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Cellulase Producing Bacteria Isolation, Screening and Media Optimization from Local Soil Sample

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Abstract Cellulase enzyme earns consecutively increasing demand in industries of Bangladesh. This experiment has been planned to develop the enzyme industry in our country rather than import enzymes from other countries and conclude with isolating and screening cellulose-degrading bacteria from local land samples and ensuring maximum enzyme production through media optimization. After collecting soil samples from various Tangail areas, a typical dilution of series was performed on a Carboxymethylcellulose plate (CMC) to produce cellulase-producing microorganisms. Based on the hydrolysis zone of the ratio, four isolates were chosen and labeled CBM21, CSG8, CSR35, and CHT8 for further morphological and biochemical analysis. Then subjected to cellulase production in 250 ml of Erlenmeyer flask using CMC broth for 48 hours of fermentation at 37°C at an agitation rate of 140rpm, and cellulase enzyme assay methods evaluated crude enzymes. Eventually, those confirmed isolates were optimized using different parameters by submerged fermentation process for enhanced cellulase production. Among four isolates, CSG8 and CBM21 exhibited the maximum clearance zone with the hydrolytic values 6.96 and 8.46. These isolates were identified as *Pseudomonas* spp. (CSG8). According to the screening process, CBM21 (0.156U/ml), CSG08 (0.106U/ml), and *E. coli* (negative control) have been used for optimization with their initial enzyme productivity. After optimization, an amazing enhancement was noticed for CBM21 (1.35 U/ml) and CSG8 (1.23 U/ml) compared to *E.coli* (0.44 U/ml). The enzyme was characterized in isolates with an effective temperature of 40 and 35°C for CBM21 and CSG8, respectively, and pH 7, glucose 5% with the incubation time 24h was optimal. Aftermath, this type of optimization can be a significant initiative in the enzyme industries of Bangladesh.

Keywords: *ellulase, isolation, screening, carboxymethylcellulose, optimization, hydrolysis*

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1. Introduction

In biotechnological processes, enzymes are at the core. Cellulase is one of the most usable enzymes that act on cellulosic material. Also, the most abundant form of biomass on Earth is cellulose, consisting of a linear polysaccharide of glucose residues with -1, 4-glycosidic linkages [1]. Generally, cellulose is a significant plant component that accounts for 50 percent of the plant biomass's dry weight, and agricultural wastes, for example, make up about half of the dry weight of secondary biomass sources [2]. As decades of research have shown, cellulase enzyme has long been involved in producing fermentable sugars from lignocellulose biomass. Cellulases are usually made up of three groups of enzymes that work together: endoglucanases hydrolyzed the polymeric chain of cellulose, exoglucanases that aid in the release of cellobiose from the cellulose polymer, and β-glucosidases, which assist in the conversion of cellobiose to glucose [3]. Cellulase is generally secreted by various microorganisms, including fungi, bacteria, and Actinomycetes, which can dissolve cellulose [4]. Overall, many scientists noticed fungi mainly because they produce cellulases that are plentiful and easy to obtain. In recent years, specific fungal sources, including Trichoderma, Aspergillus, Penicillium, etc., have been widely used for industrial purposes.

In comparison to fungi, researchers have taken into consideration various bacteria which produce cellulases because of the exponential rise, production of a multi-enzymes complex with their susceptibility to severe conditions [5,6,7]. Some genera of bacteria, including Pseudomonas sp., Cellulomonas, and Cellvibrio [8], *Micrococcus*, and *Bacillus* [9], exhibit cellulolytic properties. Forest, rural areas, and agro-industrial waste have a considerable quantity of unused or underused cellulose that causes environmental pollution. By using enzymes including cellulase, amylase, fatty lipase, protease, etc., beneficial products like sugars, biofuels, animal feed, and human nutrition are now being produced [10].

Cellulase can help to protect seeds and plants against plant pathogens, as a biocontrol [11]. Many cellulolytic microorganisms have been known for improved germination, the rapid growth of plants, and a boost in crop yields [12]. For instance, this enzyme that degrades cellulose may be used to produce washing powders, extract fruit and vegetable juices, and refining starch [13]. In the textile sector, cellulases are used to make cotton and denim finishes; for the maintenance of color in laundry detergents; to increase drainage and fiber alteration in the pulp and paper industries, and very recently applied in the biomedical research industry [14]. Cellulases convert cellulosic material to glucose and other products, which can be used as a microbial substrate material for single-cell protein and bioethanol production [15].

Some potential experiments were performed to explore the aerobic bacterial development in carboxymethyl cellulase (CMCase) medium. In the late logarithm step, *Acinetobacter anitratus* shows the highest activity for CMCases media [16]. The cellulase productivity appears to depend on a complex relationship with certain parameters such as pH, temperature, aeration, inoculum volume, and incubation time. The researchers conclude that Paenibacillus sp's strain shows high potential for the optimal development of cellulase in a 1.0% CMC containing medium [9]. Besides, some cellulose-decomposing bacteria showing CMCase activity within the 0.162–0.400 U/mL range were isolated from the Gupta et al. experiment [17].

Deka et al.' used a surface reaction technique for optimizing cellulase development by aerobic bacteria, observing that the maximum CMCase activity of *Bacillus subtilis* AS3 is 0.43 U/ml [18]. The CMCase activity of the ACCA2 strain of Enhydrobacter sp. was continuously optimized in order to optimize medium components such as carboxymethyl cellulose, peptone, sodium chloride, (NH4)2SO4, K2HPO4, and MgSO4, which resulted in a 2.39 fold increase in CMCase activity. [19]. In addition, setting up an optimized condition, Da Vinha et al. calculated that the maximum activity of CMCase is 2.0 U/ml. While using the *Streptomyces viridobrunneus* SCPE-09 and *Pseudomonas* sp. Hp207 strain, Sheng et al. reported 1.432 U/ml [20,21].

Global Cellulase's Market Report for 2018 reveals that Asia Pacific is the largest purchaser of cellulases and added that cellulase application would hit USD 2300 million by 2025, which will increase by 5.5% over the 2018-2025 period, at an annual CAGR (compound annual growth rate) [4]. The production of cellulase enzymes in the future most exciting advanced field of research by biotechnology with several factors, including cost, genetic engineering, a particular activity, and purification steps that are feasible in research interests.

The degradation of cellulosic materials has been identified in numerous investigations. Still, few studies have examined which microorganisms, especially bacteria, could be used as the best source of cellulase enzyme production for industrial purposes. For this reason, there is a growing demand for the isolation of bacterial aerobic strains, which reasonably produce higher cellulase activity.

Our study has conducted by isolating aerobic bacteria from a local soil sample that could hydrolyze cellulosic material. Besides, further identified those bacterial isolates by the growth of selective media. Current work was also done to optimize media parameters for improving the output of cellulose employing identified bacterial isolates. In conclusion, the bacterial isolates CBM21 produces 1.35 U/mL CMCase activity after modifying the cultural condition compared to others isolates.

2. Materials and Methods

2.1. Study Place and Design

This investigation was carried out in the Biotechnology and Genetical Engineering laboratory, Mawlana Bhashani University of Science and Technology, Santosh, Tangail, Bangladesh. The flow diagram of the study plan was shown in supplementary Figure 1.

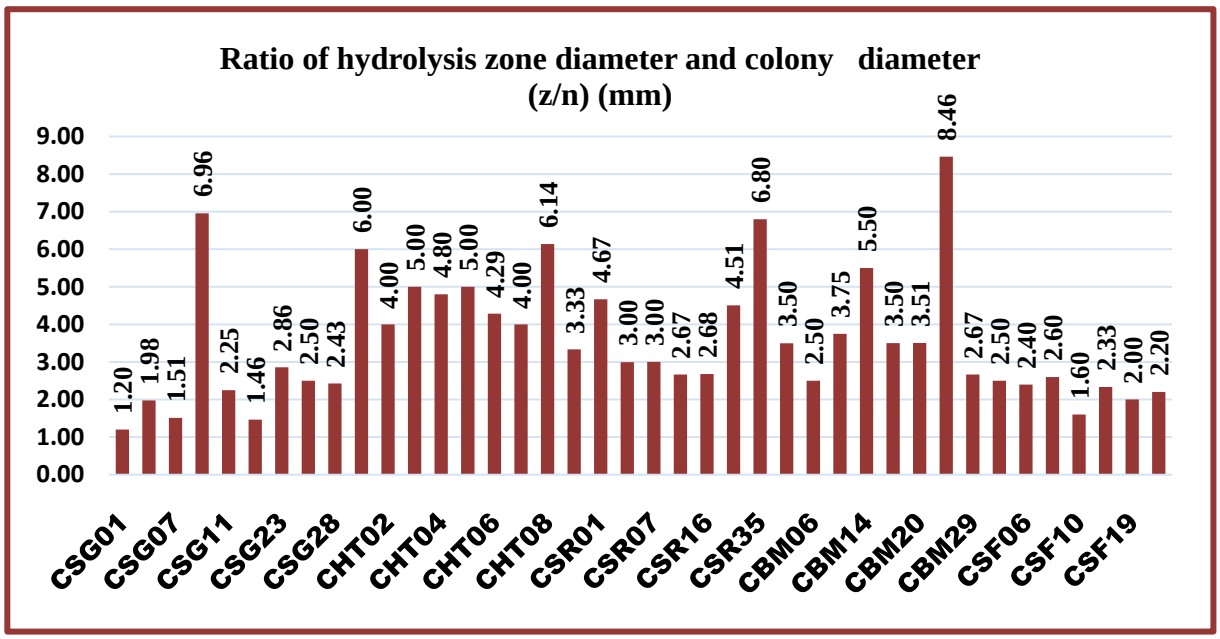

Figure 1. Ratio of hydrolysis zone diameter and colony diameter (z/n) (mm)

2.2. Isolation of Bacteria

The soil samples used in this experiment were collected from the various regions such as green wastes, tannery wastes, garden soil, tannery industry wastes, rice straw wastes, forest soil and stored at 4°C until used. Traditional serial dilution technique has been used to isolate cellulolytic bacteria through agar plate about 10^{-1} to 10^{-7} dilution factor. From the test tubes labeled 10^{-6} and 10^{-7}, 0.1 ml is applied on the media of carboxymethyl cellulose (CMC) containing: 1.0% carboxymethyl cellulose (CMC), 1.0% peptone, 0.2% $(NH_4)_2SO4$, 0.2% K_2HPO_4, 0.03% MgSO4.7H2O and 0.2% gelatin and 1% agar [22]. The CMC agar plates were subsequently incubated for 24 hours at 37°C. Different discrete colonies were found on an agar plate and were selected further.

2.3. Primary Screening of Cellulose Degrading Bacteria

CMC agar plates had been soaked with iodine solution for approximately 5 minutes and then allowed to stand at room temperature. Several colonies were shown a clear circular zone on the agar plate. The respective broth culture which were shown clear zone were streak on CMC agar media containing 1.0 % carboxymethylcellulose, 0.1% KH_2PO_4, 0.005% NaCl, 0.04 % $MgSO_4.7H_2O$, 0.1 % K_2HPO_4, 0.000125% $FeSO_4$, 1.8 % agar, pH 7.0 and incubate at 37°C for 72 hours [23]. The plates were further flooded with iodine and allowed to stand at room temperature for around five minutes. The CMC hydrolysis clear zone and colony diameter ratio were measured and reported.

$$Clear\ zone\ ratio = \frac{Clear\ zone\ diameter}{Colony\ diameter}$$

For cellulase development in a submerged environment, the bacterial colonies with the largest ratio were selected.

2.4. Identification of Bacteria

2.4.1. Morphological and Biochemical Characteristics

Bacterial isolates were pre-sumptuously characterized by morphology, as well as some biochemical analysis. The parameters investigated included colonial morphology (evaluated for size, pigmentation, form, margin, elevation, and texture), gram reactions, citrate utilization test, MIU (Motility- Indole- Urease) test, catalase production, Kligler's Iron Agar (KIA) test.

2.4.2. Identification of Bacterial isolates on Selective Media

Pseudomonas sp. is isolated and identified using Cetrimide Agar, a selective and differential medium. Cetrimide media composition pancreatic digest of gelatin 2.0%, K_2SO_4 1.0%, MgC_{l2} *0.1%*, $C_{19}H_{42}BrN$ 0.03%, glycerin 1%, agar 1.36%. The selective agent of cetrimide inhibits most bacteria as it functions as a detergent. For several years, therefore, 0.03 % cetrimide agar has been used and found to be efficient in isolation of *Pseudomonas* sp. [24].

2.5. Secondary Screening for Cellulase Enzyme Production

Potential isolates for enzyme efficiency have been examined. The maximum cellulase production isolates were considered for the following study.

2.5.1. Inoculum Development

The maximum hydrolysis zone of these isolates is grown in 20 ml inoculum production medium [composition: Carboxymethylcellulose (CMC) 0.5%; Tryptone 0.20%; KH_2PO_4 0.4%; $MgSO_4.7H_2O$ 0.02%, Na_2HPO_4 0.04%, $CaCl_2.2H_2O$ 0.0001%; $FeSO_4.7H_2O$ 0.0004% and pH adjusted to 7.0] separately and cultivated 24 hours at 37°C. It was used as an inoculum medium subsequently the source of production medium [22].

2.5.2. Crude Enzyme Production by Submerged Fermentation Process

CMC broth containing Carboxymethylcellulose (CMC) 1.0%; Tryptone 0.20%; KH_2PO_4 0.4%; Na_2HPO_4 0.04%; $MgSO_4.7H_2O$ 0.02%; $CaCl_2.2H_2O$ 0.0001%; $FeSO_4.7H_2O$ 0.0004% was prepared and allocate 50ml in 250 ml Erlenmeyer flasks for each of the selected isolated strains. The media were inoculated with 2.5ml (5%) of the selected bacterial isolate from the inoculum media and incubated at 37°C for 48hrs at 150 rpm. After fermentation, the fermented broth was centrifugated to remove undesired materials at 12000 rpm for 10 minutes at 4°C. At the end of centrifugation, the clear supernatant was collected to serve as a crude enzyme source and used to determine enzymatic activity [25].

2.5.3. Cellulase Enzyme Assay

The DNSA method was used to determine how much reducing sugar was liberated during the hydrolysis. Under the following standard assay conditions, the enzyme unit (EU) was calculated as the amount of cellulase required to release 1 mole of reducing sugar per ml per minute [25].

The cellulase activities of each of the bacterial strains were assayed in triplicate. The reaction mixes included a buffer of 1ml 0.05M citrate (pH 4.8), 1 ml substrate solution (1% CMC dissolved in citrate buffer), and 1 ml crude enzyme solution. Instead of the enzyme, the blanks had 1 ml of distilled water for 30 minutes at 50°C. Following incubation, 1.5 ml DNS reagent was applied to the test tubes to end the reaction, waited 10 minutes, and then boiling in water baths at 100°C for 10 minutes. The absorbance was measured at 575nm. Glucose release was measured with the glucose calibration curve [26], and enzymatic activity was determined by the following equation [27].

$$Enzyme\ activity\ (U/ml) = \frac{\left[\begin{array}{l} Reducing\ sugar\ (product\ concentration) \\ X\ 1000\ X\ dilution\ factor \end{array}\right]}{\left[\begin{array}{l} Molecular\ weight\ of\ glucose \\ X\ incubation\ time\ (minute) \end{array}\right]}$$

2.6. Optimization Process for Maximum Cellulase Production

Cellulase activity of the culture supernatant of bacteria was grown at different temperatures, pH, carbon sources, and incubation periods.

2.6.1. P^H

The optimal substrate and carbon source concentration are taken, and the broth pH is balanced at 6.0, 7.0, 8.0, 9.0, and 10.0 in the various conical flask using 1 N HCl and 1 N NaOH as well as autoclaved. Cultures are inoculated and cultivated at specific temperatures. The cell mass was separated by centrifugation after 24 hours of fermentation at 37°C with 150 rpm shaking, and cell-free culture filtrate was obtained as an enzyme source [28].

2.6.2. Incubation Temperature

The initial pH 7 of the production medium was adjusted and then inoculated with selected bacterial isolates that had been grown overnight. At 150 rpm, the broth was incubated at various temperatures ranging from 30, 35, 40, 45, and 50°C. The filtrate of cell-free culture is collected after 24 hours of incubation and used as a source of crude enzymes.

2.6.3. Concentration of Glucose

Different glucose concentrations were used to substitute the original concentration of 1 % sugar in growth media with 2 to 5 % sugar, and all parameters were set to an optimal level.

2.6.4. Carbon Sources

Several carbon substitutes, including fructose, lactose, and sugar, have been used as an initial source of carbon in growth media to compensate for glucose. The impact of these sources on the production medium was analyzed at 5% concentration when pH, temperature, incubation time was fixed at 7.0, 37°C, and 24 hours respectively, in a shaking incubator.

2.6.5. Incubation Period

The incubation period has optimized for the cellulase production and the pH of the production media broth was adjusted to 7.0. In a shaking incubator, 1 ml inoculum was then added to 50 ml of fermentation broth at 37°C with 150 rpm. The fermentation product was withdrawn at different time intervals, e.g., 16, 24, 36, 48, and 72 hours and then centrifuged at 12000 rpm for 10 minutes at 4°C. After centrifugation, for cellulase testing, the supernatant was used to approximate a standard glucose curve.

3. Result and Discussion

3.1. Isolation and Primary Screening for Cellulase Producing Bacteria

In this analysis, ten samples were collected from 5 different locations, yielding 40 isolates from each sample using the dilution 10^{-6} and 10^{-7} plates. From these, 160 out of 200 isolates were removed due to non-cellulolytic. The 40 isolates were then screened for cellulase activity on CMC agar using iodine staining, whereas congo red staining did not yield an effective result. According to Gohel *et al*. studies, the best results are obtained using grams of iodine, accompanied by congo red staining [29]. Gomashe *et al.* and Gupta *et al.* backed up our findings in the same enzymatic sense [17,30].

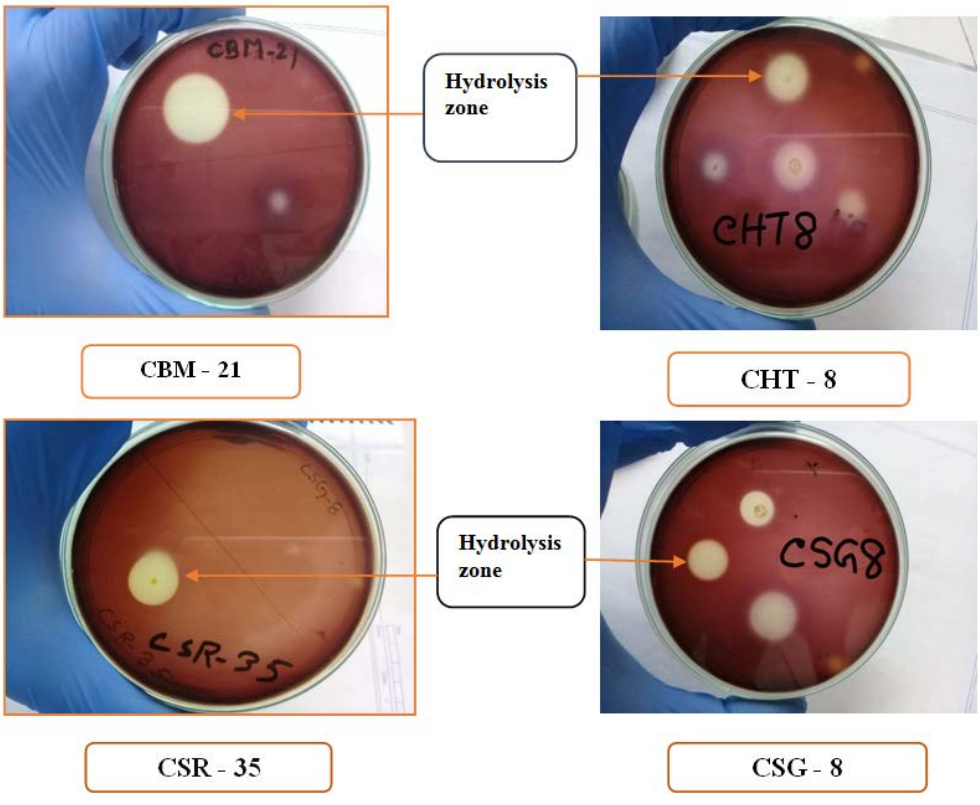

Figure 2. Hydrolysis zone after flooded with iodine

As a consequence, native microbes can be a source of cellulase that can be investigated for use in a variety of applications. The CMCase activity is shown in Figure 1; of both the four isolates, the highest ratio of the apparent zone diameter to the colony diameter on the CMC agar plate was determined compared to other isolates (Figure 2). Out of 43 isolates, four isolates were efficient cellulase producers and analyzed for secondary screening.

3.2. Colony Morphological and Biochemical Characterization

CBM21 isolates produced diffusible whitish, and CSG08 isolates green pigment with irregular and smooth translucent raised colonies on nutrient agar plate based on morphological and biochemical characteristics. On gram staining, all isolates showed gram-negative without CBM21 (Table 1). Although, four isolates were showed citrate and catalase-positive after biochemical tests. Based on morphological, cultural, and biochemical research, the CBM21, CSG08, CHT08, and CSR35 isolates were presumptively identified as a *Bacillus* spp., *Pseudomonas* spp., *Klebsiella* spp. and *Serratia* spp., respectively. The results of the tests are given in the following Table 2 and Figure 3.

Table 1. Colony Characteristics and Gram Reaction

Sl No.	Isolates no.	Colony characteristics	Gram reaction
1	CBM21	Color: Cream off Whitish Surface: Wrinkled Margin: Irregular Elevation: Convex	+
2	CSG08	Color: Greenish blue Surface: Smooth Margin: Undulate Elevation: Low convex	-
3	CSR35	Configuration: Round Margin: Entire Elevation: Raised Surface: Smooth	-
4	CHT08	Configuration: Round Elevation: Convex Margin: Entire Color: Red	-

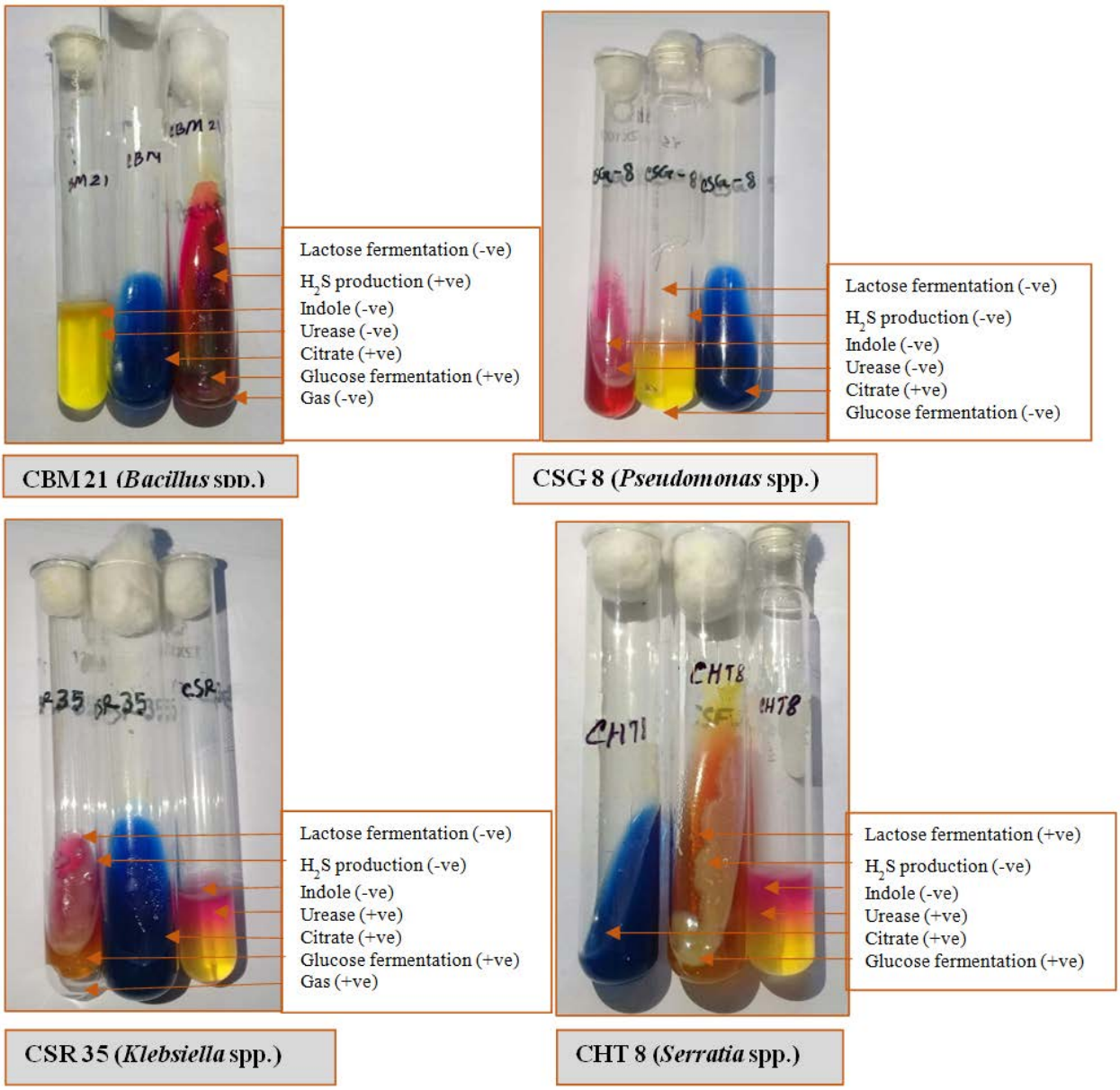

Figure 3. Results of biochemical test

Table 2. Results of Gram Biochemical Test

Biochemical Test	Bacterial isolate no			
	CBM21	CSG8	CHT8	CSR35
Catalase Test	+	+	+	+
Citrate	+	+	+	+
Indole	-	-	-	-
Motility	+	+	-	+
Urease	-	-	+	+
Glucose Fermentation	+	-	+	+
Lactose Fermentation	-	-	+	-
Gas	-	+	+	-
H_2S	+	-	-	-
Presumptive Result	*Bacillus spp.*	*Pseudomonas spp.*	*Klebsiella spp.*	*Serratia spp.*

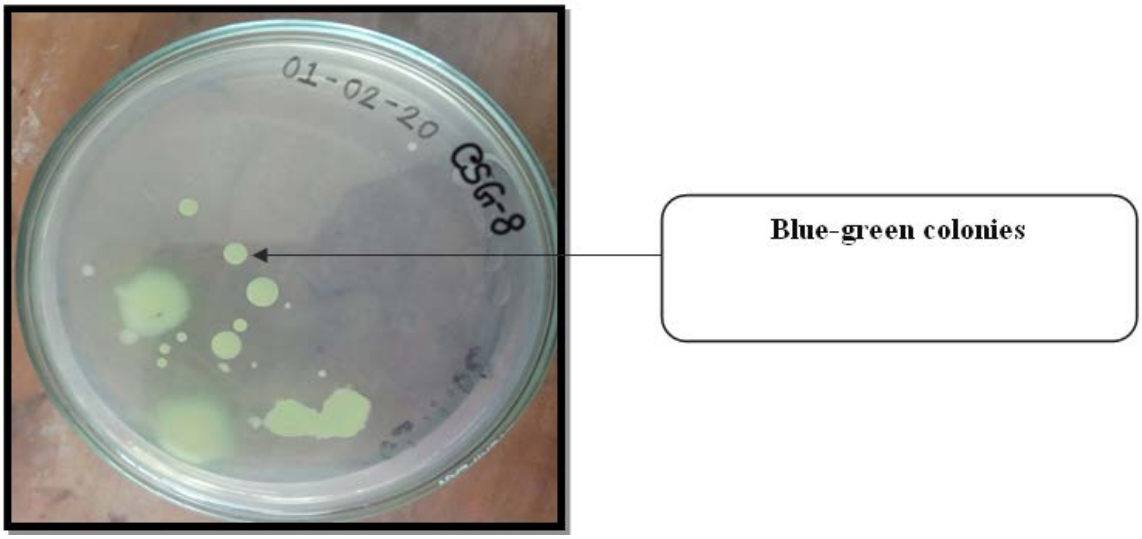

Figure 4. Blue-green colonies on a cetrimide agar plate

3.3. CSG-08 Culture on a Cetrimide Agar Plate

Colonies exhibiting blue-green pigmentation are considered presumptive positive, and it is regarded as *Pseudomonas* spp. (Figure 4). In the study of cellulolytic bacteria, Talia *et al.* [31] discovered that *Pseudomonas* spp. dominated in soil samples obtained from native Chaco soil.

3.4. Measurement of Cellulase Activity

The possible four bacterial isolates (CBM21, CSG08, CHT8, and CSR35) were identified after screening and detection to evaluate their enzyme activity in the submerged fermentation process. Crude enzyme samples were tested using the enzyme activity technique. The maximum cellulase activities of the isolate CBM21 were found 0.156 U/ml compared to other isolates, as shown in Table 3.

Table 3. Activity of enzyme from different isolates

Isolate no.	Conc. of unknown sample	Absorbance of unknown sample	Enzyme activity (U/ml of enzyme)
CBM21	0.843	0.192	0.156
CSG08	0.574	0.127	0.106
CSR35	0.186	0.0334	0.034
CHT08	0.171	0.0297	0.032

Gupta *et al.* [17] found in their studies several cellulose-degrading bacteria with cellulase activity ranging from 0.162 to 0.400 U/ml. In contrast, another study by Ekperigin M.M., 2007 demonstrated that the *A. Anitratus* and *Branhamella* spp. Produced highest enzyme activity was 0.48 and 2.56 U/ml for CMC, respectively [32]. Both findings supported our results by comparing with their studies.

3.5. Optimizing the Production of Cellulase

For cellulase development from selected isolates, the different optimal parameters were calculated.

3.5.1. The Effect of pH on the Production of Enzymes

The media with different pH ranged from 6.0 to 10.0 allowed two isolates and *E.coli* to grow. In CBM21 and CSG8, maximum enzyme activity in pH 7.0 was observed, compared with *E.coli*. However, the enzyme activity has been reduced by increasing pH. This result was compared to a standard glucose curve, consistent with other researchers' findings that most bacteria are best suited to pH 6 to 8 [33,34]. Shown in Figure 5.

3.5.2. The Effect of Incubation Temperature on Enzyme Production

Regulation of the enzymes reported at various temperatures showed that all three bacteria produced

maximum cellulase at 35 to 40°C. This Figure 6 result showed that for optimal CBM21 and E. coli, the optimum temperature was 40°C while CSG about 35°C. The temperature was determined by adjusting the physical properties of the cell membrane to affect extracellular enzyme secretion. Bakare et al. claimed that [35] the *Pseudomonas fluorescence* produced cellulase enzyme during 30 to 35°C with showing the best result at 35°C temperature. On the other hand, *Bacillus subtilis* 115 and *Bacillus subtilis* were optimally temperature at 40°C.; also, minimum cellulase yield was observed in fermentation at 45°C [36], while *Bacillus subtilis* and *Bacillus circulans* achieved optimum yield at 40°C [9].

3.5.3. Effect of Different Concentration of Glucose

Results obtained from Figure 7 showed that 5% glucose exhibited the highest cellulase activity compared to other glucose concentrations. CBM21 and CSG8 were produced the highest amount of enzyme (1.23 and 1.31 U/ml) than *E.coli*, where it produced 0.44U/ml.

3.5.4. Effect of Different Carbon Source on Enzyme Production

To substitute 1% sugar, which was the initial concentration in growth media, with 2 to 5 % carbon sources, different concentrations of carbon sources were used. The findings revealed that 5% of the carbon source, including glucose and lactose, brought the highest cellulase production for CBM21 and CSG8 compared to other concentrations of carbon sources at 24 h incubation. Figure 8 showed that fructose produced high cellulase only for *E.coli* spp. that serves as a control group at 24 h of incubation. In comparison to other studies, the best source of cellulase activity was found to be glucose [37]. Saraswati *et al*. [38] claimed that the best carbon source of *Bacillus subtilis* was lactose extracted from cow dung to produce optimal cellulase. But in another study by Teodoro and Martins [39] revealed that maltose had been used for Bacillus spp. as the best source of carbon.

3.5.5. Effect of Different Incubation Period on Enzyme Production

Figure 9 illustrated the correlation between the incubation period and enzyme activity. It was observed that the level of cellulase increased from 24 to 48 h and after that decreased more rapidly as the fermentation its endpoint. The work currently shows that the synthesis of enzymes is reduced when the incubation time reaches the optimal time. Haq *et al*. [40] also suggested that a decrease in incubation enzymatic activity could be due to nutrient depletion and in the fermentation medium, the formation of other by-products. At the same time, another study by Ariffin *et al.* [41], concluded that the inactivation of enzyme secretion is associated with bacterial stress caused by nutrient depletion in the medium. However, the highest activity of the enzymes was observed in our research at 24 hours of bacterial culture.

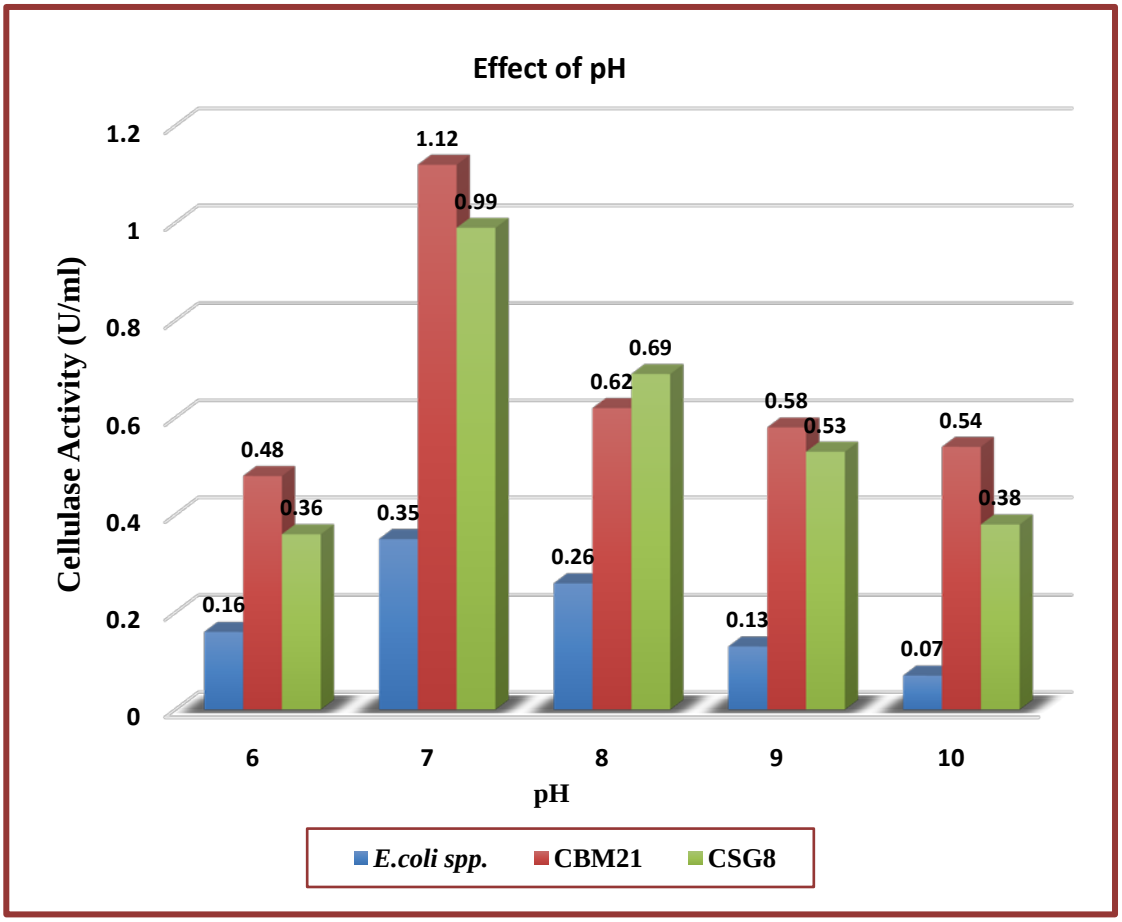

Figure 5. Effect of pH on cellulase activity

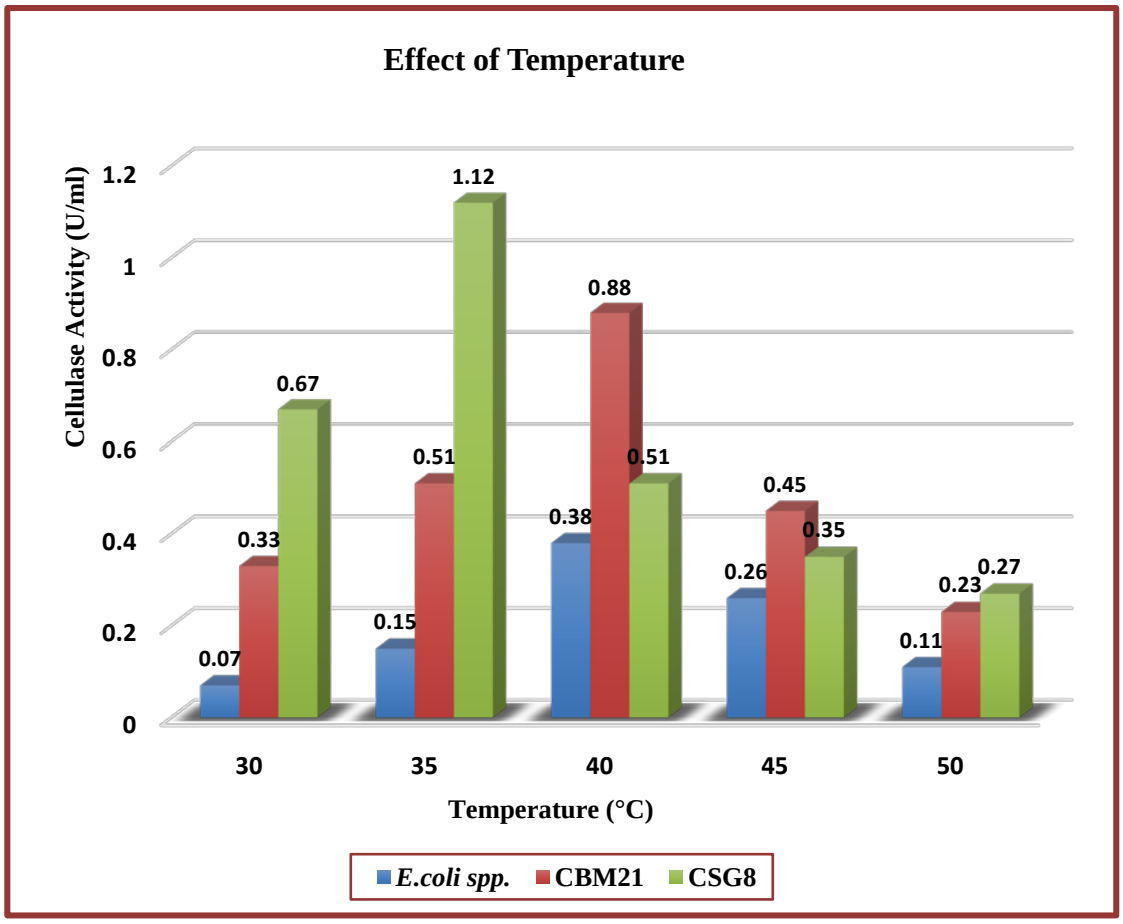

Figure 6. Effect of Incubation temperature on cellulase activity

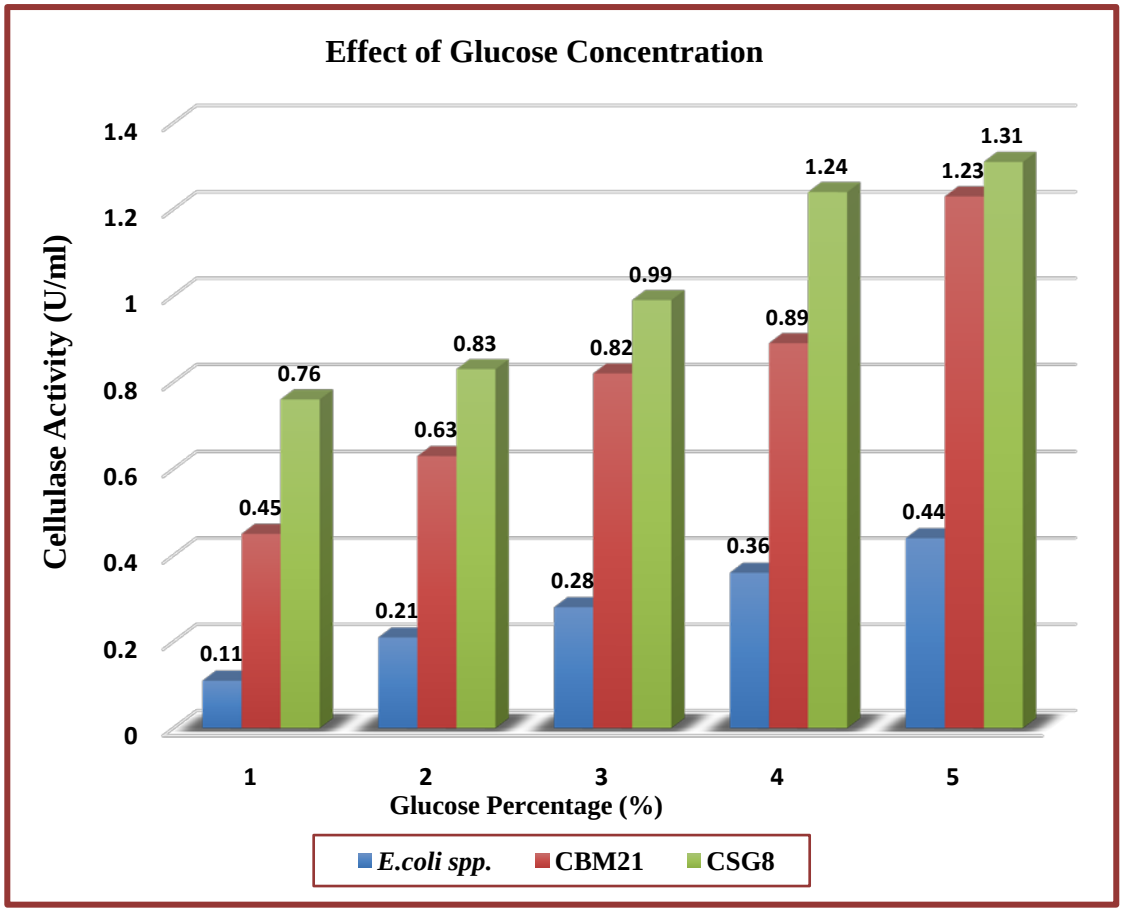

Figure 7. Effect of glucose concentration on cellulase activity

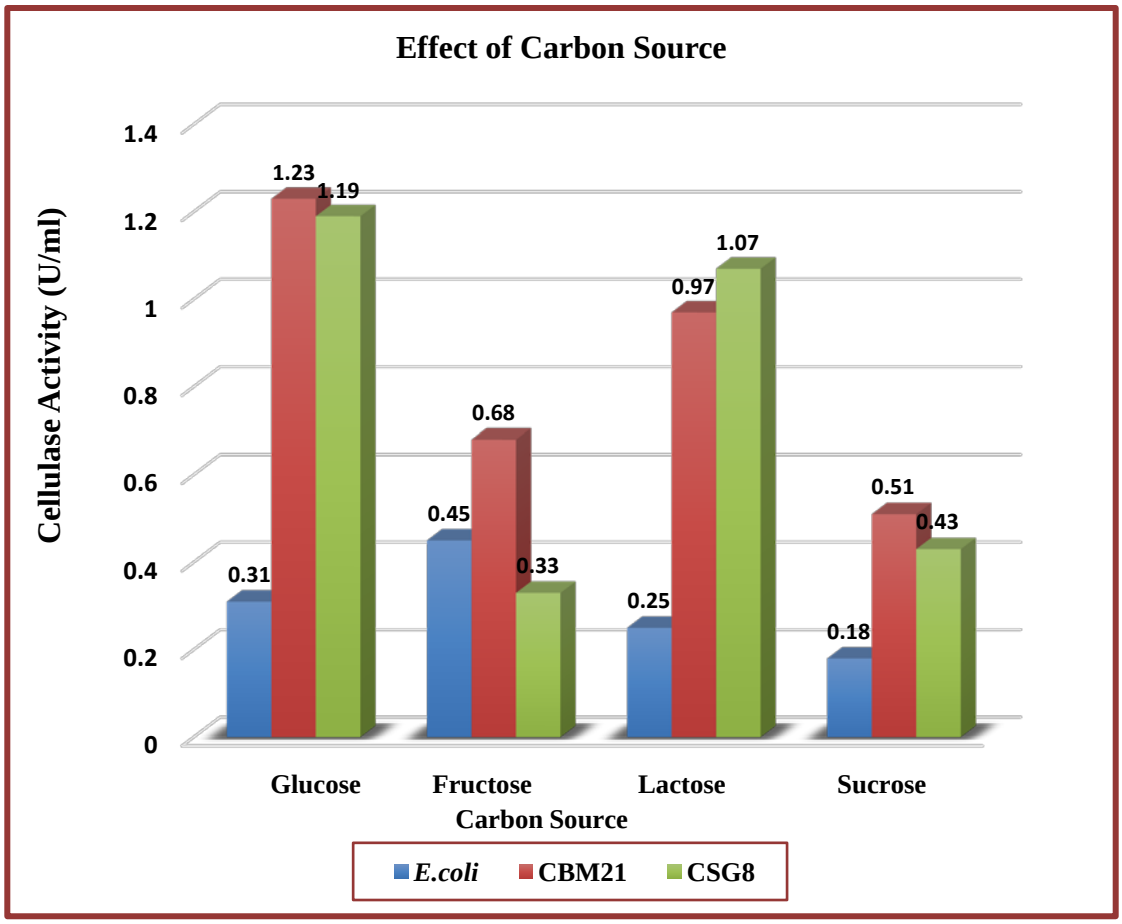

Figure 8. Effect of carbon source on cellulase activity

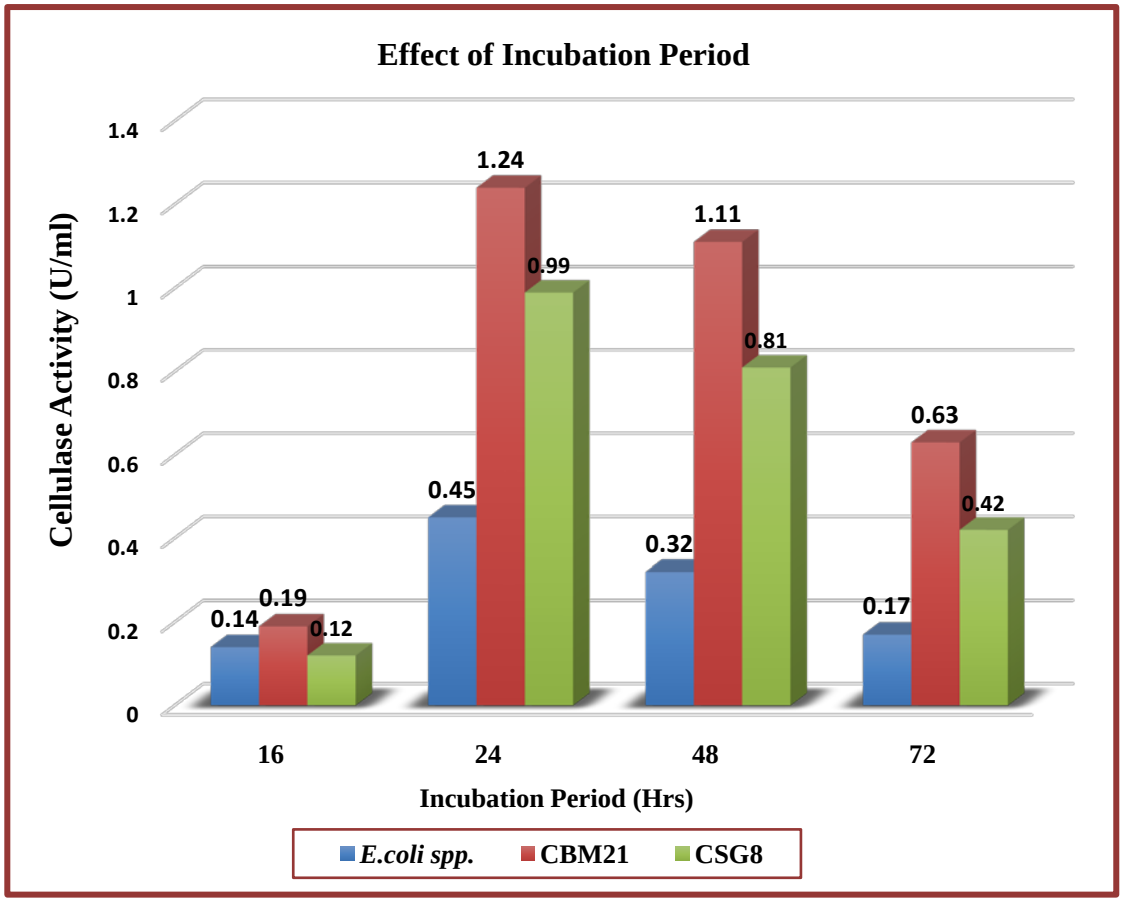

Figure 9. Effect of incubation period on cellulase activity

4. Conclusions

A wide range of cellulolytic bacteria can be found in soil, including those that have yet to be isolated. This research was done to isolate potential cellulase-producing bacterial isolates from soil samples. Specific isolates were selected due to breaking down cellulose activity and cellulolytic characteristics. Among all these four isolates CBM21, CSG08, CSR35, and CHT08 were picked for possible cellulase activity determination as their ratio of colony diameter and clear zone diameter were higher than others. Based on colony morphology, selective culture, and biochemical analysis of these four isolates, CSG08 was presumptively identified as organisms from *Pseudomonas* species. It was also confirmed after observing bluish-green colonies on particular selective media. Partial purification of the cellulase enzyme has been performed, and the function of the enzyme is determined by the DNSA method. CBM21 and CSG08 were found to be highly active in our research as their enzyme activity was determined to be 0.156 U/ml and 0.106 U/ml compared to other isolates.

The production of cellulase for each isolate has been optimized to determine optimal parameters for stability and ensure better enzyme production and enzyme activity. After optimization, an amazing enhancement was noticed for CBM21 (1.35 U/ml) and CSG8 (1.23 U/ml) compared to *E.coli* = 0.44 U/ml. The enzyme was characterized by an optimum incubation temperature of 40 and 35°C for CBM21 and CSG8, respectively, and pH seven was found to be optimal after 24 h incubation period and 5 % glucose used as a single carbon source. These optimized requirements can be seen in various industrial sectors. Further large-scale culture studies are required by mutagenesis and protein engineering techniques to optimize other parameters, including inoculum concentration, inducer presence, and medium additives.

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Supplementary

Table S1. Different sample collection site for cellulase enzyme production

Sample no	Collection Site	Total no. of CFU/gm.	Labeled as
1	Green waste	$1.49x10^9$	CSG
2	Tannery industry waste	$1.74x10^9$	CHT
3	Rice straw	$1.40x10^9$	CSR
4	Municipal waste	$2.06x10^9$	CBM
5	Forest soil	$1.22x10^9$	CSF

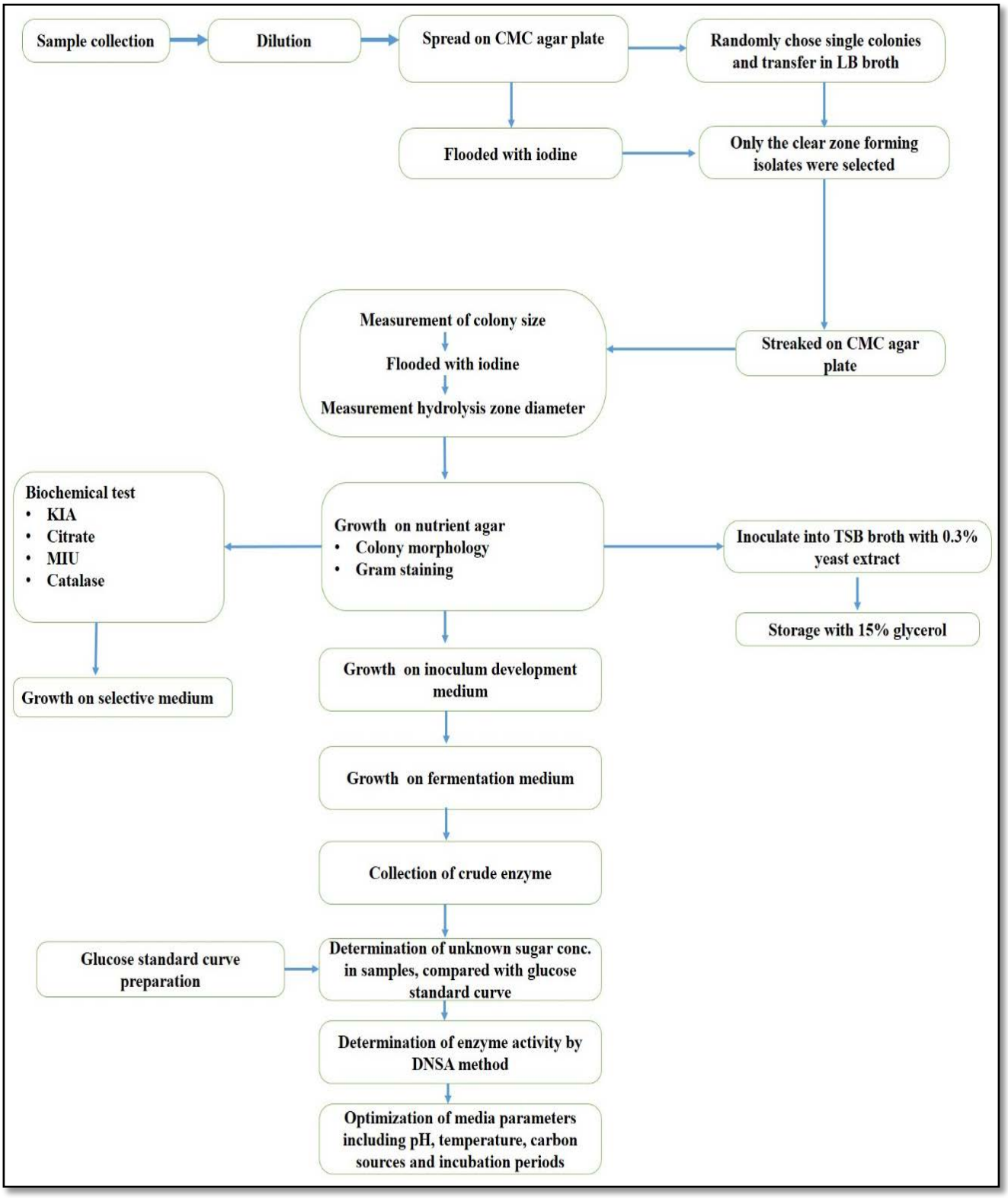

Figure S1. Flow diagram of the study design

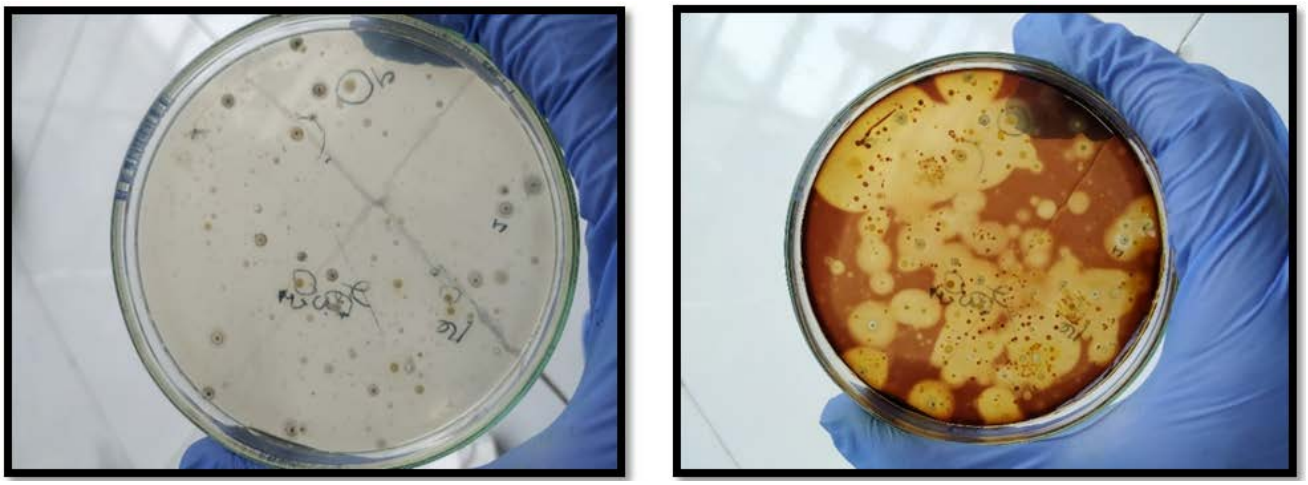

Figure S2. Dilute sample culture on CMC agar plate and flooded with iodine solution