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Research Article

Eco-Friendly Enzyme Production: Utilizing Invasive *Wedelia trilobata* L. for Cellulase Synthesis with *Aspergillus niger*

Rejitha Lalitha Rajappan*, Chacko Suju Skaria, Elsa Mariam Vinu

Department of Botany, Mar Ivanios College, Thiruvananthapuram, Kerala, INDIA.

ABSTRACT

Aim: This study explores the dual potential of *Wedelia trilobata* L., an invasive plant species, for cellulase enzyme production and antibacterial activity. The aim was to utilize *Wedelia trilobata* as a substrate for cellulase production using *Aspergillus niger* through Solid-State Fermentation (SSF) and evaluate the antibacterial properties of its extracts against clinically relevant pathogens**.** **Materials and Methods:** Fresh aerial parts of *Wedelia trilobata* were collected, processed, and used as a substrate for SSF. *Aspergillus niger* was cultured and screened for cellulase production using a zone clearance test on Carboxymethyl Cellulose (CMC) agar. Cellulase activity was quantified using the DNS method, and protein content was estimated via Lowry's method. Antibacterial activity was assessed using the well diffusion method against Gram-negative (*Escherichia coli, Klebsiella pneumoniae*) and Gram-positive (*Staphylococcus aureus, Bacillus cereus*) bacteria. **Results:** It was observed that *Aspergillus niger* effectively produced cellulase, with maximum enzyme activity observed at 120 hr of incubation. The protein concentration peaked at 831 µg/mL at the same time point. The cellulase enzyme demonstrated practical applications, such as ink stain removal from cotton cloth. Antibacterial assays revealed significant activity, with the highest zone of inhibition (15 mm) against *E. coli* and 13 mm against *S. aureus*. The extracts exhibited greater efficacy against Gram-negative bacteria compared to Gram-positive strains. **Conclusion:** This study highlights the potential of *Wedelia trilobata* as a sustainable substrate for cellulase production and a source of antibacterial compounds. The findings underscore the value of invasive species in biotechnological and pharmaceutical applications, offering eco-friendly solutions for resource utilization and environmental management.

Keywords: *Wedelia trilobata*, *Aspergillus niger*, Solid State Fermentation, Antibacterial activity.

Correspondence:
Dr Rejitha Lalitha Rajappan
Assistant Professor, Department of Botany, Mar Ivanios College, Thiruvananthapuram-695015, Kerala, INDIA.
Email: rejitha.lr@mic.ac.in

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INTRODUCTION

Plants are a cornerstone of biodiversity, exhibiting remarkable adaptability to diverse environmental conditions due to their genetic variability.[1] Each plant species possesses unique medicinal properties, attributed to specific chemical compounds that vary in concentration among individuals. Among these, *Wedelia trilobata* L., an invasive species listed among the world's worst by the IUCN, has garnered significant attention. Known for its aggressive growth and ecological impact, *Wedelia trilobata* often outcompetes native vegetation, posing threats to local ecosystems.[2] However, it also holds a long history of traditional use in Ayurveda, Unani, Siddha, and Traditional Chinese Medicine, highlighting its medicinal potential.

Recent studies have shifted focus toward exploring the biochemical properties of invasive species like *Wedelia trilobata*, particularly their antibacterial activity and enzymatic potential.[3] The rise of antibiotic resistance has intensified the search for novel antimicrobial agents, and plant-derived secondary metabolites offer a promising avenue.[4] *Wedelia trilobata* produces compounds with antimicrobial properties, making it a candidate for combating clinically significant pathogens such as *Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus*, and *Bacillus cereus*.[5,6] These pathogens are associated with a range of infections, including urinary tract infections, pneumonia, and foodborne illnesses, and the emergence of multidrug-resistant strains underscores the urgent need for alternative treatments.

In addition to its medicinal potential, *Wedelia trilobata* is a source of cellulase enzymes, which play a critical role in hydrolyzing cellulose into fermentable sugars.[7] Cellulases have wide-ranging industrial applications, including biofuel production, textile processing, and waste management.[8] Solid-State Fermentation (SSF) using invasive plant species as substrates has emerged as a cost-effective method for enzyme production, with fungi like *Aspergillus niger* demonstrating high cellulase yields.[9,10] Exploring the cellulolytic potential of *Wedelia trilobata* not only

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provides insights into its ecological strategies but also opens avenues for sustainable industrial applications.[11]

This study aims to investigate the antibacterial activity of *Wedelia trilobata* extracts against clinically relevant pathogens and estimate its cellulase production potential. By elucidating the dual potential of this invasive species-its medicinal properties and enzymatic capabilities-this research seeks to contribute to addressing global challenges such as antibiotic resistance and the development of sustainable biotechnological processes. This exploration, would uncover new applications for invasive species, transforming ecological threats into valuable resources for medicine and industry.

MATERIALS AND METHODS

Fresh aerial parts of *Wedelia trilobata* L. were collected from the Pallichal region, Thiruvananthapuram. It was identified in the Department of Botany, University of Kerala, Kariyavattom, Thiruvananthapuram. The voucher specimen was kept in the herbarium of the Botany Department, Mar Ivanios College, Thiruvananthapuram for further reference.

Cellulase production from *Aspergillus niger* using *Wedelia trilobata* as substrate

Pure culture of *Aspergillus niger* was obtained from the Department of Biotechnology, Kariavattom. The organism was maintained in Sabouraud Dextrose Agar (SDA) medium.

Preparation of *Wedelia trilobata* leaves as substrate

The aerial parts of *Wedelia trilobata* were cut into small pieces and washed under running top water to eliminate dust and other foreign particles. Later the cut pieces, were kept for shade dry. Thereafter, they were ground and used as substrate in media formulation for cellulase enzyme production.

Microscopic view and morphology of *Aspergillus niger*

The *Aspergillus niger* was obtained and slide cultured, lactophenol staining was performed, followed by observation under a microscope at 40x.

Lactophenol cotton blue staining

Lactophenol Cotton Blue (LPCB) staining was used for the microscopic examination and identification of *Aspergillus niger*. On a clean microscopic glass slide, a drop of 70% alcohol was added. Further, *Aspergillus niger* sample was tested using inoculation loop to ensure the proper mixing of sample in the alcohol. Two drops of Lactophenol Cotton Blue Solution were added using a dropper before the ethanol dries off. The stain was carefully covered with a sterile coverslip. The fungal spores and other fungal structures were observed under the microscope of power 40X.

Screening of cellulase production

A zone clearance test was conducted on Carboxy Methyl Cellulose (CMC) agar plate to identify the cellulase enzyme produced by the organism. The CMC agar plate was prepared by adding 2 g of CMC, 1 g of K_2HPO_4, 1 g of $NaNO_3$, 1g of KCL, 0.5 g of $MgSO_4$, 0.01 g of $FeSO_4.7H_2O$, 5 g of yeast extract and 15 g of agar in 1000 mL distilled water. Spot plating was performed with *Aspergillus niger*. The plate was then kept for 48 hr incubation. At the end of the incubation day, the agar media was flooded with aqueous solution of Congo red (0.2%) and kept for 15 min. Later the agar plate was flooded with 1M NaCl for 10 min. The zone of clearance indicating the presence of enzymes was observed and measured.

Cellulase production via solid state fermentation

In the present work production of cellulase using *Aspergillus niger* with *Wedelia trilobata*, as substrate was carried out. For the pre-inoculum, a 25 mL SDB was prepared in a 100 mL Erlenmeyer flask, sterilized at 121°C for 20 min, and cooled. It was inoculated with *Aspergillus niger* and incubated at 30°C for 72 hr. Further, one gram of *Wedelia trilobata* was moistened with basal media (49.29% moisture), sterilized, and cooled. The substrate was inoculated with 500 µL of *Aspergillus niger* culture and incubated at 30°C for 120 hr under static conditions. Finally, enzyme production was monitored at 72, 120, and 168 hr. Crude enzyme was extracted by adding 10 mL of 0.1M citrate buffer and shaking at 120 rpm for 10 min. The sample was centrifuged at 8000 rpm for 10 min, and the supernatant was used for cellulase analysis.

Cellulase Assay

The cellulase concentration was determined using Whatman No. 1 filter paper as the substrate. A mixture of 0.5 mL enzyme extract and 32 mg filter paper was incubated at 50°C for 1 hr. After incubation, 0.5 mL DNS reagent was added, and the mixture was heated in a boiling water bath for 5 min. Subsequently, 1.0 mL of 40% Potassium Sodium Tartrate solution was added while the tubes were warm. The mixture was cooled, diluted to 5 mL with water, and absorbance was measured at 540 nm. A standard curve was prepared using glucose solutions of known concentrations.

Estimation of Protein

A series of test tubes containing different concentrations of Bovine Serum Albumin (BSA) working standard were prepared. To each tube, 0.2 mL of the sample was added, and the volume was adjusted to 1 mL with water. A blank was prepared using 1 mL of water. Then, 5 mL of reagent C was added to all tubes, mixed thoroughly, and allowed to stand for 10 min. Subsequently, 0.5 mL of reagent D was added, mixed well, and incubated at room temperature in the dark for 30 min. The developed blue colour was measured at 660 nm. A standard graph was plotted, and the protein concentration in the sample was calculated.

Applications of cellulase enzyme

Determination of Ink stain removal

The sample was centrifuged at 8000 rpm for 15 min and the supernatant was taken. A cotton cloth was dipped in petri dish containing 5 drops of ink and 10 mL water for staining. The stained cloth was then dipped in petri dish containing 5 mL of the supernatant and kept for 30 min. After washing and drying result was observed.

Determination of Antibacterial activity

Well Diffusion Method as described in European pharmacopoeia with slight modifications was used for antibacterial testing. Pure culture of 100 µL of the test strains (two Gram-negative bacteria viz., *Escherichia coli* and *Klebsiella pneumonia* and two Gram positive bacteria viz., *Staphylococcus aureus* and *Bacillus cereus*) were swabbed uniformly using a sterile swap on the surface of nutrient agar to obtain an even inoculum. Swabbing was repeated two or more times, rotating the plates approximately at 60°C to ensure an even distribution of inoculum. The plates were allowed to dry for 5 min. Then, a hole with a diameter of 8mm was punched aseptically with a sterile cork borer and 100 µL test sample at the desired volume was introduced into the well. The antibacterial activity was observed after incubating the plates for 24 hr at 37°C and the zone of inhibition surrounding the well was noted in mm.

RESULTS

In the present study, *Wedelia trilobata*, an invasive species was used for the production of Cellulase enzyme. The fungal species *Aspergillus niger* was observed as a rapidly cellulose degrading fungi and was used for the study.

Lactophenol Cotton Blue Staining

Aspergillus niger exhibited white to yellow coloration during initial growth, turning black after 7 days with the formation of conidial spores. Lactophenol cotton blue staining revealed blue-colored septate hyphae, conidiophores, and conidia, confirming the fungal morphology under microscopic examination (Figure 1).

Zone Clearance Test

The zone clearance test demonstrated the cellulase activity of *Aspergillus niger*. The fungus was inoculated onto a CMC agar plate and incubated for 48 hr. Post-incubation, the plate was flooded with Congo red solution, revealing a clear zone of cellulose hydrolysis. This confirmed the secretion of cellulase by *Aspergillus niger*, capable of effectively hydrolyzing cellulose (Figure 2).

Cellulase Assay

Cellulolytic activity in the culture supernatant was assessed after 72, 120, and 168 hr of incubation at 37°C. Enzyme activity was quantified using glucose as a standard (Table 1). A standard graph was plotted, showing glucose concentrations of 20 µg, 40 µg, 60 µg, 80 µg, and 100 µg corresponding to absorbance values of 0.091 nm, 0.135 nm, 0.197 nm, 0.260 nm, and 0.323 nm, respectively.

Protein Estimation

The protein content of the sample was estimated using Lowry's method with BSA as the standard. A standard graph was plotted, showing protein concentrations of 20 µg, 40 µg, 60 µg, 80 µg, and 100 µg corresponding to absorbance values of 0.128 nm, 0.169 nm, 0.253 nm, 0.323 nm, and 0.390 nm, respectively. The protein concentration of the sample was calculated from the standard graph, and the values obtained for different days are presented in Table 2.

Application of Cellulase Enzyme

Stain removal was done by using the supernatant (Figure 3). After washing it was observed that ink stain was removed effectively from the cotton cloth (Figure 4).

Antibacterial Activity of Wedelia trilobataThe supernatant from cellulase production was evaluated for antibacterial activity using the Well Diffusion Method against Gram-negative bacteria (*Escherichia coli, Klebsiella pneumoniae*) and Gram-positive bacteria (*Staphylococcus aureus, Bacillus cereus*). Antibacterial activity was observed in all tested strains. Streptomycin served as the positive control, while double-distilled water was used as the negative control. For *E. coli*, the zone of inhibition was 15 mm for 100 µL, 11 mm for 50 µL, and no activity was observed for 25 µL. In *K. pneumoniae*, the zone of inhibition was 13 mm for 100 µL, with no activity for 50 µL and 25 µL. Among Gram-positive bacteria, *S. aureus* exhibited a zone of inhibition of 13 mm for 100 µL and 11 mm for 50 µL, with no activity for 25 µL. For *B. cereus*, the zone of inhibition was 12 mm for 100 µL, with no activity for 50 µL and 25 µL. The maximum inhibitory activity of *Wedelia trilobata* extract was observed against *E. coli* (Gram-negative) and *S. aureus* (Gram-positive). The extract demonstrated greater antibacterial activity against Gram-negative bacteria compared to Gram-positive bacteria, and summarized in Table 3.

Table 1: Enzyme activity of cellulase.

hr	OD	Concentration (µg/mL)	Concentration (µM)	Enzyme activity (units/mL)
T 72	0.140	248	1.37	0.022
T120	0.210	372	2.06	0.034
T 168	0.125	221.42	1.22	0.020

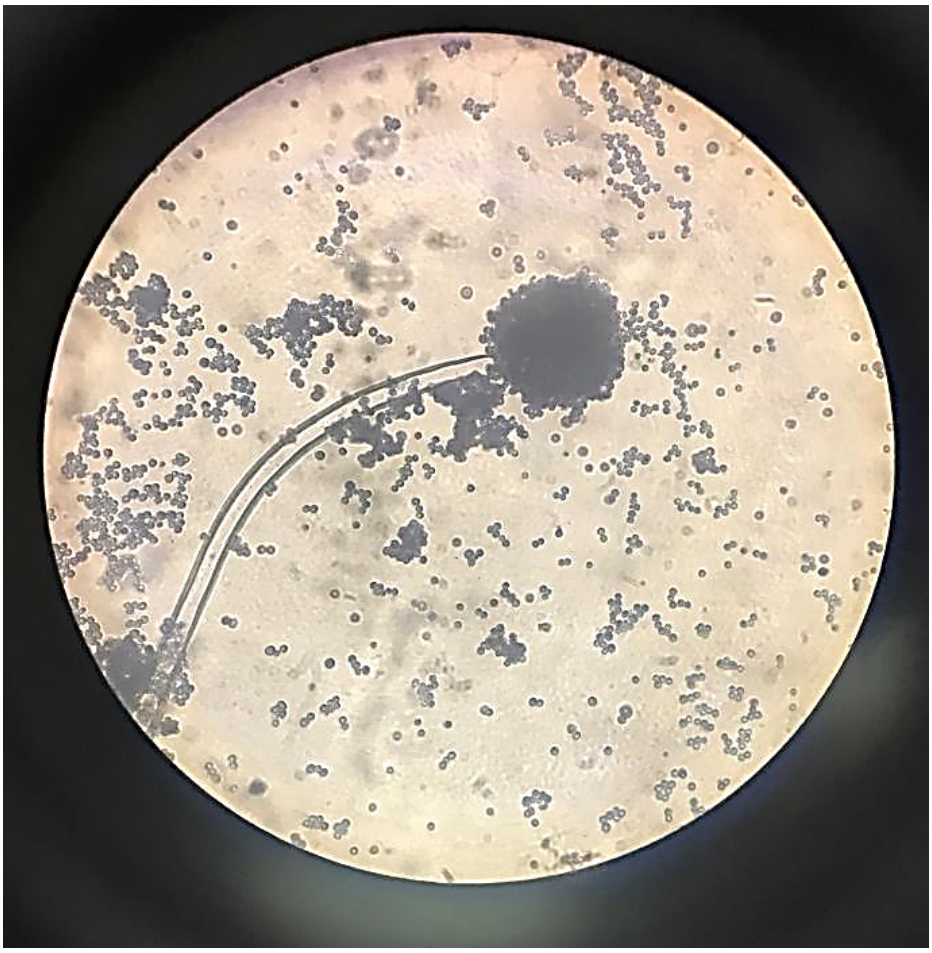

Figure 1: Lactophenol cotton blue staining of *Aspergillus niger*.

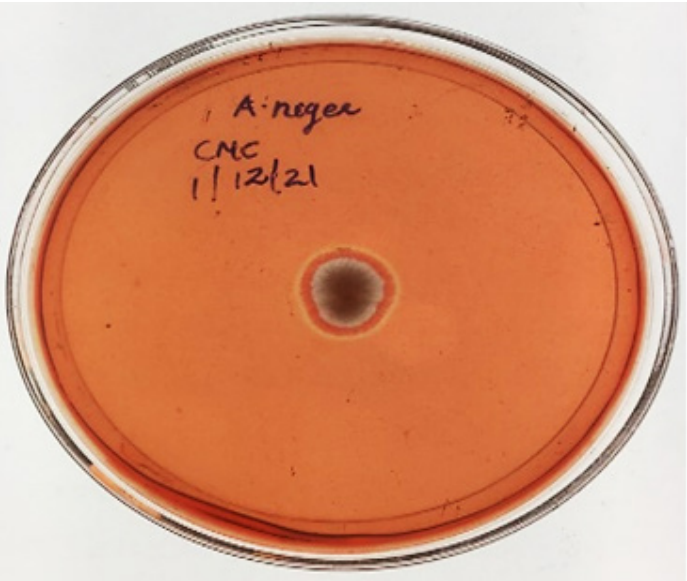

Figure 2: Zone test for cellulase production by *Aspergillus niger.*

DISCUSSION

Wedelia trilobata L., an invasive plant species rich in cellulose, serves as a sustainable and renewable substrate for cellulase production. Its utilization not only valorizes invasive biomass but also offers an eco-friendly alternative to conventional chemical processes, reducing environmental pollution and resource depletion.[12,13] *Aspergillus niger*, a robust cellulase producer, efficiently degrades cellulose into soluble sugars through its enzymatic machinery, including endoglucanases, exoglucanases, and β-glucosidases.[14,15] Factors such as substrate composition, pH, temperature, and fermentation conditions significantly influence cellulase production. In this study, Solid-State Fermentation (SSF) was employed, which demonstrated higher enzyme yields compared to Submerged Fermentation (SmF), with SSF producing 14.6 times more cellulase under optimal conditions.[16,17]

Table 2: Protein concentrations on different days.

Incubation time (hr)	Protein Concentration (µg/mL)
T 72	521
T 120	831
T 168	432

Optimization of parameters such as moisture content (49.29%), temperature (30°C), and pH (6.0) was crucial for maximizing cellulase production. Among various substrates, coir waste

Table 3: Zone of inhibition of gram +ve and gram -ve bacteria.

Samples	Zone of Inhibition (mm in diameter)			
	Staphylococcus aureus (mm)	*Bacillus cereus* (mm)	*Escherichia coli* (mm)	*Klebsiella pneumonia* (mm)
100 µL	13	12	15	13
50 µL	11	NA	11	NA
25 µL	NA	NA	NA	NA
+ve	25	21	20	20
-ve	NA	NA	NA	NA

NA: No Activity, +ve: Positive Control, -ve: Negative Control.

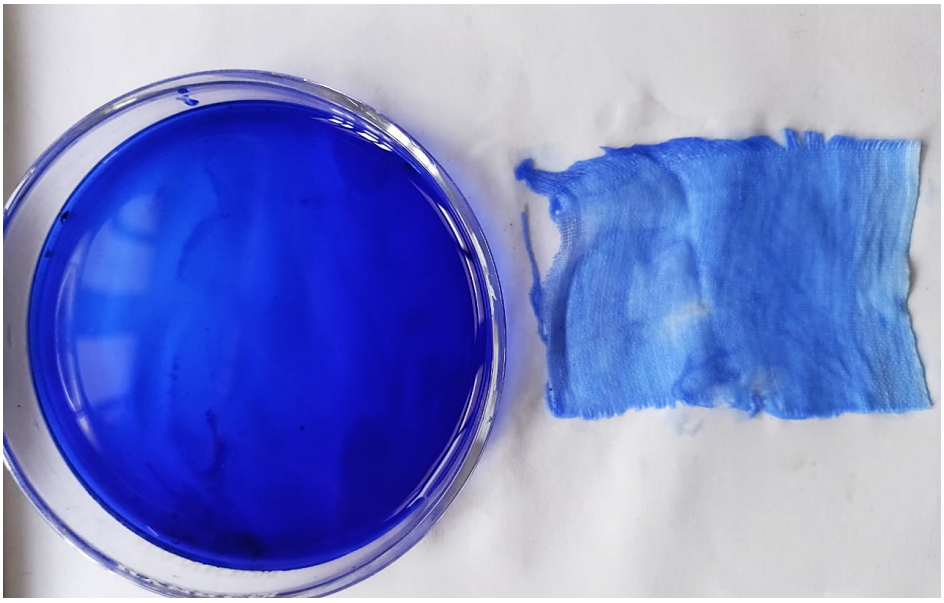

Figure 3: Ink stained petri and cloth.

Figure 4: Ink stain removed cloth.

yielded the highest cellulase activity, followed by wheat bran and rice bran.[18] The cellulase assay using Whatman No. 1 filter paper and protein estimation via the Lowry method confirmed enzyme activity and protein content in the crude extract.[19] A practical application of cellulase was demonstrated through effective ink stain removal from cotton cloth, highlighting its industrial potential.

Additionally, *Wedelia trilobata* exhibited significant antibacterial activity against both Gram-negative (*Escherichia coli, Klebsiella pneumoniae*) and Gram-positive (*Staphylococcus aureus, Bacillus cereus*) bacteria. The maximum zone of inhibition (15 mm) was observed against *Escherichia coli*, while *Staphylococcus aureus* showed a minimum inhibition zone of 11 mm. The greater inhibitory effect against Gram-negative bacteria may be attributed to differences in cell wall thickness, with Gram-positive bacteria having thicker peptidoglycan layers.[20,21] The antibacterial properties are likely due to bioactive compounds such as flavonoids, alkaloids, and phenols present in the plant.[22-24] Recent studies have further confirmed the potential of *Wedelia trilobata* extracts as natural antibacterial agents, with applications in pharmaceuticals and agriculture.[13]

These findings underscore the dual potential of *Wedelia trilobata* as a substrate for cellulase production and a source of antibacterial compounds, contributing to sustainable biotechnological and pharmaceutical advancements. Further research is needed to optimize production processes and elucidate the mechanisms of antibacterial activity for broader applications.

CONCLUSION

In conclusion, the study demonstrates the successful isolation of cellulase enzyme from *Wedelia trilobata* using *Aspergillus niger* by SSF, as well as the significant antibacterial activity of *Wedelia trilobata* extracts. This research highlights the potential of invasive plant species as valuable resources for biotechnological and pharmaceutical applications, offering eco-friendly solutions for sustainable resource utilization and environmental management. Future research should focus on further optimizing the SSF process for enhanced cellulase enzyme production and extracting and characterizing the bioactive compounds responsible for the antibacterial activity of *Wedelia trilobata* extracts.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

IUCN: International Union for Conservation of Nature; **SSF:** Solid State Fermentation; **SDA:** Sabouraud Dextrose Agar; **LPCB:** Lactophenol Cotton Blue; **CMC:** Carboxy Methyl Cellulose; **BSA:** Bovine Serum Albumin.

SUMMARY

The study successfully isolated cellulase enzyme from *Wedelia trilobata* using *Aspergillus niger* through SSF and demonstrated significant antibacterial activity in *Wedelia trilobata* extracts. It underscores the potential of invasive plants for biotechnological and pharmaceutical applications, promoting sustainable resource use.

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