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Nature Environment & Pollution Technology

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Type of paper: Original research

Intervention and Optimization of Urea (CH_4N_2O) for Enhancement of Lignocellulolytic Enzyme Production in Modified Potato Dextrose Media

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Key Words	Potato dextrose broth, Lignocellulolytic enzyme, Urea, Optimization
DOI	https://doi.org/10.46488/NEPT.2025.v24i04.B4299 (DOI will be active only after the final publication of the paper)
Citation for the Paper	Parshad, J., Kumar, R., Kumar, A., Chhokar, V., and Kumar, A., 2025. Intervention and optimization of urea (CH_4N_2O) for enhancement of lignocellulolytic enzyme production in modified potato dextrose media. *Nature Environment and Pollution Technology*, 24(4), p. B4299. https://doi.org/10.46488/NEPT.2025.v24i04.B4299

Abstract

The management of agriculture waste material or lignocellulogic biomass is a big task for the scientist as well as the farmers. There are many options for managing agricultural waste through *ex-situ* and *in-situ* technology but the rate of degradation of crop residues is very slow during either of the processes. Utilization of the lignocellulolytic fungi can help to enhance the rate of degradation of crop residues. This study investigates the optimization of urea concentration for lignocellulolytic enzyme production using modified Potato Dextrose Broth (PDB) media. The four lignocellulolytic fungal strains (*Aspergillus niger* GKH2, *Aspergillus flavus* GHR4, *Aspergillus terreus* GD2, and *Trichoderma harzianum* JLB) were used for the optimization of varying urea concentration to enhance enzyme production. The effect of different concentrations of urea on various enzyme productions was checked. Results revealed that supplementation of media with 2% urea concentration found to best for the enzyme production by all fungi. The study was taken in view of the wide C: N ratio of rice straw, which restricts fungal growth due to the immobilization of nitrogen source. When nitrogen source is applied appropriately in the form of urea, it can balance the C: N ratio, encourage fungal growth and facilitate *ex-situ* and *in-situ* degradation of paddy straw. The results demonstrate that 2% urea concentration significantly enhances enzyme activity, suggesting a cost-effective strategy for boosting fungal lignocellulose-degrading capabilities, which could contribute to sustainable agricultural residue management and soil health.

1. Introduction

India is an agrarian country, with 54.6% of its people engaged in agriculture and other allied activities (Chandramouli 2011). The accumulation of lignocellulosic agricultural residues, such as rice straw, sugarcane bagasse, and wheat straw, presents an environmental challenge due to limited disposal options and the associated risks of soil degradation and pollution. Rice straw constitutes 60% of the

burned crop residues, with an estimated 50 Mt of rice residue discarded through open burning (Bhattacharya et al. 2021). This traditional disposal method causes significant environmental pollution, contributing to greenhouse gas emissions and poor air quality (Kumar et al. 2019a; Kumar et al. 2019b). Additionally, burning crop residues exacerbates issues like deteriorating soil health, declining groundwater levels, and rising greenhouse gas (GHG) (Bhatt et al. 2021). The management of lignocellulosic agricultural residues is a critical aspect of sustainable agriculture and requires the adoption of eco-friendly alternatives. The residue management practices include both *ex-situ* (composting) and *in-situ* (incorporation in soil) approaches using microbial consortia, which can enhance the degradation rates and may offer an effective solution for agricultural waste management (Parshad et al. 2024). The agricultural waste have been used for the production of hydrolytic enzymes by using different types of microorganisms (Yadav et al. 2024; Morbia et al. 2024).

Biodegradation of lignocellulosic biomass by microorganisms, particularly fungi, represents a promising approach for managing agricultural waste. The degradation of waste material whether it is exsitu or in situ is time consuming process if relied upon the natural decomposition so supplementation of the microbial consortium can enhance the rate of degradation process. Fungi are known for efficiently producing a variety of lignocellulolytic enzymes, such as cellulase, hemicellulase, xylanase, laccase, cellobiase, and lignin peroxidase. These enzymes are essential in breaking down complex plant residues and converting lignocellulosic materials into simpler organic molecules. This bioconversion process effectively degrades tough plant biomass, promotes nutrient cycling, and improves soil fertility. But the optimization of hydrolytic enzymes production by potential fungi is need to be done to enhance the enzyme production and to make more effective fungal consortium in sustainable agricultural waste management and soil health restoration (Gupta et al. 2016). Fungi, particularly those from the *Aspergillus* and *Trichoderma* genera, are widely recognized for their ability to produce lignocellulolytic enzymes. However, optimizing media ingredients is crucial for enhancing enzyme production. Nitrogen, in particular, plays a vital role in fungal growth and enzyme production. It has been observed that applying nitrogen, especially in the form of urea, during sowing and at the crown root initiation stage in wheat farming has a notable impact on soil microbial dynamics, including the growth of lignocellulolytic fungi. Nitrogen encourages fungal growth and the production of enzymes that facilitate the breakdown of the complex lignocellulosic structures. However, excessive nitrogen application might lead to an imbalance, where fungi prioritize nitrogen metabolism over lignocellulose breakdown, potentially slowing down the decomposition process. Nitrogen availability significantly impacts fungal growth and enzyme production, influencing primary metabolic pathways and secondary metabolite synthesis. As a nitrogen source, urea is cost-effective and readily available, supporting fungal growth. While adequate nitrogen levels are essential for enzyme synthesis, excess nitrogen can inhibit enzyme production due to nitrogen catabolite repression mechanisms. Thus, achieving a balance between nitrogen levels is necessary to maximize enzyme yields while minimizing inhibitory effects (Arif et al. 2024). Urea is an inexpensive and readily available nitrogen source that, when hydrolyzed, provides ammonia, which is an effective nitrogen source for promoting fungal growth and enzyme synthesis. Additionally, previous studies have demonstrated that urea supports higher lignocellulolytic enzyme production in certain fungal species, including *Penicillium* and *Aspergillus*, making it a suitable choice for optimizing enzymatic degradation of lignocellulosic materials (Xie et al., 2015; Li et al., 2018). In comparison to ammonium sulfate, urea's slower release of nitrogen ensures a more controlled growth environment, preventing excessive ammonium accumulation that could inhibit enzyme activity.

Therefore, the present study aims to explore the optimization of varying urea concentrations in four promising lignocellulolytic enzyme-producing fungi (*Aspergillus* spp. and *Trichoderma* spp.) to enhance their efficiency in breaking down complex plant residues. The findings contribute to

developing improved fungal-based systems for agricultural residue degradation and soil health restoration, providing an environmental friendly alternative to traditional waste disposal practices.

2. Materials and methods

2.1 Fungal isolates and cultivation

Four fungal isolates (*Aspergillus niger* GKH2, *Aspergillus flavus* GHR4, *Aspergillu sterreus* GD2, and *Trichoderma harzianum* JLB) were selected based on their lignocellulolytic potential. These isolates were cultivated and maintained on Potato Dextrose Agar (PDA) before being transferred to PDB media for enzyme production studies.

2.2 Preparation of modified PDB media

Standard potato dextrose broth (PDB), consisting of potato and dextrose ingredients, was modified by adding urea as a nitrogen supplement. Urea concentrations varied across different treatment groups, *viz*. 1%, 2%, 3%, and 4% w/v. The control was maintained without the addition of urea for comparison.

2.3 Enzyme activity assays

Each fungal isolate was subjected to submerged state fermentation in urea-amended PDB media. After incubation, using standard protocols, enzyme activity assays were conducted for cellulase, xylanase, laccase, and lignin peroxidase. CMCase (Cellulase) and FPase activity were measured using the dinitrosalicylic acid (DNS) method, based on the release of reducing sugars (Miller et al. 1959 & Ghose, 1987). Xylanase activity was determined by measuring xylose released from the xylan substrate (Ghose & Bisaria (1987). The substrate ABTS was utilized to assess laccase activity.

The 100 µl of culture media was incubated with 100 µl of 10 mM ABTS in 800 µl of 50 mM sodium acetate buffer (pH 5) at 30°C for 25 minutes. A spectrophotometric measurement at 420 nm was used to determine the appearance of green color, which indicated ABTS oxidation. Laccase activity was measured by estimating the amount of enzyme needed to oxidize 1 µmol of ABTS per minute at 30°C. Each unit of activity represents a specific enzyme amount (Ire & Ahuekwe 2016). Lignin peroxidase activity was carried out using veratryl alcohol as a substrate (Daljit & Paramjit 2001). Cellobiase activity was checked using p-nitrophenyl-β-D-glucopyranoside (pNPG) as a substrate. The enzyme activity is expressed in terms of U/mL, with one unit of activity defined as the quantity of enzyme that releases one micromole of p-nitrophenol in a minute (Wood & Bhat 1988).

2.4 Data analysis

The results were statistically analysed using OPSTAT software and beta version to determine the optimal urea concentration for each enzyme activity, using two-way ANOVA for treatment comparisons.

3. Results

3.1. CMCase production

The results showed that 2% urea yielded the highest activity, with *A. flavus* GHR4 (0.581 IU/mL) followed by *A. niger* (0.367 IU/mL), *T. harzianum* (0.304 IU/mL) and *A. terreus* (0.287 IU/mL) (Fig.1).

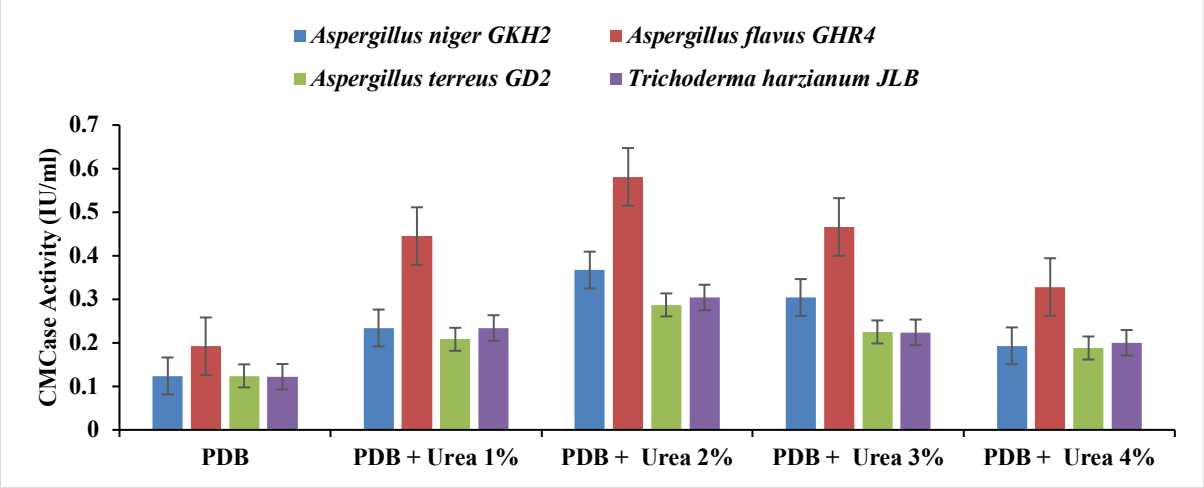

Fig. 1: Effect of the varying urea concentration on CMCase production, error bars represent the variability or uncertainty in the measured acitivity.

The lower activities were observed at 1% and 4% urea, indicating that urea concentration significantly affects enzyme production. The 2% urea concentration maximized cellulase production across all four fungal isolates, with *A. flavus* GHR4 showing the highest activity, indicating an efficient cellulase production response to nitrogen supplementation. The interaction effects between fungal isolates and urea concentrations are statistically significant, suggesting that each isolate responds differently to the urea treatments

3.2. Effect of urea on FPase activity

The highest activity was observed in *T. harzianum* JLB (0.386IU/mL) followed by *A. terreus* GD2, *A. niger* GKH2, and *A. flavus* GHR4. The 1% and 3% urea showed similar activities, suggesting a moderate effect. The 4% urea had the lowest FPase activity (Fig. 2).

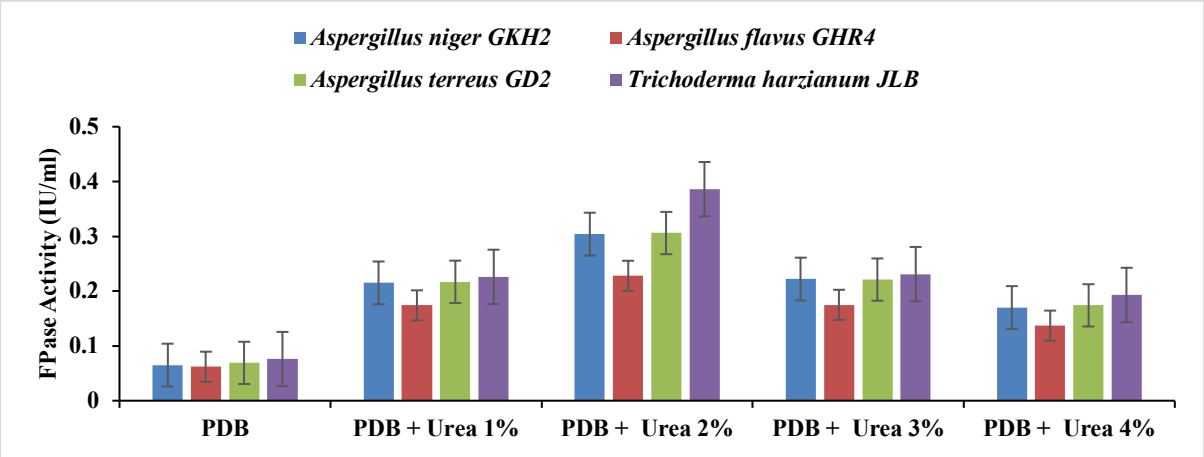

Fig. 2: Effect of the varying urea concentration on FPase production, error bars represent the variability or uncertainty in the measured acitivity .

The results showed that supplementation of 2% urea increased the FPase activity across all the isolates, indicating it was the most effective concentration.

3.3. Effect of urea on xylanase activity

The xylanase activity varies with different urea concentrations. Xylanase activity at 2% concentration increases for all isolates compared to 1% urea. *T. harzianum* JLB continues to show the highest activity (1.185 IU/mL), followed by *A. niger* GKH2, *A. terreus* GD2, and *A. flavus* GHR4. *A. flavus* GHR4 still has the lowest activity among the isolates but had improved from 1% urea.

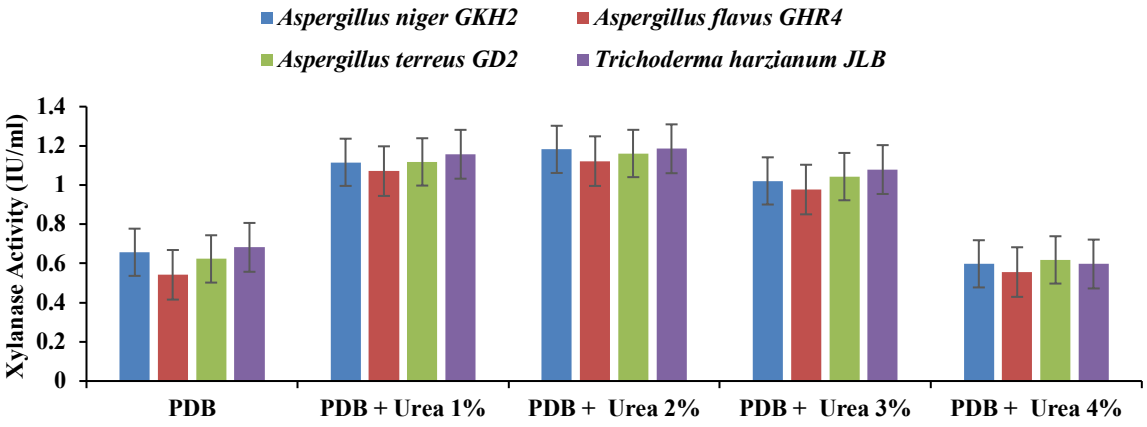

Fig. 3: Effect of the varying urea concentration on xylanese production, error bars represent the variability or uncertainty in the measured acitivity.

The activity was the lowest across all isolates at 4% urea**.** This significant drop suggests that a high level of urea adversely affects xylanase production. *A. flavus* GHR4 shows the lowest activity (0.556 IU/mL), while *T. harzianum* JLB retains the highest activity among the isolates at this concentration (0.597 IU/mL). The decline in activity was observed at higher concentrations in all isolates. *T. harzianum* JLB demonstrated the most significant increase in xylanase production, which highlights its adaptability to nitrogen-rich environments (Fig. 3).

3.4. Effect of urea on laccase activities

Laccase activity generally declined with increasing urea concentrations at 3 and 4% across all fungal isolates. It suggests that higher urea concentrations inhibit the enzyme activity.

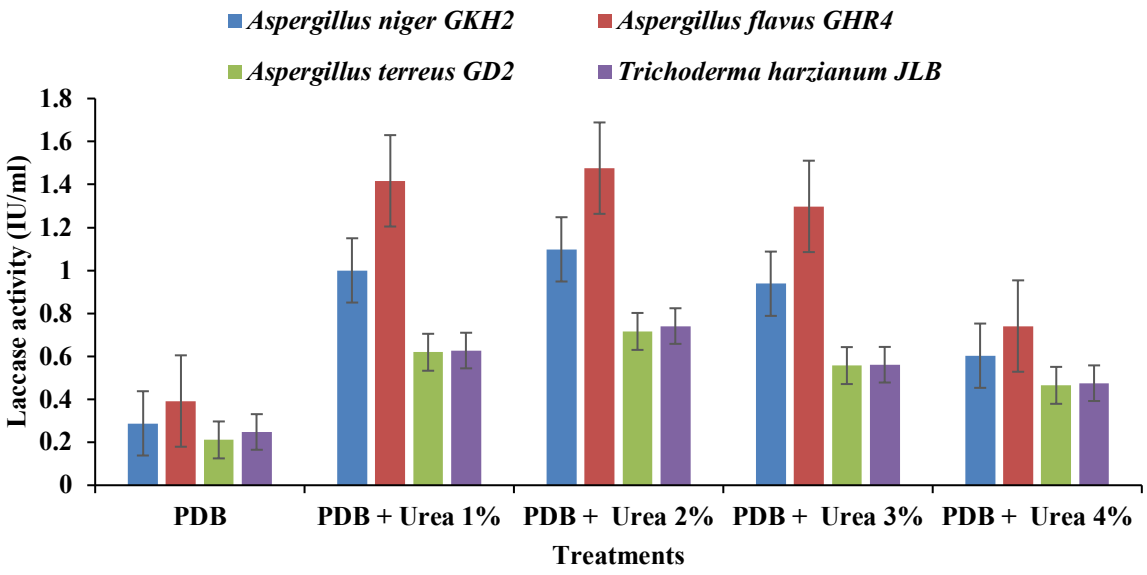

Fig. 4: Effect of the varying urea concentration on laccase activity, error bars represent the variability or uncertainty in the measured activity.

Laccase activity increases slightly for most isolates compared to 1% urea, with *A. flavus* GHR4 still showing the highest activity (1.476 IU/mL) at 2% urea concentration (Fig. 4).

3.5. Lignin peroxidase Production

The highest lignin peroxidase activity was observed with a 2% urea concentration, which suggested that a 2% urea concentration might be optimal for maximizing lignin peroxidase activity in submerged fermentation. The activity decreased at 4% urea concentration, indicating a potential inhibitory effect or diminishing returns at higher concentrations.

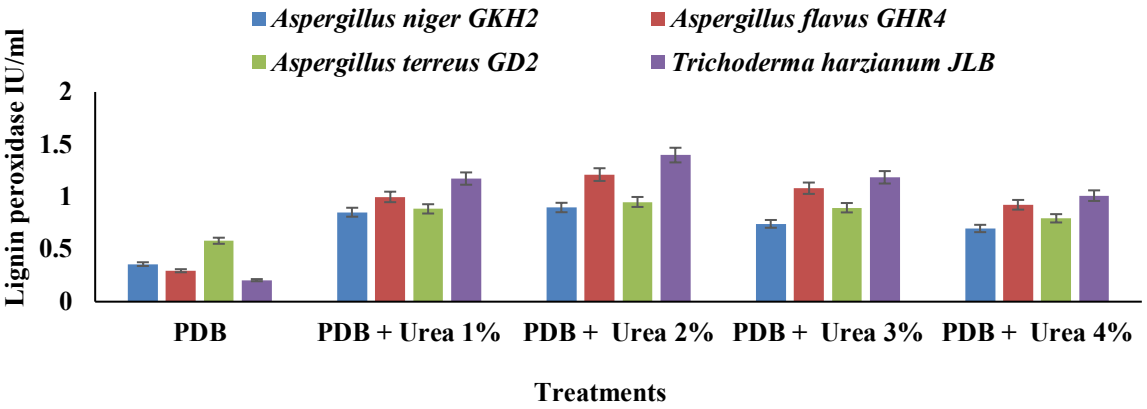

Fig. 5: Effect of the varying urea concentration on lignin peroxidase activity, error bars represent the variability or uncertainty in the measured activity

T. harzianum JLB showed the highest lignin peroxidase activity (1.398 IU/mL), indicating it was the most efficient strain for lignin degradation among all the isolates. Laccase and lignin peroxidase activities were highest at 2% urea, though the response was less pronounced than cellulase and xylanase.

A. terreus GD2 was observed as the highest lignin peroxidase producer, while *A. flavus* GHR4 led in laccase production, suggesting strain-specific responses to nitrogen optimization (Fig. 5).

3.6. Effect of urea on cellobiase activity

A. niger GKH2 showed the highest activity at 2% urea concentration (0.990 IU/mL) and the lowest at 4% urea concentration (0.526 IU/mL). *A. flavus* GHR4 activity was relatively lower than the other strains, with a highest at 2% urea (0.599 IU/mL) and a low at 4% (0.297 IU/mL).

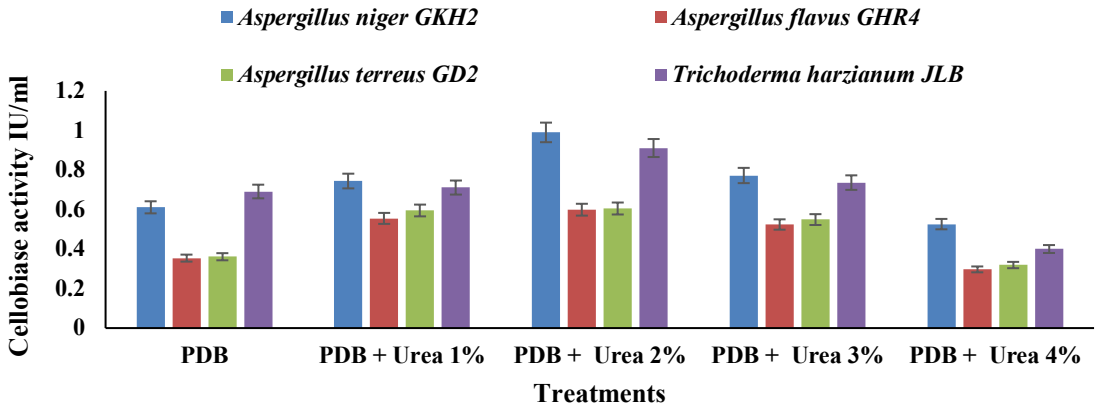

Fig. 6: Effect of the varying urea concentration on cellobiase activity, error bars represent the variability or uncertainty in the measured activity.

A. terreus GD2 showed a more consistent activity level with a slight peak at 2% urea (0.605 IU/mL) and a lower value at 4% (0.319 IU/mL). *T. harzianum* JLB showed higher cellobiase activity (0.911 IU/mL) than the other strains, especially at 2% urea concentration (Fig. 6).

3.7. Statistical analysis and optimal urea concentration

Statistical analysis confirmed that 2% urea concentration was optimal for enhancing lignocellulolytic enzyme activities without causing inhibitory effects. The results were statistically significant ($p < 0.05$), supporting the hypothesis that nitrogen optimization is critical for enzyme production.

4. Discussion

Fungal diversity in the soil can produce different types of lignocellulolytic enzymes and enhance the decomposition rate. The activity of different enzymes depends on the existing soil's environmental conditions, such as temperature, moisture, pH, and availability of nutrients. So, there is a need to understand different factors and their optimization, which can stimulate enzyme production. Rice residues face challenges during the *in-situ* decomposition, such as a high C: N ratio, and researchers have investigated there that nitrogen content enhances fungal growth and enzyme production. Farmers also use the recommended dose of nitrogen at the time of sowing as well as during the crown root initiation stage of wheat, that is why nitrogen may affect the lignocellulolytic fungal growth and enzyme production (Kumar et al. 2019; Kumar et al. 2022; Korav et al. 2024). The present study includes the optimization of varying urea concentrations in four promising lignocellulolytic enzyme-producing fungi. In this study, the variable enzyme production observed across four lignocellulolytic fungi at varying urea concentrations aligns with previous research, suggesting that urea can significantly enhance enzyme activity compared to control in modified potato dextrose broth. It was observed that adding 2% urea in potato dextrose broth enhanced the cellulase, FPase, cellobiase and xylanase enzyme production. However, the high urea concentration (3, 4%) inhibited the enzyme production (Fig. 1-3 &

6). Urea optimization for cellulase production by *Penicillium funiculosum* NCIM 1228 led to a significant increase in enzyme activity, particularly highlighting urea as a critical factor influencing enzyme production and reported a remarkable 3.82-fold enhancement in FPase activity, a 3.61-fold increase in β-glucosidase, and a 3.29-fold rise in xylanase activity (Chavan et al. 2024). Similarly, Abena and Simachew (2024) observed various nitrogen sources and found that yeast extract led to the highest xylanase production, followed by ammonium sulfate, with urea showing moderate xylanase activity. However, its impact may vary depending on the specific enzyme and fungal strain. The findings from this study suggest that urea plays a role in promoting enzyme production, which is consistent with earlier research, particularly in the context of optimizing fermentation media. However, it is worth noting that other factors, such as the presence of additional nutrients or environmental conditions, may also contribute to the overall enzyme yield. Further investigations into the interaction between urea and other medium components could provide valuable insights into maximizing enzyme production across various fungal strains. Similarly, another study by Shahriarinour et al. (2011) highlighted the inhibitory effects of higher urea and ammonium sulfate ($(NH_4)_2SO_4$) on cellulase production beyond 3 g L^{-1} nitrogen sources pointing to a threshold beyond which the compounds become detrimental. Interestingly, β-glucosidase activity was absent when ammonium sulfate was used, suggesting its unsuitability as a nitrogen source for the synthesis of this specific enzyme component. The highest cellulase production was observed when yeast extract was used as a nitrogen source, followed by peptone, urea, and ammonium sulfate in *Aspergillus terreus,* supporting the highest production levels for all three primary cellulase components such as FPase, CMCase, and β-glucosidase. The superior performance of yeast extract may be attributed to its complex nutrient profile, providing not only nitrogen but also growth factors and amino acids essential for enhanced enzyme production (Shahriarinour et al. 2011).

Maeda et al. (2010) found that urea supplementation significantly increased cellulase activities, particularly CMCase, with an over 100% increase from 2,260 to 5,143 U/L, while FPase activity showed a 46% increase. In this way, urea acts as a key nitrogen source, which enhances enzyme production by improving fungal growth and metabolic activity. However, excessive urea can inhibit enzyme synthesis, likely due to feedback inhibition or shifts in metabolic priorities. This study confirms that a 2% urea concentration in PDB optimally supports lignocellulolytic enzyme production, providing a balanced nitrogen input that enhances enzyme yield without detrimental effects.

Ansari et al. (2023) investigated the impact of different nitrogen sources on fungal growth and enzyme production. High hydrolytic enzyme producing fungal isolates were tested for enzyme production in a basic mineral medium with various nitrogen sources, including ammonium sulfate (AS), ammonium nitrate (AN), urea (U), and combinations of these, at a final nitrogen concentration of 0.3 g/L. The results revealed significant variations in enzyme activities across the isolates and nitrogen treatments. The highest ligninase activities were observed with AS (99.94% CR decolorization) and urea (89.82%), with isolate VC85 performing best in the presence of AS. For cellulolytic activity, isolates C200 and C184 exhibited the highest cellulase activities of 8.8 and 6.5 U/mL, respectively, in the presence of AS and AN+U, with the overall highest cellulase activity seen in the AN+U combination (3.90 U/mL). The findings emphasize the importance of selecting suitable nitrogen sources to improve fungal performance in composting and highlight the potential of *A. fumigatus* species in accelerating the degradation of lignocellulosic waste for more efficient compost production. The present study revealed that all four fungi strains showed increased in lignin peroxidase and laccase activity at 2% urea addition (Fig. 4 & 5). Mehboob et al. (2011) used the five different nitrogen sources to evaluate the impact on lignin peroxidase production in *Ganoderma lucidum* during fermentation. The results revealed that *G. lucidum* exhibited the best lignin peroxidase production when $(NH_4)_2SO_4$ was used as the nitrogen source. The highest enzymatic activity was recorded in the medium containing $(NH_4)_2SO_4$, which achieved 1996.6 U/ml followed by urea (1830.6 U/ml) and NH_4NO_3 (1792.3 U/ml), indicating that ammonium sulfate

was the most effective nitrogen source for enhancing lignin peroxidase production in *G. lucidum* under the tested conditions.

Kheiralla et al. (2013) optimized the cultural conditions for lignin peroxidase production by *Phanerochaete chrysosporium* and *Pleurotus ostreatus*. The study found that urea was the most effective nitrogen source and fructose the most effective carbon source for lignin peroxidase production. The optimal concentrations for these substances were 0.30% urea and 3.0% fructose, which resulted in the highest enzymatic activity from both *P. chrysosporium* and *P. ostreatus*.

Reis et al. (2015) optimised cellulase and xylanase production by *Penicillium echinulatum* S1M29 using salts and urea, which indicated significantly enhanced enzyme production. Lower concentrations of salts, including KH_2PO_4 (2.0 g/L), $(NH_4)_2SO_4$ (1.4 g/L), $MgSO_4 \cdot 7H_2O$ (0.375 g/L), and $CaCl_2$ (0.375 g/L), along with a higher urea concentration of 0.525 g/L, led to a notable increase in enzyme production. These optimised conditions increased enzyme activity levels to 87% higher than the standard medium. Specifically, the highest activities recorded were for FPase (1.5 U/mL), endoglucanase (7.2 U/mL), xylanase (30.5 U/mL), and β-glucosidase (4.0 U/mL), which were 87%, 16%, 17%, and 21% higher, respectively, than the activities obtained with the standard medium. The study demonstrated the effectiveness of response surface methodology in adjusting the mineral and urea concentrations. This improved enzyme yields and helped minimise the waste of medium components, making the process more efficient.

5. Conclusions

In conclusion, this study demonstrates that urea is an efficient nitrogen source for enhancing the production of lignocellulolytic enzymes by fungi, particularly *Aspergillus* spp. and *Trichoderma* spp. The optimization of urea concentrations significantly improved enzyme yields, with 2% urea being identified as the optimal concentration. This finding has practical implications for both agricultural waste management and industrial applications. Farmers can adopt the use of nitrogen supplements like urea to enhance the microbial degradation of agricultural residues, such as rice straw, directly in the field. This approach can reduce the environmental impact of open burning, improve soil health, and promote nutrient cycling. In industrial the finding can be used in the fermentation processes for the production of valuable enzymes used in biofuel production, waste management, and the bioremediation of lignocellulosic materials. The ability to enhance enzyme production through urea supplementation offers a promising strategy for scaling up fungal-based systems to meet the increasing demand for sustainable agricultural waste management solutions. Future research may be explored to analyse the impact of urea in different fermentation systems, particularly solid-state fermentation (SSF), which more closely mimics industrial conditions. Further studies could also evaluate the field-scale application of optimized nitrogen treatments to assess their real- impact on crop residue management and soil microbial dynamics. The interactions between urea and other nitrogen sources or organic additives may provide in-depth knowledge into enhancing fungal enzyme production under diverse environmental conditions.

Author Contributions:

Jagdish Parshad-Conceptualisation and visualisation, writing original draft, methodology, literature review, data curation,

Anil Kumar-Conceptualisation and visualisation, writing original draft, methodology, literature review, data curation, formal analysis, supervision, writing- reviewing & editing

Ravinder Kumar-Writing (original draft preparation) writing (review and editing),

Ajay Kumar-Visualisation, resources, software, grammar editing and data analysis
Vinod Chhokar-Conceptualisation and visualisation, writing original draft, methodology, literature review, data curation, formal analysis,
All authors have reviewed and authorized the final version of the manuscript.
Funding: Outside sources did not support this study.
Data Availability Statement: The manuscript contains the data used in this study.
Acknowledgements: The authors thank you for reviewing and providing feedback on the manuscript.
Conflicts of Interest: The authors disclose no conflicts of interest.

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