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Research article

Bioconversion of agricultural waste into cellulase enzyme using *Bacillus subtilis*

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ABSTRACT

Green chemistry offers a promising alternative to toxic chemicals in the industrial sector, particularly in the production of renewable energy and plant-based products. Cellulase, a vital biocatalyst, breaks down cellulose into simple sugars and glucose molecules, which are used in various industries such as brewing, textiles, paper, pulp, and biofuel generation. Present study is focusing on the bio-remedial activity of cellulase enzyme in the degradation of agricultural waste. Bacterial cultures were isolated from soil samples and optimized to produce cellulase from various agrowaste substrates. Bacterial cultures were incubated for 7 days in an orbital shaker incubator, and the Dinitrosalicylic Acid (DNS) method was used to detect and measure cellulase activity. The study found that optical density of *Bacillus subtilis* showed the highest cellulase activity (0.60) when rice bran was used as the substrate as compared to wheat bran (0.46) and corn cob (0.38). Hereby our research concludes that microbial cellulase is effective at bioconverting agricultural waste and may eventually replace synthetic cellulase in a sustainable manner.

1. Introduction

The expanding human population has led to the requirement of increasing agricultural production. According to some estimates, agricultural productivity has been increased more than three times during the past fifty years. Moreover, technological advancements toward the green revolution and the growth of agriculturally rich soil are further two variables contributing greatly to agricultural productivity (Tomme et al., 1995). It is believed that agricultural residues are excellent sources of renewable hydrocarbons. Leveraging agro-waste as raw materials would not only lower industrial production costs but also reduce environmental pollution levels. Agricultural wastes are used for the manufacturing of biodiesel, biogas generation, antioxidants, bioplastics, animal feed, and antibiotics. The demand of organic wastes based green fuel is very high, and this is due to the limited life-span, high cost and pollution intensive nature of the existing fossil fuels, and therefore global reformations in the biowastes to biofuels production policies are needed to made urgently (Areeshi, 2022).

Cellulose degradation is vital to the global carbon cycle, representing a major carbon flow from the reservoir of fixed carbon to atmosphere (Liu et al., 2021). Approximately 50% of the dry weight of agricultural waste is made up of cellulose. Cellulosic waste from industry and agriculture has been building up in the environment recently. Cellulose is commonly degraded by the enzyme cellulase by carrying out depolarisation of cellulose into fermentable sugar. The synergistic action of cellulolytic enzymes during cellulose degradation is crucial for the release of glucose (Singh et al., 2023). Endo-β-1, 4-glucanase, exoglucanase or cellobiohydrolase and β-glucosidase are the components of cellulase enzyme which work simultaneously on cellulose and convert it in to glucose via the enzymatic hydrolysis (Vaid et al., 2021). Hence, cellulases play key role in biomass

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conversion, and have gained tremendous industrial interests in biotransformation of cellulose (Singh et al., 2023). Numerous microorganisms, primarily bacteria and fungi, produce this enzyme (Immanuel et al., 2006). Given their fast growth rate, ability to produce multi-enzyme complexes, stability at extremely high temperatures and pH levels, reduced feedback inhibition, and capacity to survive a variety of environmental stressors, bacteria have been thoroughly studied for the manufacture of cellulase (Bhat et al., 2000). *Bacillus subtilis* continues to be an important bacteria due to its ability to generate and secrete huge amounts of extracellular enzymes (Mawadza et al., 1996). Microbial activity is influenced by temperature, pH, agitation speed, and dissolved oxygen (Jo et al., 2008). Jo et al. (2008) observed that cellulase activity was inhibited by a higher speed of agitation. Hence for improved enzyme synthesis, it is required to optimise the growing conditions.

Wastes containing cellulose may be industrial, urban, or agricultural in origin. Sewage sludge may also be regarded as a source of cellulose because it contains the cellulosic carbon that is required for the formation of methane during the anaerobic digestion of sludge (Nandimath et al., 2016). The anaerobic digestion process can address waste to energy and nutrient recycling challenges efficiently and circularly, for instance, the production of biogas utilises organic, natural materials that can be reused (Zamri et al., 2021). The by-product of a biogas plant can be used as a useful complement or alternative to chemical fertilisers that are harmful and hazardous (Zamri et al., 2021). The previous negative perspective, in which wastes were seen as worthless and even undesirable and for disposal only, has been largely replaced by a positive mindset, in which wastes are acknowledged as raw materials with potential value (Soliman et al., 2013).

In the current research, our research aimed to study the bio-remedial activity of cellulase enzyme to degrade the waste generated in the agricultural sector. A pH range from 7-8 and the physical parameters were optimized for the CMCase (Carboxy-methyl cellulase) activity from newly isolated *B. subtilis*. This bacteria used in the study is a gram +ve rod-shaped bacterium and is widely distributed in soil, waste biomass of plant material, air, and compost prepared from waste biomass. For future improvements of cellulases in the genetic sphere, genomics, proteomics, metabolomics and metabolic pathway, engineering should play an eminent role in cost-effectiveness of enzymes production (Paul et al., 2021). Cellulase recovery aspects that have gained significant progress in recent years (Cai et al., 2020; Dong et al., 2019) should be considered as one of the most effective routes for economical and sustainable use of cellulases.

2. Materials and methods

2.1 Isolation and Characterization of bacterial colonies

One gram of soil was dissolved in 100 mL of water and suspension was prepared. The selective media was used in the isolation of cellulolytic bacteria such as Carboxy methyl cellulose (CMC) agar media containing CMC 1%, 2.0 g of KH_2PO_4, 1.4 g of $(NH_4)_2SO_4$, 0.3 g of $MgSO_4.7H_2O$, 0.1 g of $CaCl_2$, 1.0 g of peptone and 2.0 g of agar (Soeka and Sulistiani, 2019). According to (Soeka and Sulistiani, 2019) CMC agar plates were inoculated by spreading 100 µL of the prepared suspension and incubated at 37°C for 24 hours (Fig. 1a). After staining the culture plates for 30 to 60 minutes with 0.1% 16 congo red (Fig. 1b), they were rinsed with 2% NaCl. Clear zones (Fig. 1c), that developed around the colonies suggested the presence of bacteria that produced cellulase, which were further separated and purified through further streaking.

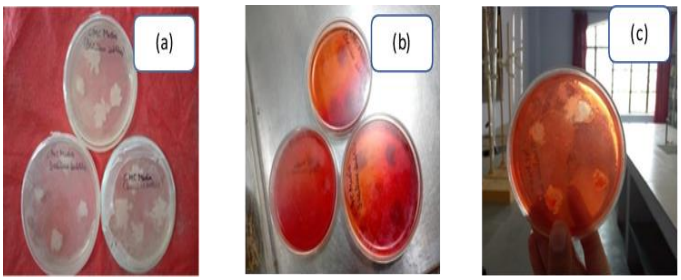

Fig. 1 showing staining before (a), after (b) and after deataining (c)

The screened-out cellulase-producing cultures were identified and characterized. Bacteria were cultured for 24 hours on nutrient agar media at 37°C. The morphology was observed after gram staining under the light microscope and then biochemical analysis was performed. The most promising bacterial isolate was used for further cellulase-producing studies.

2.2 Culturing of Bacteria for cellulase production

The broth media used for the production of enzyme cellulase consisting of 0.5 (g/L) glucose, 0.75 (g/L) peptone, 0.01 (g/L) $FeSO_4$, 0.5 (g/L) KH_2PO_4, and 0.5 (g/L) $MgSO_4$. 100 mL conical flasks were filled with 10 mL of medium each. The flasks were autoclaved at 121°C for 15 minutes and inoculated with overnight-grown bacterial culture. The uninoculated flask was kept as control. At 37°C and 100 rpm, each flask was incubated on an orbital shaker incubator. The culture mixture was centrifuged at 5000 rpm for 15 minutes after 7 days to extract the supernatant, which was then utilised as a source of crude enzymes.

2.3 Cellulase enzyme activity using agro-waste as substrate

The basal media was used for enzyme production consisted of 5 (g/L) lactose, 5 (g/L) NH_4NO_3, 1 (g/L) KH_2PO_4, 0.6 (g/L) $MgSO_4.7H_2O$, 0.1 (g/L) $CaCl_2$, 0.01 (g/L) $FeCl_3$. Different agro-waste substrates were used in the study which contain rice bran, wheat bran, and maize cob. 100 g of each of these substrates were desiccated in the oven at 70°C for 3 days and converted into fine powder form by grinding and sieving (Soeka and Sulistiani, 2019). 200 mL of basal media was taken in three Erlenmeyer's flasks each containing 4 g of substrates maize cob, wheat bran, and rice bran respectively which were autoclaved at 121°C and 15 lbs for 15-20 minutes. *Bacillus subtilis* was added to the flasks, and they were then cultured for 5 days at 37°C and 120 rpm on an orbital shaker incubator.

2.4 Estimation of cellulase enzyme

The estimation of enzyme cellulase produced by bacteria *Bacillus subtilis* using different agro-waste substrates rice bran, wheat bran, and maize cob was done by performing a DNS test.

2.4.1 DNS Test

The method described by Miller, 1959 was used to measure cellulase activity. 50 mL of reagent-grade water, 1.0 g of 3,5-dinitrosalicylic acid, and 30 g of $KNaC_4H_4O_6.4H_2O$ were combined to prepare DNS. 20 mL of 2 N NaOH were then added, stirred on a magnetic stirrer, and placed in an amber container for storage.

In a shaking water bath, a 2 mL reaction mixture comprising 1.8 mL of 0.5% CMC and 0.2 mL of crude enzyme solution was incubated for 30 minutes at 37°C. The DNS reagent (3 mL) was then added. The mixture became yellow after five minutes of boiling. Samples were compared to a blank that contained all the reagents without crude enzyme. The optical density of samples and blank were measured at 575 nm.

3. Results and discussion

3.1 Screening of cellulase

After de-staining the petri plates, clear zones were observed indicating cellulose degrading activity of the bacteria. Clear zones were observed after de-staining the petri plates with NaCl, which indicates cellulose degrading activity of the bacteria. The positive screened bacteria were then further identified using biochemical tests.

3.2 Biochemical Characterization

According to the observed results (Table 1), it was concluded that the bacteria isolated was *Bacillus subtilis*.

Table 1 Biochemical Tests of *Bacillus subtilis*

Test Performed	Observed Results	Standard Results
Gram Staining	Positive	Positive
Oxidase	Negative	Negative
Catalase	Positive	Positive
Motility	Negative	Negative
Methyl Red	Negative	Negative
Voges-Proskauer	Positive	Positive
Indole	Negative	Negative
H_2S Production	Positive	Positive
Urease	Negative	Negative

3.3 DNS Test

The DNS Method measures the total quantity of reducing sugar to provide a quick and easy estimate of the degree of saccharification (Fig. 2). After conducting the test, optical densities of different substrates that were observed and recorded at 575 nm are shown in (Table 2). *Bacillus subtilis* can convert natural waste such as wheat bran, rice ran and corn cob as carbon source and protein for its growth and production of cellulase enzyme. The development of sustainable enzymes for enhancing the bio-conversion efficiency of cellulosic biomass into various value-added products is without doubt one of the emerging strategies, as this offers better specificity with eco-friendly and green products and minimum substrate loss (Thapa et al., 2020).

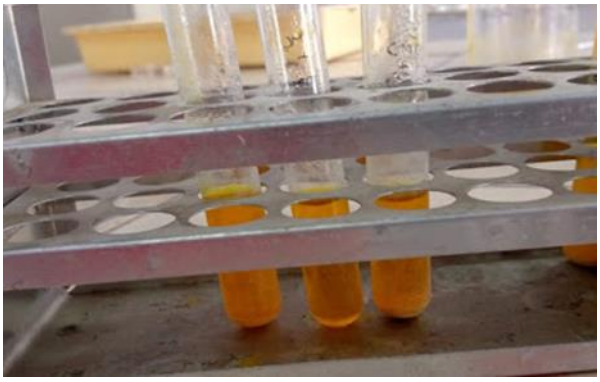

Fig. 2 Pictorial view of DNS Test of *Bacillus subtilis*

Table 2 Optical densities of different agro-waste substrates with DNS reagent

S.No	Substrates	Optical densities
1.	Wheat Bran	0.46
2.	Corn Cob	0.38
3.	Rice Bran	0.60

For instance, it is possible to hydrolyze cellulose and hemicellulose to release sugars that can then be further fermented to produce ethanol. In enzymatic hydrolysis, the heat-tolerant cellulase could expedite the process and lower production costs. However, further studies are required to scale up cellulase production for the improved utilization of the strain at the industrial level (Bhardwaj et al., 2021). Future perspectives of the study may involve identifying new strains with the potential of multi-enzyme production and further developing pre-treatment methods for effective utilization of lignocellulosic feedstocks (Bhardwaj et al., 2021). The demand for cellulase in complex substrates like agricultural waste has increased, making it suitable for fermentation to produce bioenergy. Bacterial cellulase is an option due to their synergistic multi-enzymatic complexes and ability to function in various pH, salinity, and temperature conditions, including composting processes, where thermostable enzymes accelerate bioconversion rates.

4. Conclusion

Results of the present study suggested that the bacterium *Bacillus subtilis* isolated from the soil sample which was utilised for the conversion of agricultural waste as a substrate for cellulase synthesis, demonstrated greater activity when rice bran was used rather than wheat bran and maize cob. Microbial cellulase has been in steadily rising demand as an appealing option for developing cleaner and more sustainable industrial applications.

Author contributions

The following roles were performed by the given authors during the completion of manuscript. Aditi Sharma: Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Writing- Original draft preparation, Visualization. Himanshi Bhagoria: Formal Analysis, Writing-Original draft preparation, Visualization. Janvi Sharma: Graphical abstract, Writing – review and editing,

Visualization. Rupanki Sisodia: Writing – review and editing, Visualization. Lavi Dhiman: Writing- Reviewing and Editing, Supervision.

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