Research Article GU J Sci 39(2): 677-686 (2026) DOI: 10.35378/gujs.1771790

Gazi University

Journal of Science

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A Novel Strategy for Increasing Heterologous Protein Production in *Escherichia coli*

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Highlights

- Corn steep liquor (CSL) is a nutrient-rich bacterial growth media similar to Luria-Bertani (LB).
- *E. coli* strains produced high levels of recombinant peptide in CSL without the need for an inducer.
- The CSL is suitable medium for use in the pilot- and large-scale production of recombinant proteins.

Article Info

Received: 25 Aug 2025
Accepted: 5 Feb 2026

Keywords

Protein expression
Cell density
Industrial byproduct
Escherichia coli
SDS-PAGE

Abstract

The utilization potential of corn steep liquor (CSL), an industrial byproduct, as an alternative culture medium for the heterologous production of a GST/Snakin-Z fusion peptide was investigated in *Escherichia coli* BL21, DH5α, and Mach1 strains by cultivating in Luria-Bertani (LB) and CSL at low incubation temperature (25°C) with and without Isopropyl-β-D-1-thiogalactoside (IPTG) induction. The highest OD_{600} values were measured as 1.25 and 1.26, respectively, in Mach1 cultured in CSL at 25 °C and 37 °C, while the highest wet biomass (6.33 mg/ml and 6.67 mg/ml, respectively) was obtained from BL21 grown under the same conditions. No significant difference was observed between OD_{600} values of CSL and LB cultures, nor between wet weights ($p>0.05$). Results indicated that CSL contained suitable nutrients for bacterial growth, and it could provide growth equivalent to the cell density obtained from LB cultures. The BL21, cultivated in LB at 25 °C, produced recombinant peptide, and IPTG induction enhanced expression but no peptide production was observed at 37 °C, even in the presence of IPTG. In contrast, high production levels were detected in BL21 cultivated in CSL at both temperatures, regardless of IPTG induction. DH5α also produced recombinant peptide in both media and at both temperatures, and IPTG enhanced production. Mach1 produced recombinant peptide in CSL, while it did not produce it in LB at both temperatures. The IPTG did not significantly affect protein expression at both temperatures. This study demonstrates that sole CSL can be nutrient-rich and an economical medium for inducer-free pilot and large-scale recombinant protein production in biotechnological processes.

1. INTRODUCTION

Bioproduction of various chemicals, such as antimicrobial peptides, proteins, antibiotics, enzymes, and antibodies, contributes to sustainable industry [1]. Thus, there is no need to use substantial quantities of animal and plant tissues or biological fluids for extracting and purifying small amounts of target proteins. The recombinant protein production involves cloning a gene of interest into a vector, introducing the recombinant vector into a suitable microbial host, inducing expression, and subsequently purifying the protein [2,3]. This technology has enabled the expression of the protein in microbial hosts, thus significantly improving production efficiency. Bacteria, yeast, filamentous fungi, and unicellular algae are used as microbial host systems, and each system has distinct advantages and limitations. So, the suitable host should be selected carrying the optimal expression system for related protein production [4,5]. Bacteria are the most commonly used host systems; however, they have some challenges, such as protein misfolding, aggregation as inclusion bodies, proteolytic degradation, and metabolic burden for the host of recombinant protein. The *Escherichia coli* BL21 (DE3) strain has been genetically modified to enable high-level transcription by T7 promoter and has low protease activity to minimize the degradation of expressed

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proteins [6,7]. The BL21 has a faster growth rate and more favorable metabolic characteristics for protein expression than other strains like K, DH5α, or JM109 of *E. coli* [8]. Additionally, its DH5α, Mach1, and JM109 strains are preferred for producing constructed plasmids because they carry recA and endA mutations, which enhance plasmid stability and prevent unwanted recombination [9].

The protein production efficiency is directly related to the promotor. An ideal promoter should enable high protein production in the absence of an inducer. The lac promoter, a key component of its operon, has been widely used to regulate recombinant protein expression [10]. Although this promoter induced by lactose or lactose analogs is used to activate protein production, its catabolite repression by glucose can limit protein expression [11,12]. Another commonly used promoter system is T7, which enables strong transcriptional control and high protein yields. Both promoters can exhibit leaky expression in high-copy plasmids, leading to premature protein production, metabolic stress, reduced cell viability, and lower protein expression. This issue has been solved by the development of lacUV5 and its variant promoters [13,14]. The recombinant protein yield and solubility can also be significantly enhanced by optimizing the culture conditions, such as temperature, inducer concentration, media composition, and oxygen levels [3,15]. Furthermore, the supplements added as constituents of culture media can promote protein folding and activity [16]. Despite the development of alternative expression systems (baculovirus, mammalian cells, and cell-free systems), *E. coli* remains the host of choice for many recombinant protein production applications [17,18]. To enhance the efficiency of recombinant protein production and reduce costs, there is a necessity for continuous advancements of strain engineering, promoter design, and culture media. Thus, the potential applications of recombinant proteins will expand in the fields of biotechnology and medicine.

Corn steep liquor (CSL) is a by-product of the starch industry and is produced during the wet milling of corn. Corn kernels are steeped in a sulfur dioxide solution for 30-48 h to break down corn components in this process. As a result, the steep water becomes enriched with soluble nutrients. After starch separation from steep water, the remaining liquid is concentrated to achieve a solids content of 45-55 %, yielding a nutrient-rich CSL that contains essential amino acids, vitamins, minerals, reducing sugars, organic acids, and enzymes. CSL's nutrient composition led to its utilization as an alternative to traditional carbon and nitrogen sources in fermentation processes [19,20]. CSL was first utilized for penicillin production in 1947 [21], and since then it has been used as an additive for culture media in the biosynthesis of antibiotics, enzymes, and organic acids [22].

It was aimed in the present study to optimize the heterologous production of the plant-originated glutathione S-transferase (GST)/snakin-Z peptide in different *E. coli* strains grown on a variety of culture media and incubation temperatures. CSL was directly used as the sole culture medium for the first time. It was found that cell density reached that of those grown on Luria-Bertani (LB) when the bacterial host was cultivated on sole CSL. Furthermore, recombinant peptide production was accomplished without the utilization of Isopropyl-β-D-1-thiogalactoside (IPTG). Outputs demonstrated the feasibility of using it as a cost-effective product in heterologous protein production, enabling host cell density at least as high as in commercial media in the expression system, where industrial CSL is used directly as culture medium instead of commercial alternatives.

2. MATERIAL METHOD

2.1. Media Preparation, Plasmid Vector and Bacterial Strains

Media and their ingredients used in this study are given in Table 1. The CSL was provided by Pendik Starch Factory. pGEX-6P-1-SNK plasmid containing GST/snakin-Z fusion peptide obtained from Teker et al. [23]. *E. coli* DH5α, Mach1, and BL21 (DE3) strains were used as host cells for subcloning of the plasmid vectors and GST/snakin-Z expression. Bacteria containing pGEX-6P-1-SNK were cultured for 16 h in LB supplemented with 100 µg/mL ampicillin at 37 °C, 180 rpm in a shaker. For long-term storage, all bacterial stocks were maintained at -80°C in LB medium with 25% glycerol.

Table 1. *Media used in this study*

Media	Compound	Concentration
Luria-Bertani (LB)	Tryptone	10 g/L
	Yeast extract	5 g/L
	NaCl	5 g/L
	Ampicillin	100 µg/mL
Corn steep liquor (CSL)	Corn steep liquor	20-40 g/L
	$MgSO_4$	0.25 g/L
	NaCl	0.5 g/L
	NH_4Cl	1 g/L
	KH_2PO_4	3 g/L
	$Na_2HPO_4.7H_2O$	6 g/L
	Ampicillin	100 µg/mL

2.2. Transformation of *E. coli* Cells

Transformations of plasmid vectors into competent *E. coli* cells were carried out using the heat-shock method. Colonies carrying pGEX-6P-1-SNK were checked by colony PCR using pGEXF/R (GGGCTGGCAAGCCACGTTTGGTG/CCGGGAGCTGCATGTGTCAGAGG) primers. Positive colonies were cultured in LB containing 100 µg/mL ampicillin at 37°C and 180 rpm for 16h. *E. coli* strains without a plasmid were used as negative controls.

2.3. Culture Conditions for the Expression of Fusion Peptides in *E. coli*

50 µL of bacterial stocks (frozen at -80°C) were inoculated into 5 mL of LB medium (1/100, v/v) and incubated at 37°C for 16 hours with shaking at 180 rpm. After incubation, 50 µL of saturated culture was added to 5 mL of LB and/or CSL medium (1/100, v/v). The cultures were incubated at 25-37°C for 16 hours, shaking at 180 rpm. Recombinant peptide production was induced by adding 100 µM IPTG to the cultures and incubating additional 4 hours. The uninduced group was cultured under the same conditions without the addition of IPTG.

2.4. Monitoring of Bacterial Growth

The bacterial growth in the cultures was monitored by measuring OD_{600} with a spectrophotometer and by determining the wet weight of the cells in a certain volume. 200 µL of the cultures were transferred to a 96-well plate, and OD_{600} was measured with a spectrophotometer. For the determination of wet weight, 1 mL of the cultures was centrifuged at 8 000 rpm for 5 minutes. The supernatant was then removed, and the weight of the pellets was determined.

2.5. Protein Production Analysis

The fusion peptide expressions were analyzed using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). A 30% acrylamide solution **(**acrylamide/bis, 29:1) was used to prepare 10-15% acrylamide gels. 1 mL of culture was centrifuged at 8000 rpm for 5 minutes, and the cells were lysed in 1x SDS sample loading buffer. The lysates were centrifuged at 10000 rpm for 5 minutes to precipitate cell components. After denaturation of proteins at 95°C for 5 minutes, the samples were loaded onto the gel, and electrophoresis was performed at 40 V for 15 minutes, followed by 80 V for 75 minutes for each gel (10×10×1 mm). The separated proteins were visualized using silver staining method [24].

2.6. Statistical Analysis

Data was statistically analyzed using GraphPad Prism 9.0 software. Normality was determined using the Shapiro-Wilks test. Student's t-test and one-way analysis of variance (ANOVA) with Tukey's post-test were

used for pairwise and multiple comparisons of OD_{600} and wet weights, respectively. The standard error was calculated via column statistics. The confidence interval was 0.05.

3. RESULTS

3.1. Effect of Culture Conditions on Bacterial Growth

OD_{600} and wet cell weights of *E. coli* BL21, DH5α and Mach1 strains cultured in LB and CSL media are given in Table 2. While the highest OD_{600} values among the cultures were detected in Mach1 cultures (in saturation conditions; OD_{600}/1.25 at 25°C and OD_{600}/1.26 at 37°C), the highest wet cell weights were observed in BL21 cultures (in saturation conditions; 6.33 mg/ml culture at 25°C and 6.67 mg/ml culture at 37°C). However, no statistically significant difference was detected among the growth profiles of different *E. coli* strains that reached maximum saturation in LB and CSL media at 25°C or 37°C (18-20 hours for 25°C; 12-16 hours for 37°C) ($p>0.05$) according to both t-test and ANOVA. These findings revealed that sole CSL as a culture medium can be used as effectively as LB in the growth of bacterial strains (Figure 1).

Table 2. *OD_{600} values and wet weights of three strains*

Strain	Media	Temperature (°C)	OD_{600}	Wet Weight (mg/mL)
BL21	LB	25	1.13 ± 0.01	5.67 ± 0.33
		37	1.20 ± 0.02	6.00 ± 0.58
	CSL	25	1.14 ± 0.04	6.33 ± 0.33
		37	1.22 ± 0.02	6.67 ± 0.33
DH5α	LB	25	1.15 ± 0.03	5.83 ± 0.44
		37	1.22 ± 0.01	5.67 ± 0.33
	CSL	25	1.13 ± 0.02	5.67 ± 0.33
		37	1.20 ± 0.05	6.00 ± 0.57
Mach1	LB	25	1.08 ± 0.02	5.00 ± 0.57
		37	1.17 ± 0.03	5.33 ± 0.33
	CSL	25	1.25 ± 0.01	5.00 ± 0.57
		37	1.26 ± 0.02	6.00 ± 0.57

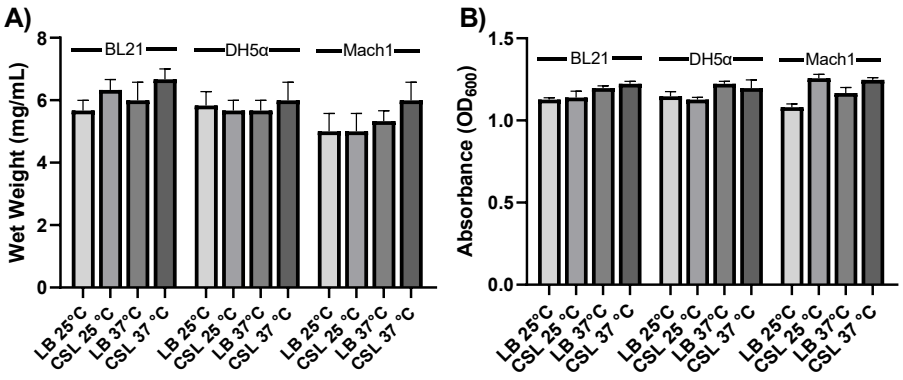

Figure 1. *Graphical representation of the changes in wet weights (A) and OD_{600} values (B) depending on the culture medium and temperature. T-test and ANOVA revealed that no statistically significant differences between the experimental groups ($p > 0.05$)*

3.2. Effect of Culture Conditions on Recombinant Peptide Production

The fusion peptide expression abilities of *E. coli* strains harboring pGEX-6P-1-SNK plasmid cultured in LB and CSL media at 25°C and 37°C were analyzed by SDS-PAGE/silver staining method. It was observed that *E. coli* BL21 strain cultured in LB medium at 25°C produced the fusion peptide without induction by IPTG. In cultures grown at 37°C, BL21 cells did not produce detectable fusion peptide on the polyacrylamide gel even after IPTG induction. In contrast, BL21 strain grown in CSL medium at both 25°C

and 37°C produced high levels of fusion peptide. The IPTG induction had no significant effect on peptide production of the cells (Figure 2A). Unlike *E. coli* BL21, it was determined that the DH5α strain grown in both LB and CSL medium at 25°C or 37°C was able to produce the fusion peptide independently of IPTG induction. However, IPTG induction had a positive effect on peptide production in these cultures (Figure 2B). *E. coli* Mach1 cells transformed with pGEX-6P-1-SNK grown in LB medium at 25°C and 37°C did not produce detectable amounts of fusion peptide. In contrast, fusion peptide synthesis was detected in Mach1 cells cultured in CSL. In these cultures, peptide production was not significantly affected by induction with IPTG (Figure 2C).

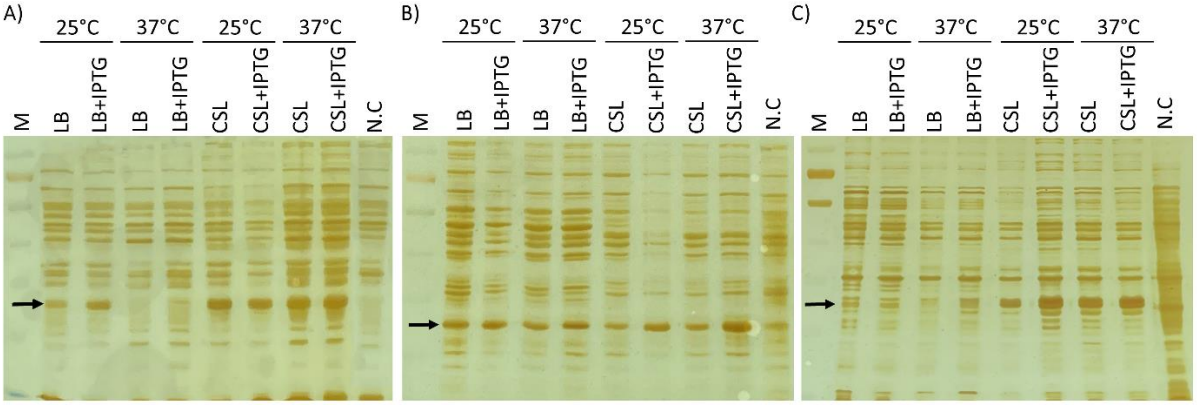

Figure 2. *SDS-PAGE gel images of BL21 (A), DH5α (B) and Mach1 (C) strains cultured in LB and CSL media with and without IPTG at two temperatures. N.C: negative control*

4. DISCUSSION

E. coli is the most commonly used host for recombinant protein production in various industrial sectors, particularly in the pharmaceutical industry. The choice of growth medium plays a critical role in the successful production of recombinant proteins, as it provides the essential nutrients to for bacterial growth and efficient protein synthesis [25]. The high cost of complex media components, including yeast extract and peptone, with rich nutritional composition, limits their utilization in large-scale industrial applications [26, 27]. For this reason, there is growing interest in identifying cost-effective alternative nutrition sources such as cheese whey, molasses, and CSL and bringing them into use [22, 28]. Unlike other studies, CSL was directly used as a culture medium for bacterial growth, and more recombinant protein production was achieved without the utilization of an inducer in the current study. Although both cell densities and wet weights obtained from all three strains used were found at similar levels between LB and CSL, it was revealed that CSL contains the essential components required for effective bacterial growth, induces recombinant production, and also reduces growth costs compared to commercial culture media. Although not statistically significant, a slightly better growth profile of BL21 and Mach1 compared to DH5α in the CSL indicates that strains may utilize this nutrition component as a medium more efficiently than DH5α.

BL21 is a genetically engineered bacterial strain for high-yield protein production. Its efficient central metabolism facilitates optimal energy utilization for protein synthesis rather than the accumulation of byproducts such as acetate, which can inhibit bacterial growth. Additionally, its deficient protease activity reduces recombinant protein degradation [7, 8]. López-Cano et al. [29] demonstrated that this strain was suitable for producing recombinant host defense peptides; its effective utilization for recombinant peptide production was emphasized in numerous subsequent studies [30-32]. BL21 produced a high level of GST/snakin-Z, particularly in the CSL. The achievement of high levels of GST/snakin-Z production by using BL21 was consistent with previous reports. Similarly, it was found that Mach1 effectively produced GST/snakin-Z peptides in CSL, which it did not produce in LB, and showed that it could be used like BL21in contrast to its general use, which involves amplification of plasmid vectors. The DH5α exhibited basal protein expression levels when cultured on both LB and CSL. It was stated in the literature that this strain has already been preferred in the amplification and maintenance of recombinant plasmid vectors [33-35].

Temperature is one of the most important growth parameters that influences the metabolism and the activity of bacterial cells in culture. It also significantly affects protein solubility and stability. However, high temperature often leads to the formation of inclusion bodies [36, 37]. When Azaman et al. [36] tested the effects of temperatures on recombinant peptide production by using 16, 30, and 37°C, they found that the highest amount of recombinant peptide was found in the cultures incubated at 30°C in LB. They also reported that as the incubation period progressed, the amount of recombinant peptide at 16°C reached the same amount as that of 30°C. Huang et al. [37] determined that reducing the incubation temperature from 37°C to 25°C resulted in a threefold increase in recombinant peptide production. They stated that low temperature improved 3D folding of protein, and thus contributed to an increase the protein solubility. In the present study, 25 and 37°C were examined for the determination of the most effective condition in bacterial growth and recombinant protein expression. BL21 produced recombinant peptides only at 25°C in LB, whereas no peptide production was detected at 37°C, despite IPTG induction. It was also demonstrated that lower temperature enhanced protein solubility, consistent with the report of Huang et al. [37]. Similar to BL21, 25°C was the temperature inducing recombinant peptide production in LB cultures of the Mach1 strain. Peptide production remained unaffected by temperature in DH5α grown in this medium. It was revealed that temperature was not an affecting factor on recombinant protein production for all *E. coli* strains cultured in CSL; as a result, effective protein production was carried out in both conditions. Moreover, recombinant peptide production was not detected in Mach1 cultured in LB at both temperatures, whereas protein expression in CSL was observed independently of temperature. Determination of a high level of proteins in all strains indicates that this industrial byproduct provides essential nutrients, cofactors, and/or metabolites required for growth and protein expression independent of temperature and strain. In this study, experiments were conducted using a single CSL batch obtained from the Starch Factory. Using industrial by-products in cell culture and in vivo protein production has some potential limitations, such as batch-to-batch variability and impurity. Comparable cell densities and recombinant protein production levels were obtained from all batches.

Expression must be induced by adding the lactose or its synthetic analog (i.e. IPTG) when the culture reaches a certain growth phase in the recombinant system, which contains the lac promoter [38]. However, Studier, [39] indicated in their study that the culture medium with lactose might also activate the lac promoter. This system, in which there was no need to monitor bacterial growth and manually add an inducer at an appropriate time, is referred to as auto-induction. Later, auto-induction has become an effective and convenient method of both small- and large-scale recombinant protein production [40]. In this context, the present study revealed for the first time that CSL has an auto-induction effect by showing that IPTG-containing medium did not alter the amount of recombinant protein, especially depending on host strain used. And it also demonstrated that CSL may valuable and cost-effective nutrient composition inducing recombinant peptide production no need to use the inducer. Although Rafi et al. [41] previously showed that the addition of CSL at different concentrations to the culture medium resulted in an auto-induction effect, they did not directly use CSL as a medium. However, IPTG increased peptide production in DH5α–when grown in LB and CSL at both temperatures. These results demonstrated that the auto-induction property of CSL may be strain-specific. Although IPTG is an effective inducer, its usage not only increases production costs but also causes metabolic stress on bacterial cultures at high doses [39]. The outputs of the present study demonstrate for the first time that CSL is a cost-effective and valuable medium for recombinant protein production, and it also offers advantages over other media by eliminating the need for induction to trigger protein production and the possibility of cellular stress.

5. CONCLUSION

In conclusion, this study introduces a novel approach to the literature by demonstrating that CSL is a convenient medium for both bacterial growth and recombinant protein production. Its low cost and potential auto-inducing properties will provide remarkable advantages for industrial-scale applications. Evaluating the capacity of different *E. coli* strains to utilize CSL under varying culture conditions and improving its efficiency through genetic manipulations will lead to the widespread industrial use of this byproduct in large-scale bioprocesses.

ACKNOWLEDGEMENT

CSL kindly gifted by Pendik Starch Factory.

This study was funded by Scientific Research Projects Coordination Unit of Istanbul University [Project number: 38963].

This study was derived from the doctoral thesis of Mehmet Arslan titled "Scale-up Production of Recombinant Fusion Peptide Originated From Plant", conducted at the Department of Molecular Biology and Genetics, Institute of Science, University of Istanbul.

DECLARATION OF ARTIFICIAL INTELLIGENCE (AI) USE

When drafting the manuscript, neither generative AI agents nor large language models (LLMs) were used in any aspect of the study.

CONFLICTS OF INTEREST

No conflict of interest was declared by the authors.

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