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RESEARCH ARTICLE OPEN ACCESS

Screening, Analysis, and Purification of Unique Cellulase from *Bacillus subtilis* R2A Derived from Agricultural Fields and Waste Dump Sites

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Abstract

Microbial cellulase enzyme, endoglucanase, performs a significant role in various industries, including textiles, paper pulp, biofuel production, agriculture, and pharmaceuticals. The aim of this research was focused on extracting, purifying, and investigating the microbial enzyme produced by *Bacillus subtilis* strain R2A (accession number PP088049). The cellulase-producing *Bacillus* strain was isolated from agricultural fields near Gwalior in Madhya Pradesh. The bacterial isolate of *Bacillus* species was preliminarily identified via cultural characteristics and biochemical characterization, using selective media culture. *B. subtilis* isolate exhibited significant cellulase enzyme activity under optimized conditions (pH 4.0-9.0, 37 °C, 120 rpm). The cellulase enzyme was refined via salting out, utilizing Diethylaminoethyl Cellulose-52 matrix column chromatography to purify microbial cellulase enzymes. The microbial cellulase was refined up to a 5.44 enrichment factor and had an catalytic activity of 137.95 U/mg protein. Microbial cellulase was a single-unit enzyme possessing an estimated protein molecular weight of 51.4 kDa, as evaluated through SDS-PAGE. This indicates the potential of *B. subtilis* isolate as a potent resource for biotechnological and industrial processes, particularly in producing sustainable fuels and cellulose-derived bioproducts. Microbial cellulases play an essential role in drug discovery by initiating the biotransformation of complex carbohydrates into secondary metabolites, as a result expediting the development of novel therapeutics, and improving medication efficacy. Future studies could focus on upscale production and exploring its application in diverse industrial settings.

Keywords: Cellulase, *Bacillus subtilis* Isolate, Column Chromatography, SDS-PAGE

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INTRODUCTION

Cellulose constitutes 50% of plant biomass by dry weight and 50% of the secondary biomass sources, in the form of agro-industrial waste.[1-5] Cellulose is considered one of the most resistant fibers and non-crystalline polysaccharides that are resistant to hydrolysis and insoluble in water.[6-8] It consists of monomeric units of D-glucose, which are bonded through β-1,4 carbohydrate linkages.[9-11] Cellulase enzymes can break down these β-1,4 carbohydrate linkages, resulting in the synthesis of smaller oligosaccharides and ultimately glucose.[12-15] Lignocellulose biomass is the primary structural constituent of flora and is a sustainable energy source within the biosphere.[16-22]

Although carboxymethyl cellulase (endoglucanases) from *Bacillus* species are unable to degrade crystalline cellulose, certain polysaccharide-degrading enzymes, such as alkaline cellulases, have shown promising results, and have been extensively used in detergent.[23-25] Bacteria are considered efficient producers of cellulase enzymes because they can secrete substantial amounts extracellularly, which simplifies the extraction and purification processes.[26-30] Among them, *B. subtilis* has been extensively investigated for its remarkable cellulase production capabilities.[31,32] Various strains, for example, *Bacillus subtilis* JBS250, *B. subtilis* strain LFS3, CBS31 strain of *Bacillus subtilis*, *Bacillus halodurans* IND18, and BC1 strain of *Bacillus subtilis*, have demonstrated significant potential in biomass fermentation, fiber modification, and pharmaceutical applications.[33-36]

This investigation aimed to obtain, isolate, produce, and purify microbial cellulase from cellulose-degrading bacteria, with a specific focus on a *Bacillus subtilis* isolate, followed by characterization of the enzyme for potential large-scale applications.[37] The examination is concentrated on maximizing production, enzyme separation through DEAE-Cellulose 52-column chromatography methods, and determining its physiochemical properties using SDS-PAGE.[38] Microbial cellulase enzymes have various applications in the pharmaceuticals, brewing, textiles, food, paper, and pulp industry (Figure 1).[39]

MATERIALS AND METHODS

Materials

Folin-Ciocalteu (phenol) reagent, bovine albumin, and DEAE Cellulose-52 were obtained from HiMedia. Acrylamide, Bis-acrylamide derivative TEMED, diammonium persulfate (APS) was procured from Genetix Biotech Asia Pvt. Limited. The other chemicals, all of LG grade, were obtained from Sigma-Aldrich Corporation.

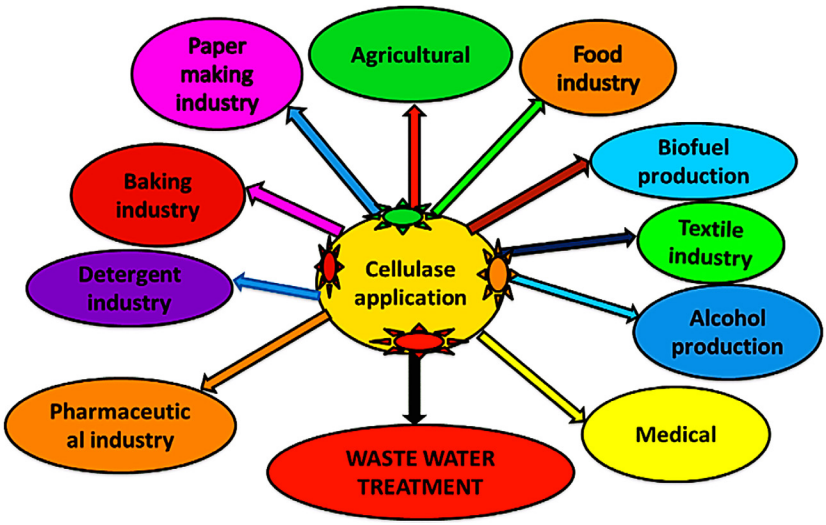

Figure 1. Cellulase: A versatile enzyme for biofuel, pharmaceutical, agriculture, and industrial applications

Isolation of microorganisms

Cellulase-degrading bacterial samples were obtained from five cellulose degradation sites, including agricultural fields. The two differential media used in the streak-plate method were: Carboxymethyl cellulose (CMC) agar media: Peptone (10 gm/litre), K_2HPO_4 (2 gm/litre), Carboxymethyl cellulose (10 gm/litre), $MgSO_4.7H_2O$ (0.3 gm/litre), gelatin (2 gm/litre), and $(NH_4)_2SO_4$ (2.5 gm/litre), and agar (15.0

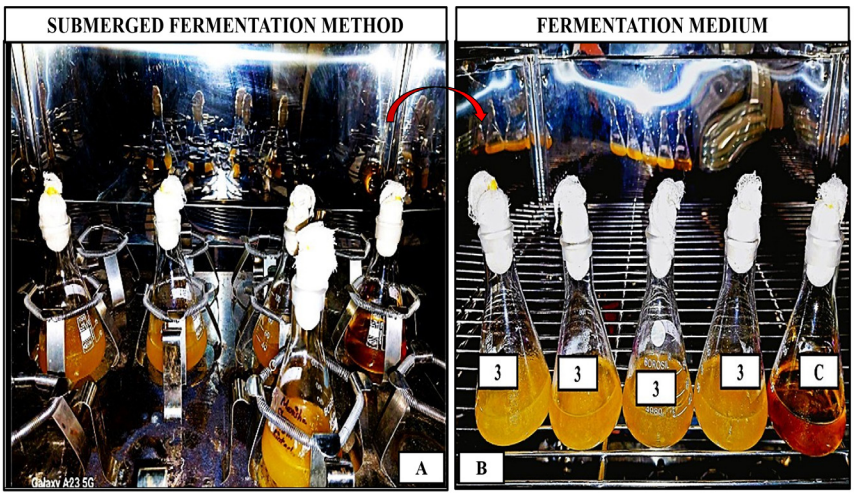

Figure 2. Submerged fermentation for cellulase enzyme production using *Bacillus subtilis* R2A. (A) Fermentation medium inoculated with *Bacillus subtilis* placed in a biological shaker incubator at 37°C and 120 rpm for 48 hours. (B) Post-incubation culture in conical flask showing enzyme production in submerged fermentation broth after 2 days

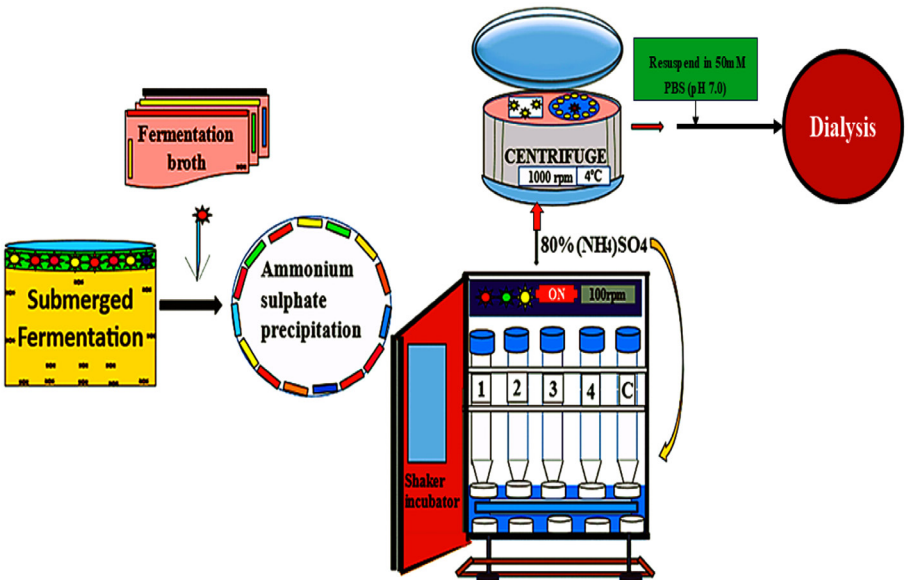

Figure 3. Ammonium Sulfate Precipitation of crude enzyme obtained from submerged fermentation. Proteins in the crude enzyme extract were precipitated by gradual addition of solid ammonium sulfate $(NH_4)_2SO_4$ to 80% saturation. The mixture was stirred at 4 °C overnight, centrifuged, and the pellet resuspended in 50 mM phosphate buffer (pH 7.0). The semi-purified cellulase was then dialyzed against the same buffer to remove residual salts. Adapted from Ibrahim et al.[41]

gm/litre). Neutral pH was maintained, and Petri dishes were kept at 37 °C for 72 hours for incubation. Sucrose agar media (SAM): yeast extracts (2.0 gm/litre), $MgSO_4.7H_2O$ (0.25 gm/litre), K_2HPO_4 (0.5 gm/litre), sucrose (2.0 gm/litre), and agar (15.0 gm/litre). Similarly, neutral pH was maintained and the Petri dishes were kept at 37 °C for 72 hours for incubation.[39,40]

Characterization of Cellulolytic bacteria

Cultural characteristics

Microorganisms were characterized based on their colony characteristics, form, aroma, edge, texture, and pigmentation. Gram staining is a microbiological technique that categorizes microorganisms into Gram-positive (+) and Gram-negative (-) microorganisms.[40,41]

Biochemical analysis

After the phenotypic and cultural properties of the bacterial strains had been recorded, further confirmation based on their biochemical analysis includes IMViC, catalase, fermentation, and Simmon's citrate test using standard biochemical methods.[41]

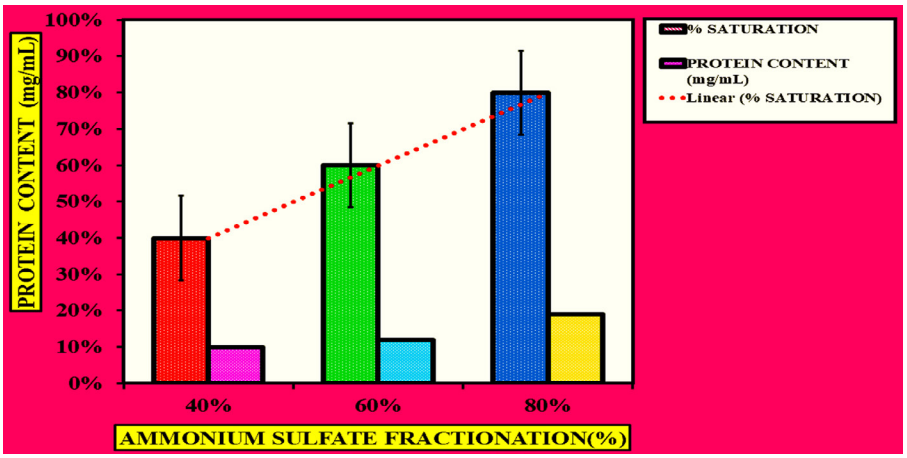

Figure 4. Impact of Enzyme Efficiency After Salting Out (Ammonium Sulfate Fractionation): Max. efficiency was observed at 80% saturation

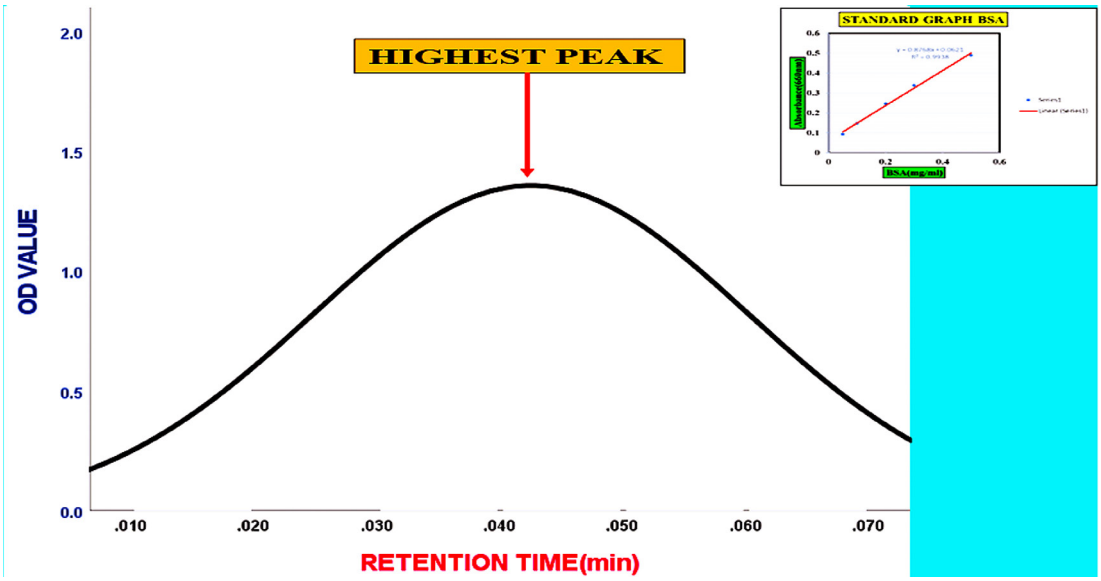

Figure 5. Elution profile of cellulase enzyme using column chromatography. The retention time is represented on the X-axis, while the OD at 660 nm is shown on the Y-axis. Analysis conducted with IBM SPSS Statistics Version 27 indicates that Fraction 4M exhibits the highest OD value, forming the peak of the Gaussian curve

Microbial cellulase enzyme is produced using the submerged fermentation method

Bacterial cultures were analyzed for cellulolytic enzyme synthesis through submerged culture techniques. CMC broth was prepared by dissolving 1% peptone, 0.3% beef extract, 3% carboxymethylcellulose, and 0.5% NaCl in 100 mL of deionized water, and sterilized at 121 °C for 15 minutes in an autoclave to ensure sterility. Sucrose broth media (SBM) was composed of 0.2% yeast extract, 0.2% Sucrose, 0.05% KH_2PO_4, and 0.025% $MgSO_4.7H_2O$ in 100 ml of deionized water and autoclaved at 121 °C for a duration of 15 min. Post-sterilization, 3.0 ml of microbial isolate broth was combined with the culture broth and maintained at 37 °C for 2 days with an intermittent shaking

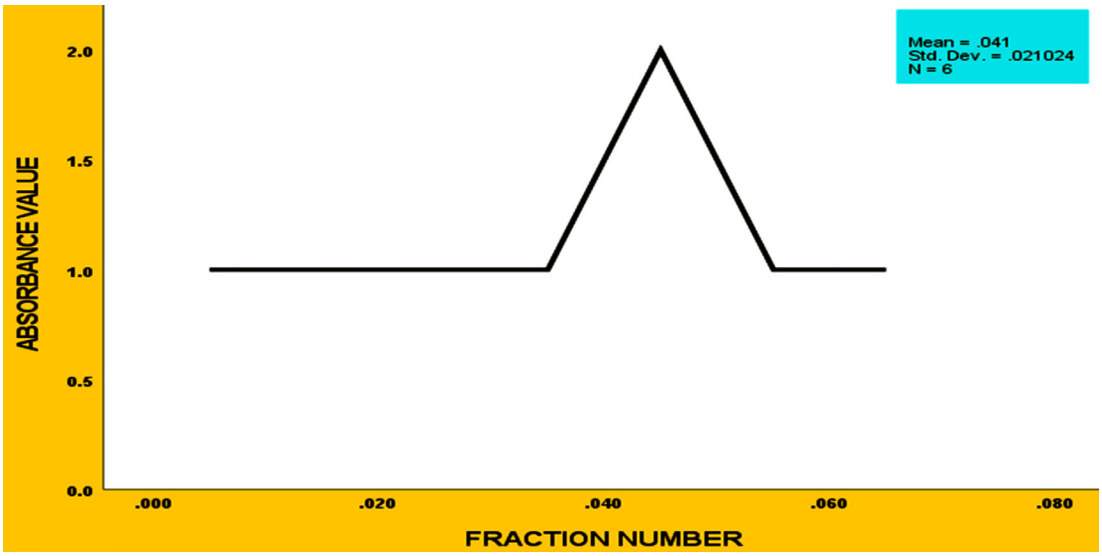

Figure 6. Elution profile of cellulase enzyme using column chromatography. The X-axis corresponds to the fraction number, while the optical density (OD) at 660 nm is plotted on the Y-axis. The analysis was performed using IBM SPSS Statistics, Version 27, highlighting the peak in the cellulase enzyme's purification curve

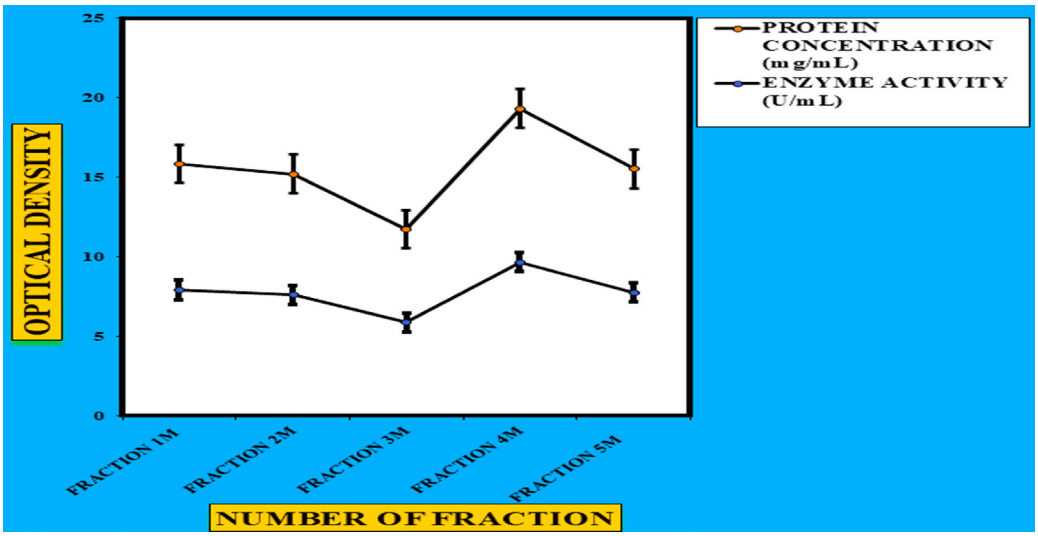

Figure 7. Separation profile of the microbial enzyme produced by *Bacillus subtilis* isolate on Diethylaminoethyl Cellulose-52, showing protein content (mg/mL) and cellulolytic activity (U/mL). The dual-axis graph was generated using Microsoft Excel, representing the correlation between protein elution and enzymatic activity across different fractions

of 120 cycles/min (Figure 2). Cellulase enzymatic activity was measured at 24 hrs intervals. The crude enzyme source was the supernatant after centrifugation.[42]

Quantification of microbial cellulase production using the DNSA method

The dinitrosalicylic acid (DNSA) method is a colorimetric technique used to quantify cellulase activity.[43]

Purification of cellulase enzyme from bacterial isolates

Ammonium sulfate fractionation (salting out)

To precipitate proteins from the crude extract, solid ammonium sulfate $(NH_4)_2SO_4$ was

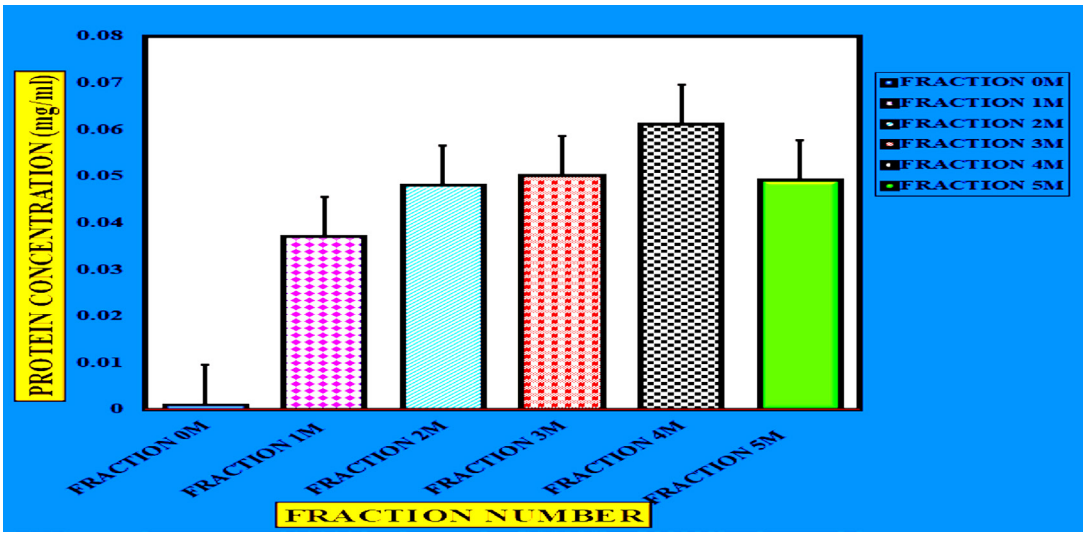

Figure 8. Elution profile of protein obtained through column purification. The X-axis shows the fraction number, while the Y-axis represents the protein amount (mg/mL). A distinct peak at fraction-4M indicates the maximum elution of the target enzyme, confirming its successful purification and separation from impurities, as analyzed using Microsoft Excel

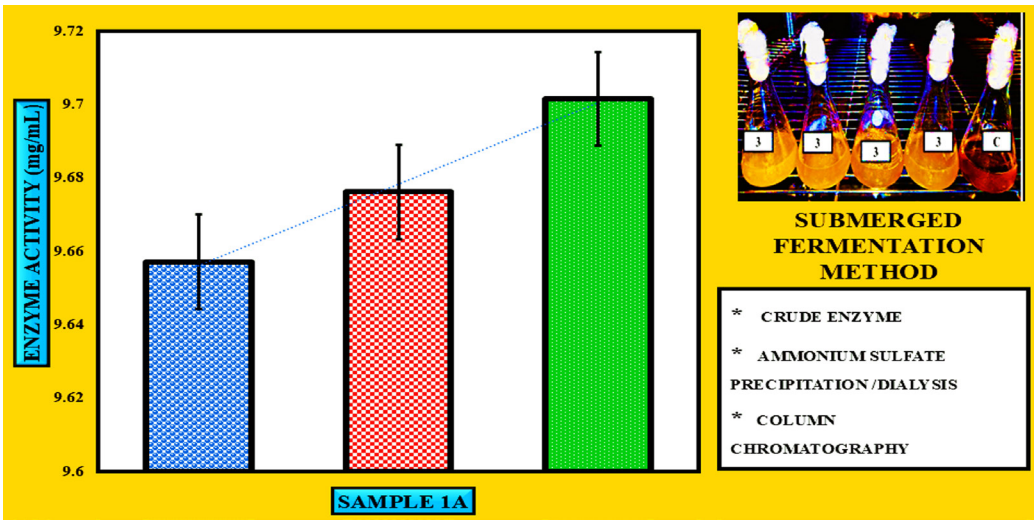

Figure 9. Purification of Microbial Cellulase Enzyme: Comparative Analysis of Crude Extract, Ammonium Sulfate Precipitation, and Column Chromatography Fractions. The X-axis represents purification stages, while the Y-axis represents cellulolytic activity. Elution profile of cellulase produce by *Bacillus subtilis* R2A on DEAE Cellulose -52 by Microsoft Excel.[4]

added to achieve 80% saturation. The solution was continuously agitated at 4 °C overnight using a mechanical stirrer to ensure uniformity and stability. After incubation, the sample was subjected to centrifugation, and the resulting precipitate was resuspended in 50 mM PBS (pH 7.0) for subsequent fractionation and analysis. The Semi-concentrated microbial cellulase was then subjected to dialysis against the same phosphate buffer to remove excess salts (Figure 3).[44]

DEAE cellulose -52 column chromatography

Diethylaminoethyl Cellulose -52 Column (1.5 × 15 cm) was vertically mounted and prepared for protein purification at a steady flow of 200 µl min^{-1}. Four-bed vol. of 50 mM Tris-HCl buffer was used to equilibrate the column. Unbound proteins were eliminated by thoroughly washing the chromatographic system. The matrix-bound proteins were released through a stepwise NaCl elution gradient (0 to 0.5 M) in Tris-HCl buffer. Six

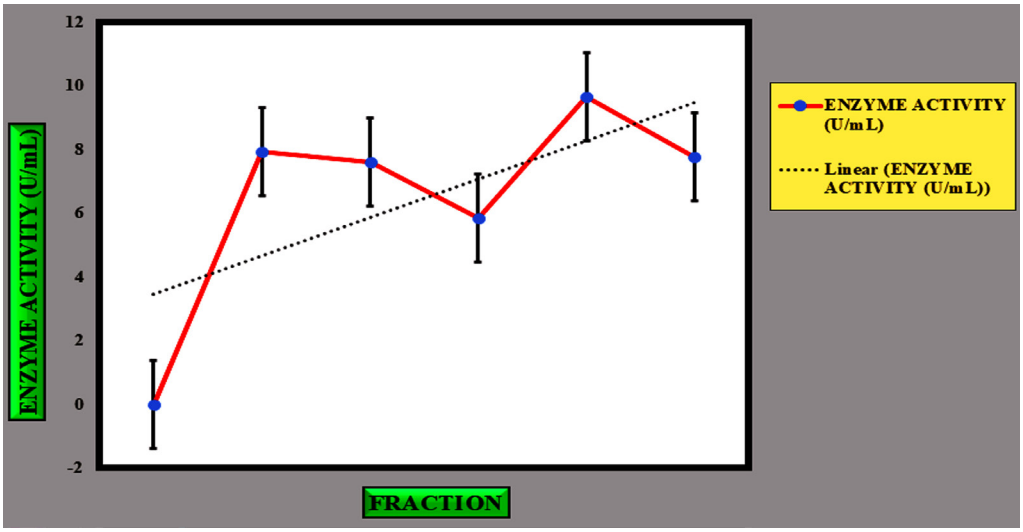

Figure 10. Chromatographic separation of cellulase produce by *Bacillus subtilis* R2A on Diethylaminoethyl Cellulose -52 by Microsoft Excel

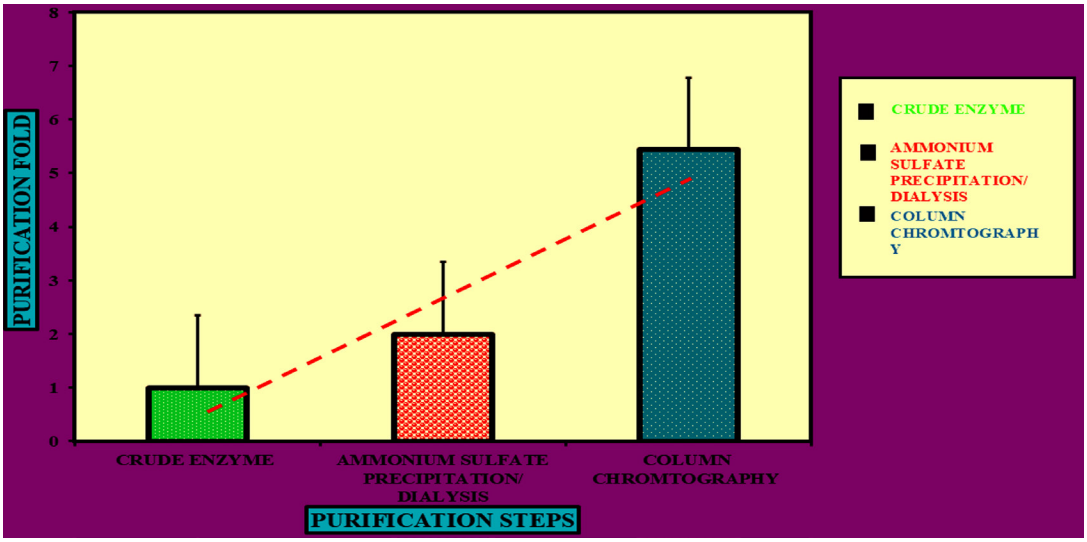

Figure 11. Comparison of purification fold at various stages of cellulase purification. The X-axis represents the various stages of purification, whereas the Y-axis indicates the purification fold. A progressive increase in purification fold was observed, reaching 5.44-fold after column chromatography.[55]

equal-volume fractions (2 ml each) were obtained at a consistent flow rate. Protein concentrations in each fraction were calculated using the Folin-Ciocalteu assay, with purified albumin derived from bovine serum as the standard.[45]

Electrophoretic analysis of purified microbial cellulase

The relative molar mass of the isolated microbial cellulase was evaluated by electrophoretic analysis. In addition to the enzyme sample, standard protein markers were added. The electrophoresis procedure was performed following Bracewell's protocol. The enzyme was combined with an equivalent aliquot of 2X Laemmli sample buffer, which included 4% SDS in 0.125 M Tris-HCl (pH 6.8), and underwent thermal denaturation at 95 °C for 10 minutes. The microbial cellulase enzymes were further analyzed using Polyacrylamide Gel Electrophoresis (PAGE), with a separating gel of 12% concentration and a stacking gel of 4% concentration, gel electrophoresis was carried at room temperature for 2 hrs at 100 V using the Mini-Protein II System (Bio-Rad, USA).

RESULTS AND DISCUSSION

Identification and molecular analysis of cellulolytic microorganisms from soil samples

Microbial cellulases are extensively studied as industrially relevant biocatalysts owing to their capacity to hydrolyze cellulose, major component of plant biomass. Due to their broad applications in industries such as biofuel production, and paper, there is a continuous demand for novel bacterial strains that exhibit enhanced cellulase activity and produce enzymes with greater stability under diverse conditions.[45,46] Microbial cellulases play a crucial role in drug discovery by facilitating the biotransformation of complex carbohydrates into secondary metabolites. This process accelerates the development of novel therapeutics, supports textile processing, enables efficient biomass degradation, and improves medication efficacy.[47] A cellulase-producing *Bacillus* strain was isolated from agricultural fields near Gwalior in Madhya Pradesh. The bacterial isolate exhibited rod-shaped morphology and Gram-positive staining.

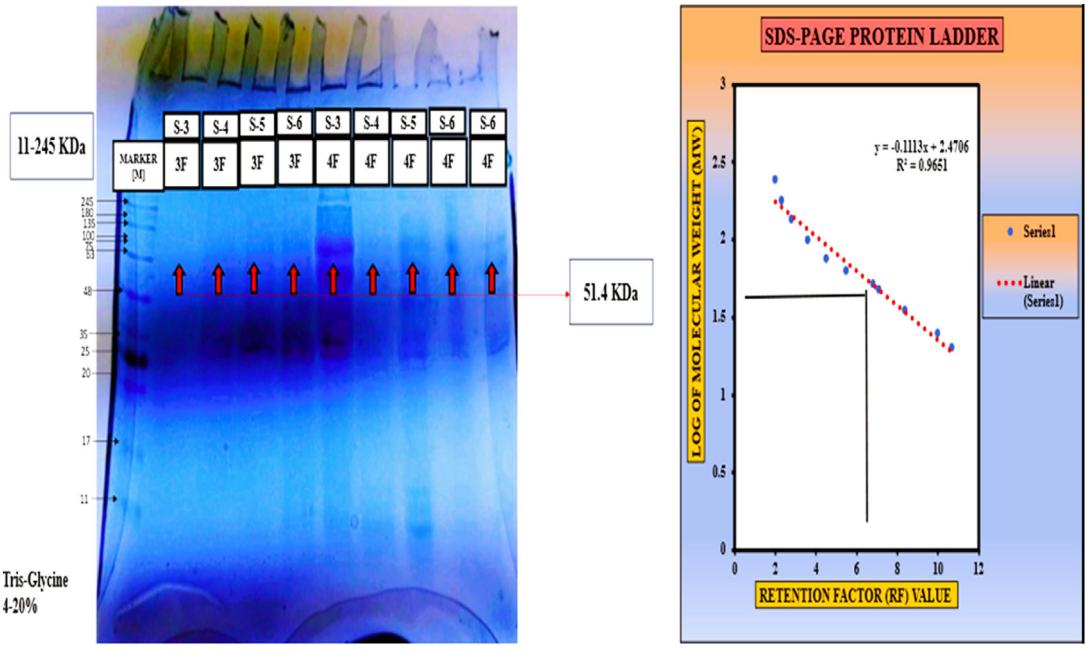

Figure 12. (A) Purified cellulase from *B. subtilis* isolate was analysed using SDS-PAGE at pH 7.0 on a 12% PAGE. Subsequently, Coomassie Brilliant Blue R-250 staining. M represents the protein marker (51.4 kDa). (B) Standard curve plotting log (MW) versus relative mobility (Rf) for molecular weight determination of unknown proteins.[55]

The colony morphology was irregular, opaque, creamy white or pale yellow, with uneven edges. Based on these characteristics, the isolate was preliminarily identified as a *Bacillus* species. Further subculturing on selective media such as Carboxymethyl Cellulose (CMC) Agar and Sucrose Agar Media (SAM), along with positive biochemical tests including Voges-Proskauer (VP), Simmons' citrate, fermentation, casein hydrolysis, and catalase, and negative results for methyl red (MR) and indole production, confirmed the identity of the bacterium as *Bacillus subtilis*.[48,49]

Extraction and identification of microbial cellulase enzyme

The *B. subtilis* isolate exhibited significant cellulase enzyme activity under optimized conditions (pH 4.0-9.0, 37 °C, 120 rpm).[50] The crude enzyme extract had a concentration of 0.383 mg/ml and showed a total cellulolytic activity of 9.7 U/ml.[51,52] The microbial cellulase produced in this study exhibited impressive pH stability ranging from 4.0 to 9.0, which is particularly advantageous for industrial applications where varying pH conditions are often observed.[53] Compared with cellulase derived from *Bacillus pumilus* and *Bacillus licheniformis*, our enzyme demonstrated superior pH tolerance.[54,55] The broad pH stability of our enzyme makes it highly suitable for diverse industrial bioprocessing applications, including biofuels, pharmaceuticals, and waste treatment. Microbial cellulases, particularly those derived from *Bacillus subtilis* R2A, have attracted considerable attention due to their broad applicability across various industries including biofuel production, and emerging roles in the biomedical and pharmaceutical sectors. The cellulase produced in this study exhibited exceptional purification efficiency, high catalytic activity, and significant stability, surpassing several microbial cellulases previously reported in the literature.

Purification

To precipitate proteins from the crude extract, solid ammonium sulfate $(NH_4)_2SO_4$ was added to achieve 80% saturation (Figure 4). The catalytic activity of the purified microbial cellulase enzyme was recorded at 137.95 U/mg, a value significantly higher than cellulases produced by other *Bacillus subtilis* strains. For instance, Msakni et al.[53] reported moderate cellulase activity following partial purification. In contrast, our study achieved a 99.54% yield with a 5.44-fold enrichment factor using a systematically optimized multi-step purification strategy. This strategy included ammonium sulfate precipitation, dialysis, and DEAE-cellulose column chromatography. During the DEAE-cellulose chromatography step, multiple fractions were collected from Sample 3 (Figure 5). Among these, Fraction 4M exhibited the highest cellulase activity, indicating successful enrichment of the active enzyme (Figure 6). A distinct peak observed at Fraction 4M represented the maximum elution of the target enzyme, confirming its successful purification and separation from impurities (Figure 7). The high recovery rate and enzyme stability achieved through this method highlight the superior efficiency of our purification process, enhancing its effectiveness compared to other methods (Figure 8). In contrast, Dikbas et al.[54] reported only 20% cellulase recovery from *Bacillus pumilus* ND8, primarily due to significant enzyme loss during purification stages. Such losses are often attributed to denaturation or enzyme adsorption to matrix surfaces common issues in enzyme purification. However, in our study, the multi-step purification protocol minimized these problems, ensuring both high enzyme purity and stability with minimal degradation during processing (Figure 9). The purification efficiency achieved in this study surpasses the results obtained by Wijayanti et al.[55] through a single-step ammonium sulfate precipitation method used for cellulase extraction from *Bacillus licheniformis* P12. Although their process retained 38% of enzyme activity, the enrichment factor was relatively low at 0.34, underscoring the limitations of relying solely on precipitation for enzyme purification at an industrial scale (Figure 10).[55] In contrast, our multi-step chromatographic approach resulted in a 5.44-fold enrichment factor and 99.54% yield with minimal loss of enzyme activity, demonstrating a significantly more efficient and scalable purification process (Figure 11).

Molecular weight and structural characteristics

SDS-PAGE analysis revealed that the purified microbial cellulase had a molecular

weight of approximately 51.4 kDa, consistent with cellulases from the GH5 family, which are known for their endoglucanase activity. SDS-PAGE was performed on the refined microbial cellulase enzyme, and its molecular weight was measured to be approximately 51.4 kDa by comparing its migration pattern to that of a protein ladder (Figure 12A). The standard curve plotting log (MW) versus relative mobility (Rf) used for molecular weight determination is shown in Figure 12B.[51-56]

CONCLUSION

This study successfully identified and characterized *B. subtilis* isolate as a potent cellulase-producing bacterium from various sources. The bacterium demonstrated optimal cellulase production under specific conditions (neutral pH, 37 °C, and 120 rpm), with the enzyme achieving a 5.44-fold increase and a catalytic activity of 137.95 U/mg. Validation of enzymatic activity was made via zymogram analysis. In conclusion, the relative molecular weight (MW) of the isolated microbial-derived enzyme was measured to be approximately 51.4 kDa through SDS-PAGE analysis. These results demonstrate the potential of *Bacillus subtilis* isolate as a valuable resource for biotechnological and industrial processes, particularly in the sustainable generation of bioenergy and other cellulose-derived bioproducts, which aligns with recent studies published by independent researchers. Future studies could focus on enhancing production efficiency and investigating its broader industrial applications to fully realize its potential.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Not applicable.

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