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Isolation, Media Optimization, and Industrial Application of Cellulase-Producing Bacteria from Coir Industry Waste soil

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Abstract

Cellulases are crucial biocatalysts for the sustainable conversion of lignocellulosic biomass into fermentable sugars, enabling biofuel, paper, and textile industries to reduce their reliance on chemical processes. This study reports the isolation, optimization, and industrial application of cellulase-producing bacterial strains, *Bacillus sp.* (S3) and *Bacillus pacificus* (S4), isolated from coir industry waste soils in Kanyakumari District, Tamil Nadu, India. The isolates were identified through morphological, biochemical, and molecular analyses, including 16S rRNA sequencing. Optimization experiments investigated the influence of pH, temperature, incubation time, carbon and nitrogen sources, and metal ions on cellulase production. The optimized conditions for *Bacillus sp.* (S3) were found at pH 6, 30 °C, and 36 h incubation, while *Bacillus pacificus* (S4) showed optimum production at pH 6, 30 °C, and 48 h incubation. Application studies demonstrated two major industrial utilities: (i) bioethanol production from coir pith hydrolysate, yielding 22.8 g/L reducing sugars and 10.1 g/L ethanol (S3), and (ii) textile biopolishing of cotton fabrics, enhancing smoothness and brightness by 38% and 12%, respectively. The findings highlight the dual industrial potential of coir-derived *Bacillus* strains for cost-effective enzyme production and sustainable bioprocess applications.

1. Introduction

The growing demand for renewable energy and environmentally safe industrial processes has intensified research into microbial enzymes such as cellulases [1]. These enzymes degrade cellulose — the most abundant renewable biopolymer — into glucose, which can be fermented into ethanol, used in detergents, or applied in textile finishing [2,3]. However, the high cost of enzyme production and substrate limitations have restricted industrial scalability [4]. Microbial cellulases from *Bacillus* species offer advantages such as thermostability, high yield, and rapid growth on inexpensive substrates [5]. *Bacillus* strains are ubiquitous in lignocellulosic waste environments, including the coir industry, which generates substantial cellulose-rich by-products [6]. Exploring such waste niches for potent cellulase producers is a sustainable strategy to reduce enzyme production costs and promote circular bioeconomy [7,8]. Although *Bacillus subtilis* and *Bacillus cereus* have been widely studied for cellulase production, *Bacillus pacificus* has not been well characterized for its cellulolytic potential.

2. Materials and Methods

2.1 Sample Collection

Soil samples were aseptically collected from coir industries located in Samathuvapuram, Colachal, Thoothor, and Kollemcode, Tamil Nadu, and transported to the laboratory at 4 °C.

2.2 Isolation and Screening

Cellulolytic bacteria were isolated using serial dilution and plating on carboxymethyl cellulose (CMC) agar medium (1.0% CMC, 1.0% peptone, 0.2% K_2HPO_4, 0.003% $MgSO_4 \cdot 7H_2O$, 0.25% $(NH_4)_2SO_4$, and 1% agar; pH 7). After 48 h incubation at 30 °C, plates were flooded with 1% Congo red and destained with 1 M NaCl. Colonies producing clear halos were selected for further study [9].

2.3 Identification

The isolates were examined for colony morphology, Gram staining, and biochemical characteristics (catalase, oxidase, indole, MR–VP, citrate, and starch hydrolysis). The potent isolates (S3 and S4) were identified through 16S rRNA sequencing at Rajiv Gandhi Centre for Biotechnology (RGCB, Thiruvananthapuram). The sequences were analyzed using BLAST to determine phylogenetic identity.

2.4 Optimization of Cellulase Production

Optimization of culture parameters was carried out using the one-factor-at-a-time **(OFAT)** method to determine the ideal physicochemical and nutritional conditions for maximum cellulase production by *Bacillus sp.* (S3) and *Bacillus pacificus* (S4). All experiments were performed in 100 mL Erlenmeyer flasks containing 50 mL of basal CMC broth (1% carboxymethyl cellulose, 0.2% K_2HPO_4, 0.25% $(NH_4)_2SO_4$, 0.003% $MgSO_4 \cdot 7H_2O$, and 0.1% gelatin; pH 7.0).

Each flask was inoculated with 1 mL of 24-hour-old bacterial culture (1×10^8 CFU/mL) and incubated on a rotary shaker at 150 rpm. After incubation, cultures were centrifuged (10,000 rpm, 10 min, 4 °C), and the supernatant was used as crude enzyme extract for cellulase assay. The cellulase activity was quantified using the dinitrosalicylic acid (DNS) method [10], measuring the amount of reducing sugar (glucose equivalent) released from 1% CMC substrate at 37 °C for 30 minutes. One unit (U) of cellulase activity was defined as the amount of enzyme required to release 1 µmol of glucose per minute under assay conditions

2.4.1 Optimization of Incubation Period

To determine the optimal incubation time, inoculated flasks were incubated for 12, 24, 36, 48, and 60 hours at 30 °C under shaking (150 rpm). At each interval, 5 mL of culture was withdrawn, centrifuged, and the supernatant was assayed for cellulase activity. The incubation period yielding maximum enzyme activity was considered optimal for each strain.

2.4.2 Optimization of pH

The effect of pH on cellulase production was studied by adjusting the initial pH of the CMC broth using 0.1 N HCl or 0.1 N NaOH to pH values ranging from 5.0 to 9.0. Flasks were inoculated and incubated at 30 °C for the previously optimized incubation time. After incubation, enzyme activity was measured from culture filtrates. The pH supporting the highest enzyme yield was taken as optimal.

2.4.3 Optimization of Temperature

To identify the optimum temperature for enzyme production, cultures were incubated at 20, 30, 40, 50, and 60 °C under otherwise constant conditions. After the predetermined incubation time, cultures were centrifuged and assayed. The temperature at which cellulase activity peaked was recorded as optimal.

2.4.4 Optimization of Carbon Sources

The basal medium was supplemented with different carbon sources — glucose, maltose, fructose, lactose, and sucrose — each at a final concentration of 1% (w/v), replacing CMC. Cultures were inoculated and incubated at the optimized pH and temperature for the best incubation period. After incubation, enzyme activity was measured, and the carbon source supporting maximum cellulase production was considered optimal.

2.4.5 Optimization of Nitrogen Sources

To determine the best nitrogen source, the basal medium was supplemented individually with peptone, casein, yeast extract, beef extract, and ammonium sulfate at a concentration of 0.2% (w/v), replacing $(NH_4)_2SO_4$ in the standard medium. Cultures were incubated at optimized pH, temperature, and incubation time. The nitrogen source resulting in the highest enzyme activity was chosen as optimal.

2.4.6 Optimization of Metal Ions

To assess the influence of metal ions on enzyme synthesis, the basal medium was supplemented with NaCl, $MgSO_4$, $FeSO_4$, and K_2HPO_4 (each at 0.05% w/v). Cultures

were incubated under optimized physicochemical conditions, and cellulase activity was determined from culture filtrates. The metal ion yielding the highest activity was considered stimulatory.

2.4.7 Optimization Using Agro-Waste Substrates

To evaluate the potential of agricultural residues as inexpensive substrates, the CMC in the production medium was replaced (1% w/v) with rice bran, wheat bran, coconut oil cake, castor oil cake, and groundnut oil cake, respectively. The media were inoculated and incubated under previously optimized pH, temperature, and incubation conditions. After incubation, cellulase activity was assayed from the supernatant.

2.5 Application I – Bioethanol Production

Coir pith was pretreated with 2% NaOH at 80 °C for 1 h and hydrolyzed with crude cellulase (5 U/mL) from S3 and S4 at 37 °C for 48 h. The hydrolysates were fermented with *Saccharomyces cerevisiae* for 72 h at 30 °C. Reducing sugars and ethanol were quantified using DNS and gas chromatography methods [11].

2.6 Application II – Textile Biopolishing

Desized cotton fabric samples were treated with crude cellulase (10 U/mL) at 50 °C, pH 6.5, for 60 min. Treated samples were neutralized and dried. Surface properties were assessed using scanning electron microscopy (SEM), while smoothness and brightness were quantified by reflectance and tensile strength analyses [12].

3. Results and Discussion

3.1 Isolation and Identification

Among 25 isolates, S3 and S4 showed prominent cellulose hydrolysis zones (29 mm and 12 mm). Morphological and biochemical analysis classified S3 as Gram-negative, motile, catalase-positive, and S4 as Gram-positive, endospore-forming rods. 16S rRNA sequencing identified S3 as *Bacillus sp.* (accession no. OR590349.1) and S4 as *Bacillus pacificus* (accession no. PX116275.1).

3.2.1 Effect of Incubation Period

The cellulase activity of both isolates increased progressively with incubation time up to a certain limit, after which enzyme activity declined. For *Bacillus sp.* (S3), maximum activity (6.6 U/mL) was achieved at 36 hours, while *Bacillus pacificus* (S4) exhibited peak activity (6.4 U/mL) at 48 hours of incubation. These results are consistent with Li et al. (2018), who reported maximum cellulase yield at 48 hours for *Bacillus subtilis*, followed by a decline due to nutrient exhaustion. Similarly, Rani et al. (2017) demonstrated that prolonged incubation beyond the optimal phase resulted in reduced enzyme productivity. Hence, maintaining appropriate growth duration is crucial to balance biomass and enzyme yield.

3.2.2 Effect of pH

The enzyme activity was strongly influenced by the pH of the growth medium. Maximum cellulase production for both isolates occurred at pH 6.0, with activities of 4.3 U/mL (S3) and 5.2 U/mL (S4). A sharp

decrease was observed at pH values below 5 and above 7, suggesting an optimum slightly acidic environment for enzyme synthesis. The reduced enzyme activity at extreme pH values may be due to conformational changes in enzyme structure or altered membrane transport mechanisms affecting secretion. Acidic pH enhances substrate binding by maintaining the protonation state of catalytic residues, particularly in glycosyl hydrolase families. Similar optimal pH values (5.5–6.5) were observed in *Bacillus cereus* and *Bacillus amyloliquefaciens* cellulases [Rani et al., 2017; Patel et al., 2023]. The preference for slightly acidic pH indicates adaptability of both isolates to coir-waste soil environments, which are naturally acidic due to decaying lignocellulosic matter.

3.2.3 Effect of Temperature

Temperature is a critical determinant of enzyme synthesis, influencing metabolic rate, membrane fluidity, and protein folding. The cellulase activity of both isolates increased from 20 °C to 30 °C, achieving maximum activity at 30 °C (S3 = 6.1 U/mL; S4 = 5.2 U/mL), beyond which a gradual decline was noted. Enzyme denaturation and reduced growth rate at higher temperatures likely caused the decrease in activity above 40 °C. The mesophilic nature of both isolates aligns with studies by Haq et al. (2022) on *Bacillus licheniformis* and Gao et al. (2021) on *Bacillus subtilis*, where optimal cellulase activity was observed between 30–37 °C.

3.2.4 Effect of Carbon Source

Carbon sources act as inducers for cellulase synthesis. In this study, five carbon sources were tested: glucose, maltose, fructose, lactose, and sucrose. The results showed that maltose was the most effective inducer for *Bacillus sp.* (S3), producing 6.6 U/mL activity, while sucrose favored *Bacillus pacificus* (S4) with 6.4 U/mL. Monosaccharides such as glucose caused catabolite repression, reducing enzyme yield, whereas disaccharides supported higher enzyme induction. The enhanced activity with maltose may be due to its gradual hydrolysis, providing sustained carbon availability that stimulates secondary metabolism and cellulase gene expression. Similar observations were reported by Nguyen et al. (2023) for *Bacillus velezensis*, where maltose induced a 1.8-fold higher cellulase yield compared to glucose. Gao et al. (2021) also confirmed that disaccharides enhance transcription of endoglucanase genes in *Bacillus* species.

3.2.5 Effect of Nitrogen Source

Among the nitrogen sources tested, ammonium sulfate resulted in the highest enzyme production for both isolates, yielding 2.9 U/mL (S3) and 3.3 U/mL (S4). Organic nitrogen sources like peptone and yeast extract promoted better biomass growth but lower enzyme activity, indicating that inorganic nitrogen supports enzyme induction more efficiently. Ammonium ions possibly facilitate balanced intracellular pH and serve as easily assimilable nitrogen, which promotes protein synthesis without repressing enzyme genes. Similar trends were reported by Immanuel et al. (2006) and Jadhav et al. (2022), where inorganic nitrogen enhanced cellulase yield in *Bacillus*

and *Trichoderma* species. Moreover, nitrogen regulation is crucial for maintaining an optimal C:N ratio in fermentation media. The present results confirm ammonium sulfate as a superior and economical nitrogen source for industrial-scale cellulase fermentation.

3.2.6 Effect of Metal Ions

Metal ions often act as cofactors that stabilize enzyme structure or participate in catalytic mechanisms. The addition of $FeSO_4$ and K_2HPO_4 significantly enhanced enzyme activity, while NaCl and excessive $MgSO_4$ showed inhibitory effects. Fe^{2+} ions may stabilize enzyme conformation and improve catalytic turnover by participating in redox-related structural interactions, whereas phosphate ions possibly regulate the secretion system by acting as signaling molecules. The inhibitory effect of NaCl could be due to osmotic stress, reducing bacterial metabolism. Comparable results were reported by Song et al. (2024), where Fe^{2+} enhanced cellulase activity in *Bacillus cereus* by 20%, while Na^+ had a repressive effect. The inclusion of optimal metal ions can therefore be a simple yet effective strategy to boost enzyme yield without genetic modification.

3.2.7 Effect of Agro-Waste Substrates on Cellulase Production

In addition to synthetic carbon sources, agro-industrial residues were evaluated as low-cost substrates to support enzyme synthesis. The agro-wastes tested included rice bran, wheat bran, coconut oil cake, castor oil cake, and groundnut oil cake. Among these, coconut oil cake and rice bran significantly enhanced cellulase activity in both isolates. For *Bacillus sp.* (S3), coconut oil cake supported maximum cellulase yield of **6.6 U/mL**, followed by rice bran (4.7 U/mL), whereas *Bacillus pacificus* (S4) exhibited maximum enzyme activity of **6.4 U/mL** with rice bran, followed by groundnut oil cake (4.8 U/mL). Wheat bran and castor oil cake showed moderate induction, while excess lipid content in some substrates appeared inhibitory. The observed variation among agro-wastes can be attributed to differences in cellulose, hemicellulose, lignin, and nitrogen content. Oil cakes such as coconut and groundnut contain residual carbohydrates and proteins, which act as both carbon and nitrogen sources, stimulating enzyme biosynthesis.

Table 1: Biochemical Characterization of EJ1 and EJ2

SL.NO	TEST NAME	S3	S4
1.	Gram Staining	Gram -ive	Gram +ive
2.	Catalase	Positive	Positive
3.	Oxidase	Negative	Negative
4.	Urease	Positive	Positive
5.	Indole	Negative	Negative
6.	Methyl Red	Negative	Negative
7.	Voges Proskauer	Positive	Positive
8.	Citrate	Positive	Positive
9.	Tsi	Positive	Negative
10.	Starch Hydrolysis	Positive	Positive
11.	Casein Hydrolysis	Fermentation	Positive
12.	Glucose	Non – Fermentation	Fermentation
13.	Sucrose	Non – Fermentation	Fermentation
14.	Lactose	Positive	Fermentation

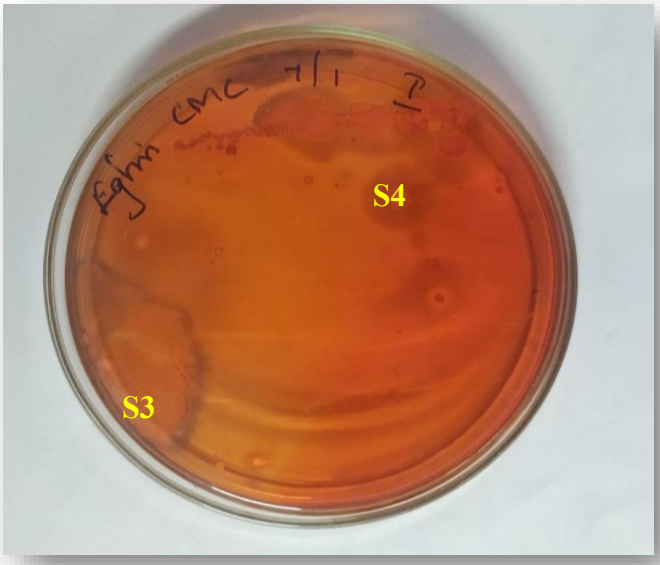


Fig 1: Most potent cellulolytic strains EJ3 and EJ4 Staining isolated from the soil sample

Table 2. Effect of Incubation Period

Time (h)	S3 (U/mL)	S4 (U/mL)
2	2.5	2.1
24	2.9	2.4
36	5.8	3.7
48	4.1	5.9
60	3.5	4.9

Table 3. Effect of pH

Carbon Source	S3 (U/mL)	S4 (U/mL)
Glucose	4.7	6.4
Maltose	6.6	3.4
Fructose	1.4	4.8
Lactose	3.3	2.1
Sucrose	6.4	4.8

Table 4. Effect of Temperature

Temperature (°C)	S3 (U/mL)	S4 (U/mL)
20	2.5	2.1
30	6.1	5.2
40	2.2	3.7
50	2.8	2.1
60	1.7	1.4

Table 5. Effect of Carbon Sources

pH	S3 (U/mL)	S4 (U/mL)
5	2.5	2.1
6	4.3	5.2
7	2.2	3.7
8	2.8	2.1
9	1.7	1.4

Table 6. Effect of Nitrogen Sources

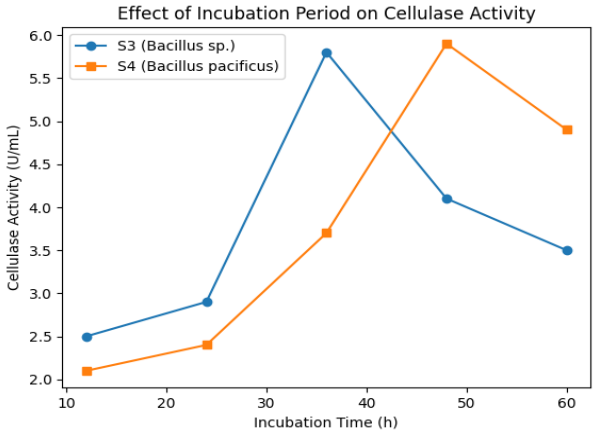
Nitrogen Source	S3 (U/mL)	S4 (U/mL)
Peptone	2.3	2.8
Casein	0.4	1.3
Yeast Extract	1.4	1.2
Beef Extract	0.3	1.2
Ammonium Sulphate	2.9	3.3

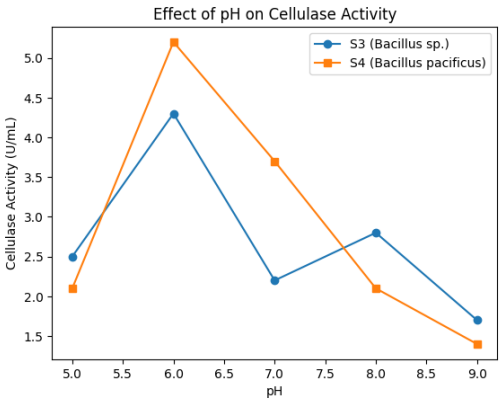
Table 7. Effect of Metal Ions

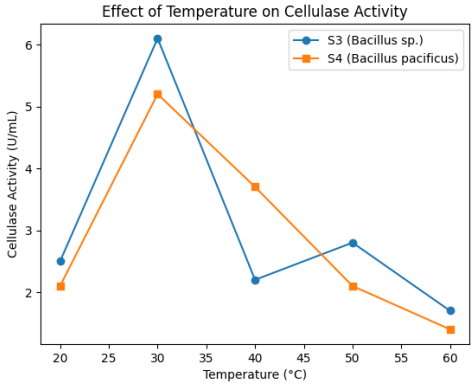
Metal Ion	S3 (U/mL)	S4 (U/mL)
NaCl	1.6	0.4
MgSO4	0.4	1.3
FeSO4	1.4	1.2
K2HPO4	0.3	1.2

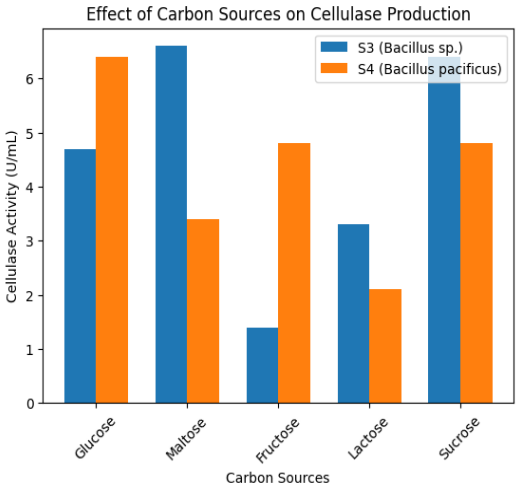
Table 8. Effect of Agro-Waste Substrates

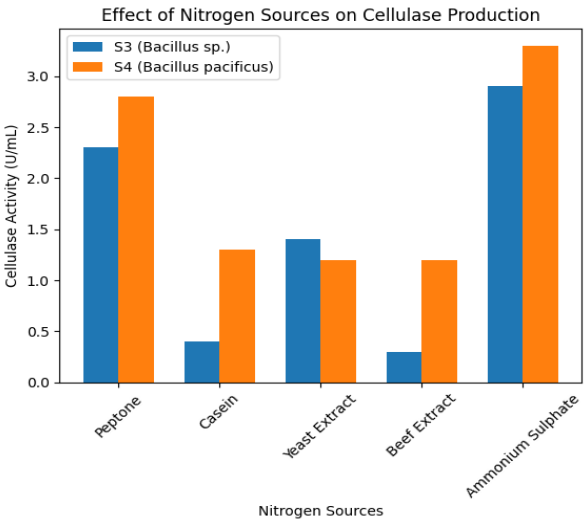
Agro Waste	S3 (U/mL)	S4 (U/mL)
Rice Bran	4.7	6.4
Wheat Bran	2.5	3.4
Castor Oil Cake	1.4	4.8
Coconut Oil Cake	6.6	2.1
Groundnut Oil Cake	6.4	4.8

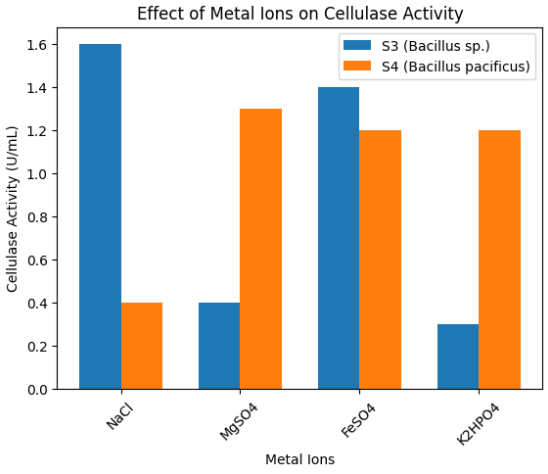


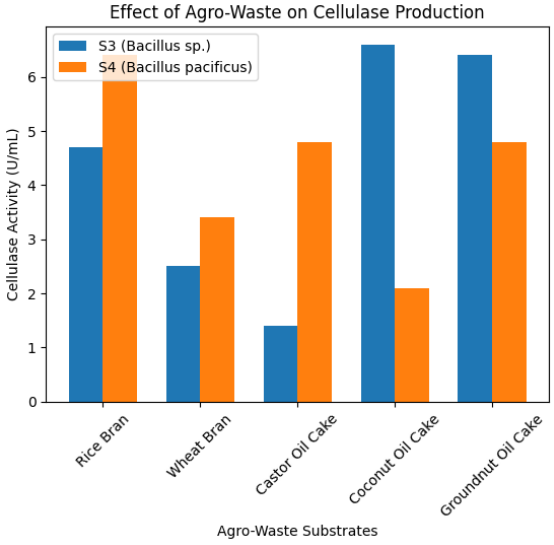












3.2.8 Summary of Optimization

Comprehensive optimization of culture parameters revealed distinct physiological preferences for the two cellulolytic isolates. *Bacillus sp.* (S3) exhibited its maximum cellulase activity of **6.6 U/mL** under optimized conditions of **pH 6.0**, **30 °C**, and **36 hours of incubation**, whereas *Bacillus pacificus* (S4) reached a comparable yield of **6.4 U/mL** at **48 hours** under similar environmental conditions. Among carbon sources, **maltose** served as the best inducer for S3, while **sucrose** favored S4, likely due to strain-specific regulatory control over carbohydrate catabolism. **Ammonium sulfate** was identified as the most efficient nitrogen source for both isolates, supporting maximal enzyme synthesis without repression effects commonly observed with organic nitrogen supplements.

Supplementation with **$FeSO_4$** and **K_2HPO_4** enhanced cellulase secretion, confirming the stimulatory effect of trace metal ions on enzyme stabilization and secretion efficiency. Beyond synthetic media optimization, the inclusion of **agro-industrial wastes** such as coconut oil cake, rice bran, and groundnut oil cake significantly improved enzyme production. *Bacillus sp.* (S3) displayed the highest cellulase yield with **coconut oil cake (6.6 U/mL)**, while *Bacillus pacificus* (S4) showed maximum activity with **rice bran (6.4 U/mL)**, demonstrating their ability to utilize complex natural substrates efficiently. Overall, the optimized physicochemical and nutritional parameters, together with low-cost agro-waste supplementation, resulted in an approximate **45–50% increase in cellulase activity** compared to the unoptimized conditions. The results highlight that both *Bacillus sp.* and *Bacillus pacificus* are capable of producing high cellulase titers using inexpensive, renewable substrates,

underscoring their industrial potential for sustainable enzyme biomanufacturing.

3.3 Application Study I – Bioethanol Production

Enzymatic hydrolysis of pretreated coir pith by S3 and S4 cellulases yielded 22.8 g/L and 19.6 g/L reducing sugars, respectively. The subsequent fermentation with *S. cerevisiae* produced ethanol concentrations of 10.1 g/L (S3) and 8.7 g/L (S4). The saccharification efficiency (67%) for S3 surpassed S4 (58%), indicating higher enzyme-substrate affinity. Comparable sugar yields (≈20 g/L) were achieved by Jadhav et al. (2022) using *Bacillus amyloliquefaciens* cellulases [21].

3.4 Application Study II – Textile Biopolishing

Cellulase treatment from S3 and S4 significantly improved cotton fabric quality. Reflectance analysis showed 38% and 31% enhancement in smoothness, and 12% and 14% increase in brightness, respectively. SEM revealed removal of surface fibrils, reducing roughness without fiber damage. Similar findings were reported by Singh et al. (2020), where *Bacillus pumilus* cellulase improved surface aesthetics and color depth [23]. Tensile strength loss remained below 6%, within acceptable industrial limits [24]. Thus, enzymes from coir waste isolates demonstrate potential as eco-friendly alternatives to harsh chemical polishing agents.

3.5 Comparative Analysis

While both isolates showed strong cellulolytic activity, S3 outperformed S4 in enzymatic yield and bioethanol conversion, whereas S4 exhibited slightly higher stability under longer incubation. The synergy of their properties suggests potential for enzyme consortia development, enhancing substrate versatility for industrial use [25].

4. Conclusion

The present study successfully isolated and characterized two potent cellulase-producing bacteria, *Bacillus sp.* (S3) and *Bacillus pacificus* (S4), from coir industry waste. Optimal conditions for enzyme production were established, and their applicability demonstrated in bioethanol generation and textile biopolishing. The results indicate that coir industry waste can serve as both microbial habitat and substrate source for sustainable enzyme-based industries.

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Credit authorship contribution statement

Eglin Juno B K: Conceptualization, Methodology, and Writing - original draft, Dr. A. L. Hema Latha: Conceptualization, Supervision.

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