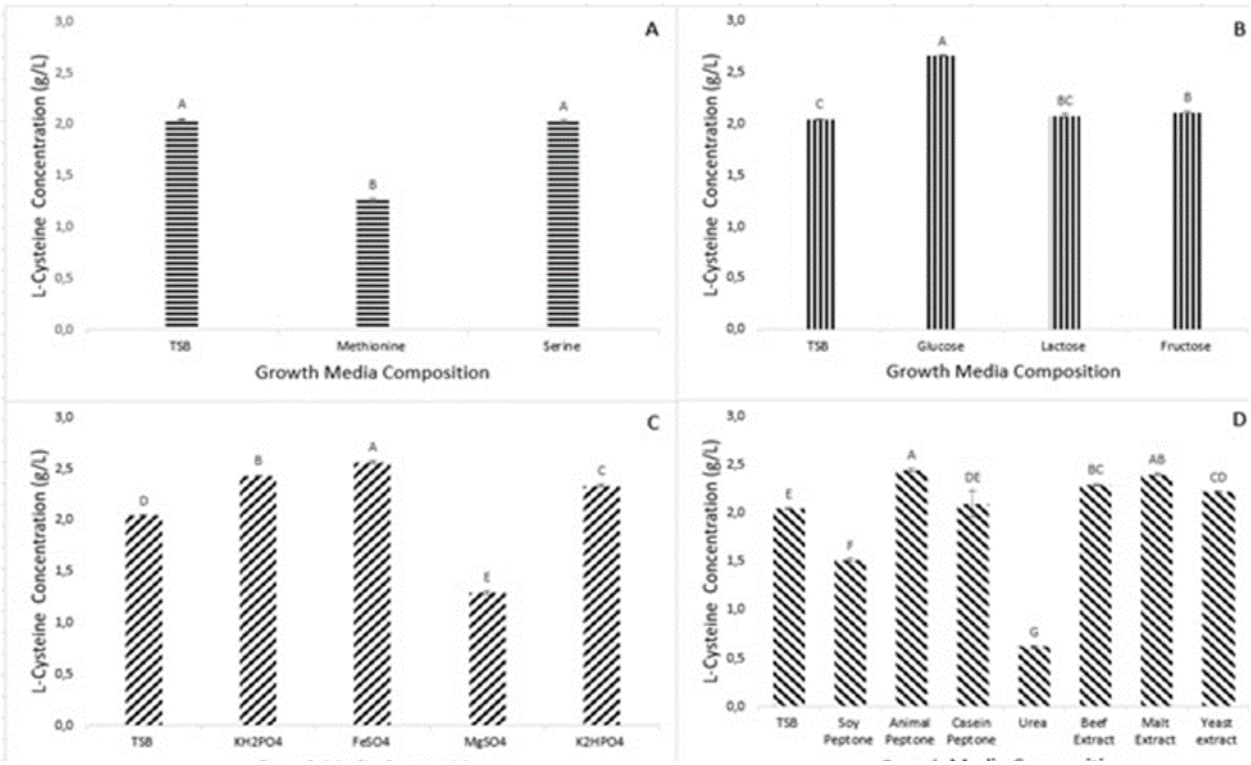

Figure 3. Effects of different variables on cysteine production of *Bacillus* sp. strain CG1. A- effects of carbohydrate sources on cysteine production, B- effects of aminoacids on cysteine production, C- effects of protein sources, on cysteine production D- effects of ingredients of growth media on cysteine production.

The peaks corresponding to functional groups of the precipitate showed identical distribution to standard cysteine except the differences in intensities of the peaks. Infrared spectra of pure standard cysteine and extracted cysteine are shown in Fig. 5. 2800 - 2400 cm^{-1} band region refers to the cysteine molecule's fingerprint (skeletal vibration) region. The carboxyl group's -C=O stretching frequency is 1581 cm^{-1}. At 1128 cm^{-1}, the -CN stretching frequency is visible. The amine group's -NH stretching frequency is visible at 1968 cm^{-1}. At 636 cm^{-1}, the –CS stretching frequency is visible [40]. Fig. 6A displays the Raman spectra of microbial and conventional cysteine.

The peak obtained at 940 cm^{-1} in the Raman spectrum belongs to the S-H band vibration to cysteine, which has sulfur in its molecular structure, unlike the other amino acids. In addition, the peak obtained at 1003 cm^{-1} indicates the characteristic peak of the interaction between these S-H and NH3 bonds. For validation, LC-MS/MS was also used, and the spectrums derived showed similar peaks, as seen in Fig. 6B.

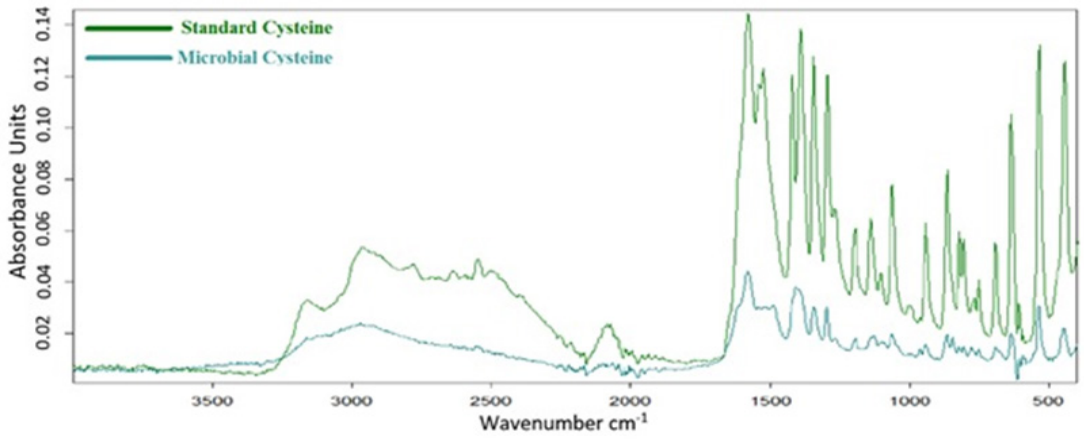

Figure 5. FT-IR-spectra of cysteine isolate vs reference

Although cysteine yields comparable to those previously reported were obtained, this study establishes *Bacillus* sp. strain CG1 as a promising environmental isolate that can serve as a basis for future metabolic improvement and industrial-scale development under sustainable, non-genetically modified conditions.

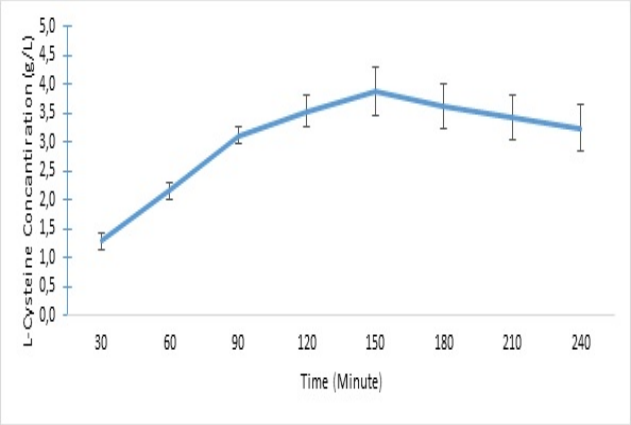

Figure 4. Recovery values over time by using ion-exchange resin

4. Conclusion

The primary focus of the current investigation was to separate bacterial strains from soil samples and look into their ability to produce cysteine in TSB. *Bacillus* sp. strain CG1 was determined to be the best-producing strain based on the data collected. The amount of cysteine produced was significantly impacted by the incubation conditions. Fortification of growth media with these ingredients at the optimal growth conditions of pH 7.5, 175 rpm, and 40°C for 84 h resulted in 4.24 ± 0.47 $g.L^{-1}$cysteine yield. The recovery efficiency was noted as around 65%, which needs to be improved by applying more efficient adsorbent and purification protocols.

Production of microbial cysteine by fermentation on the commercial scale could be possible using *Bacillus* sp. strain CG1. Although the current yield of 4.24 $g.L^{-1}$ does not exceed those reported in genetically optimized systems, this study provides a foundational framework for the development of sustainable microbial cysteine production. Future work should focus on metabolic pathway elucidation, flux analysis, and genetic improvement of *Bacillus* sp. CG1 to unlock its full biotechnological potential. Therefore, to optimize the performance of *Bacillus* sp. strain CG1, metabolic engineering techniques incorporating point mutations in genes pertinent to the cysteine production pathway should be used. To boost output and productivity, the concentration of nutrients should also be further studied.

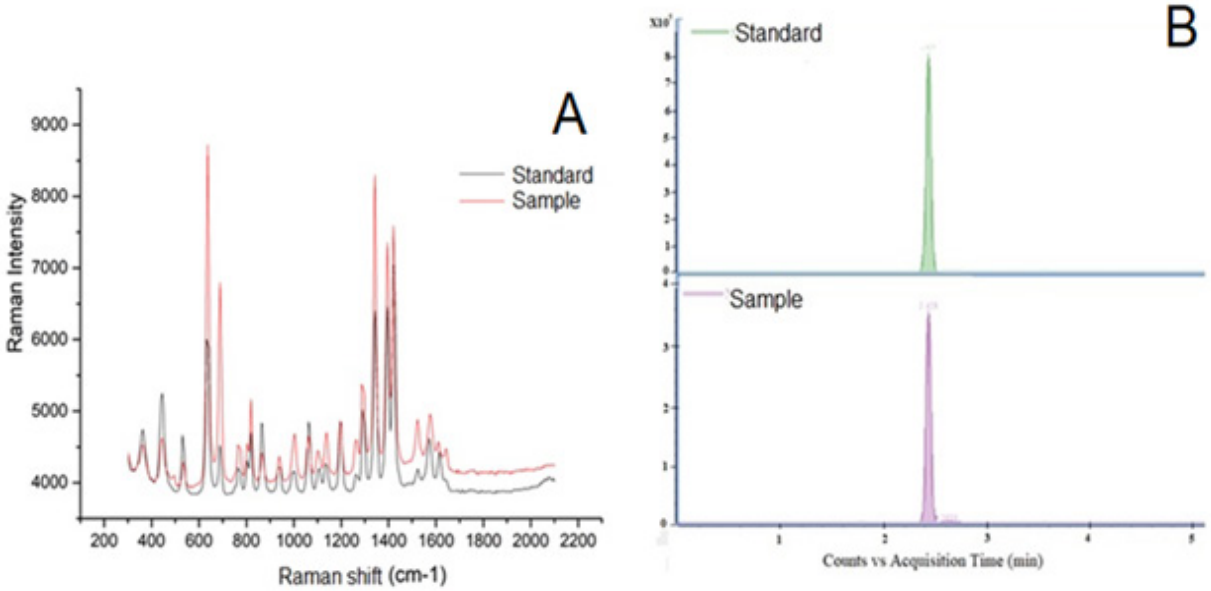

Figure 6. RAMAN-spectra and LC-MS/MS chromatogram of extracted and reference cysteine. A- RAMAN-spectra, B- LC-MS/MS chromatogram

Article Information Form

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Authors'Contribution

Conceptualization: C.G., E.E.; methodology: E.E., C.G.; formal analysis: C.G., K.Ö.G.; resources: C.G., E.E.; data curation: E.E., C.G.; writing- original draft preparation: E.E., C.G.; writing-review and editing: E.E. All authors have read and agreed to the published version of the manuscript.

The Declaration of Conflict of Interest/ Common Interest

No conflict of interest or common interest has been declared by the authors.

The Declaration of Ethics Committee Approval

This study does not require ethics committee permission or any special permission.

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