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A study on critical associations of media components on enhanced cellulase production from wild *Trichoderma viride* and cellulase immobilization on iron-oxide magnetic nanoparticles

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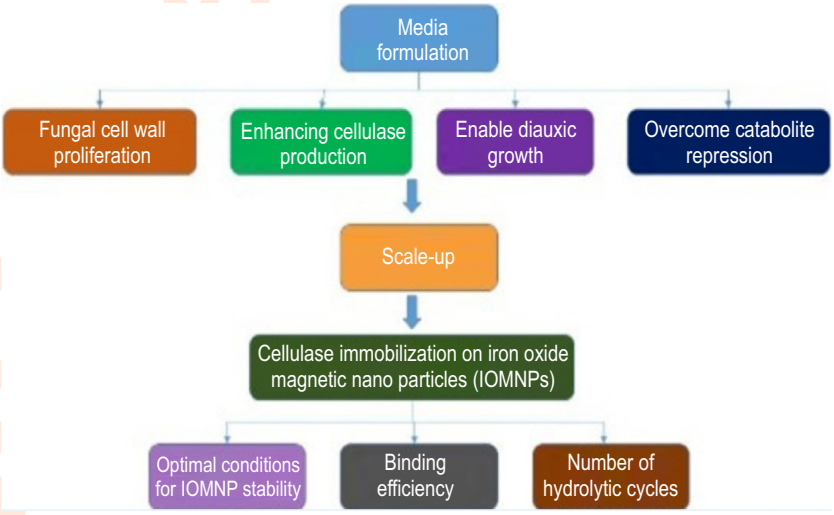
Abstract

Aim: The current study is a preliminary step towards enhancing the cellulase productivity in wild *Trichoderma viride* which will enable robust valorization of non-edible lignocellulosic biomass through co-generative enzymatic saccharification, specifically concentrating on influence of individual media components on biomass growth and cellulase productivity. Further, cellulase immobilization on iron-oxide magnetic nanoparticles was also achieved that can increase the shelf life of the enzyme.

Methodology: The cellulase production in the wild *Trichoderma viride* was enhanced using media design and formulation. EDC {1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide} functionalized iron-oxide nanoparticles were chosen to act as carriers for cellulase immobilization. The binding efficiency and relative activity were measured in addition to optimal pH and temperature for cellulase bound iron-oxide nanoparticles. Further, the hydrolysis efficiency of immobilized cellulases was also measured after which it was subjected to consecutive hydrolytic cycles to calculate the recycle rate.



Results: A maximum growth rate of 60 PCV (Packed cell volume) and total cellulase activity of 7.4 U ml^{-1} was obtained on media design and formulation. 82.5% binding efficiency was achieved on EDC functionalized iron-oxide magnetic nanoparticles which showed good stability at 5pH and 50°C. There was 44.4% activity loss after 5 consecutive hydrolytic cycles which showed steady decline with increased cycle number and finally at the end of the 10th hydrolytic cycle, 22.2% of total relative activity was retained.

Interpretation: Unprecedented total cellulase activity from a wild strain was obtained through media design. The stability of cellulases was further enhanced using iron-oxide magnetic nanoparticle immobilization.

Key words: Cellulases, Immobilization, Iron-oxide magnetic nanoparticles, Submerged fermentation, *Trichoderma viride*

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Introduction

Cellulases are a widely investigated multicomponent enzyme systems which play a crucial role in valorization of inexpensive and readily available lignocellulosic biomass in co-generative biofuel systems and provide energy security (Singh *et al.,* 2021, Ejaz *et al.,* 2021). Its production has largely been from fungal sources either through solid state or submerged fermentation which have found diverse applicability ranging over various industrial domains (Maurya *et al.,* 2012; Genc *et al.,* 2015). Some of the substrates used in submerged cultivation of wild cellulase producers are delignified oil palm empty-fruit-bunch fibre, saw dust, carboxy methyl cellulose, cellulose and sugarcane bagasse (Reese *et al.,* 1972; Gomes *et al.,* 1992; Umikalsom *et al.,* 1997; Chinedu *et al.,* 2011; Saleh *et al.,* 2021). Although, among these, a maximum of only 4.95 U ml^{-1} total cellulase activity has been reported.

The native producers of cellulase have largely shown diminished activity towards naturally available lignocellulosic biomass which makes them less desirable with a growing necessity towards genetic improvement for higher yield. However, when such genetically modified organisms (GMO) are discarded beyond the controlled and contained environment after their use, their disposition becomes unpredictable and pose potential risks to the ecosystem (Sharma *et al.,* 2017). Unlike which, one of the wild strain's highly crucial and effective contemporary application is in the integrated disease management system. *Trichoderma* being an endophytic symbiont has been proven to enhance the nutritional value of food, restore soil fertility, strengthen plant defense system towards both biotic and abiotic stress and increase the plant productivity through colonization of root system, such as in case of rice, tomato, ghost pepper, cabbage etc. (Molla *et al.,* 2012; Kumar *et al.,* 2014; Doni *et al.,* 2018; Ji *et al.,* 2020). In addition, the National Farmer Policy (2007) has opened a great market for biocontrol agents and biofertilizers like *Trichoderma*, through strong promotion of biopesticides for welfare of both farmers and the environment (National Farmer Policy, 2007).

Hence a potent wild strain can be a valuable candidate towards a wide range of industrial applications. High cellulase titer is essential for making enzyme production process industrially viable. Thus media design remains a critical necessity in enhancing the cellulase yield and biomass development in submerged fermentation. The primary media components required for high cell growth and cellulase production are carbon and nitrogen sources. *Trichoderma* (*Ascomycotina*; *Hypocreaceae*) has been known for its diverse nutritional adaptations to different substrates available in its native environment. It grows belligerently when simple carbon sources such as glucose, starch, fructose, maltose etc., is made available in the medium but the cellulase production is essentially hindered due to carbon catabolite repression (CCR). Studies have shown that cellulase, constitutive or induced, is catabolite sensitive. Hence, addition of complex carbon source such as plant cell wall polysaccharides can help bypass this issue in submerged cultivations. In addition to the choice of carbon source, the importance of various organic and inorganic nitrogen sources such as soy peptone, soybean flour, yeast extract, ammonium nitrates, ammonium salts and urea as nitrogen source in the cellulase enzyme production have largely been a subject of interest in many studies given their critical role in cell maintenance, growth, reproduction and enzyme biosynthesis. Increased volumetric enzyme activity and biomass (69.8 U l^{-1} h^{-1} and 14.7 g l^{-1}) was reported in *Trichoderma reesei* on using cellulose-yeast extract combination in their culture medium. It was found that in *Aspergillus terreus*, ammonium sulphate was found to suppress β-glucosidase activity when supplemented at concentrations higher than 3 g l^{-1}, while cellulase activity was accentuated by yeast extract and peptone (Ahamed and Vermette, 2009; Shahriarinour *et al.,* 2011; Saravanan *et al.,* 2012). Moreover, Saravanan *et al*. (2012) also reported positive effects of yeast extract and soybean cake flour in promoting cellulase production from *Aspergillus nidulan* in submerged cultivation. Besides, recent studies on the genetic level effects of ammonium ions on cellulase production has shown significant up-regulation of differently expressed genes that not only aid in metabolism of cellulose but also in various amino acid metabolism, pyruvate metabolism and glycolysis/ gluconeogenesis in *Trichoderma koningii* (Saravanan *et al*., 2012; Xiang *et al*., 2021).

Apart from enhancing productivity via optimizing the substrates, there exists a need to reduce the cost of enzyme production by enhancing its stability. Immobilization techniques will address this issue by not only enabling enzyme recycle but also in extending the shelf life of the enzyme, and improving the accessibility of substrate to the enzyme during hydrolysis with superior thermal and pH stability. In case of cellulases, various substrates in agreement to their surface properties and their compatibility with the enzyme, have been used towards making the enzyme reusable either through covalent attachment or physical adsorption. Among the many immobilization substrates, iron oxide magnetic nanoparticles (IOMNPs) have been used widely in immobilizing bioactive materials such as nucleic acids, enzymes, peptides and antibodies. Linkers, such as glutaraldehyde and aminopropyl triethoxysilane (APTES) have been employed in immobilization to make it easier for the biomolecule to bind to the substrate since linker-free immobilization results in decreased accessibility to the biomolecule (Kumar *et al.,* 2019). Direct functionalization of IOMNPs with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide have also been reported to produce competent immobilization of cellulases with minimal losses to up-to 4 cycles of hydrolysis (Jordan *et al.,* 2011; Paz-Cedeno *et al.,* 2021). This study addresses crucial facets of saccharification efficiency such as the enhancement of cellulase production aspect as well as conservation of enzyme shelf life using immobilization. Additionally the optimal pH and temperature for the cellulase conjugated iron oxide magnetic nanoparticles were assessed.

Materials and Methods

Microorganism and culture maintenance: *Trichoderma viride* stock of spore culture was obtained from Jawaharlal Nehru University, New Delhi, under the Virtual Enzyme Project, Department of Biotechnology, New Delhi. The spore culture was

maintained in Potato Dextrose agar (PDA) (Merck, Germany) slants at -4ºC. Glycerol stocks were made, maintained and stored at -20ºC. The working stock was regularly cultured in Potato Dextrose Broth at Centre for Biotechnology, Anna University, Chennai.

Inoculum preparation: *Trichoderma viride* spores were cultivated and preserved in PDA slants which were further used to prepare glycerol stocks (spores and vegetative mycelial fungal glycerol stocks). The incubation conditions for *Trichoderma viride* was 30ºC at 200 rpm for 24 hrs with multiple stage inoculum subculturing for final inoculum generation.

Scale up studies: Scale-up studies were performed in Bioengineering Lab Fermentor Type KLF2000 and New Brunswick BioFlo®/CelliGen® 115 Fermentors with running time up to 120 hrs and agitation speeds ranging from 700 to 1000 rpm.

Biomass estimation: Packed cell volume (PCV) was used to estimate the biomass growth in fungal cultures given the non-uniformity of production media. This was obtained by subjecting the sample to 3,400 x g rpm for 5 min and calculating PCV by the following equation:

$$\text{Packed Cell Volume} = \frac{\text{Total sample volume-supernatant volume}}{\text{Total sample volume}} \times 100$$

Media design: Media design studies were conducted using seven different medium, including simple and complex carbon components in addition to which different organic and inorganic nitrogen sources were considered for formulating the media that best supported biomass growth and proliferation and enzyme synthesis. All the media chemicals were procured from SRL Pvt. Ltd., India and Merck, Germany.

Determination of cellulase activity: The residual sugar was estimated using Liquizone Glucose-MR (GOD-POD) kit (Medsource Ozone Biomedicals Pvt. Ltd., Haryana, India). The supernatant of the centrifuged sample was used for residual glucose determination (Agrawal *et al.,* 2013). NREL (National Renewable Energy Laboratory) protocols were used to determine the total cellulase activity, exoglucanase activity, endoglucanase activity and cellobiase activity(Lübeck and Lübeck, 2018). While the protein concentrations were measured using Bradford Assay (Bradford, 1976).

Preparation of iron oxide magnetic nano particles (IOMNPs): Co-precipitation technique was used in the production of IOMNPs wherein the ferric and ferrous ions were combined under alkali environment with constant agitation in 2:1 ratio. After the formation of dark precipitate, it was washed with water and anhydrous ethanol to remove traces of alkali.

EDC functionalization of IOMNPs and cellulase immobilization: Fifty milligram of IOMNPs was suspended in 5 ml of 8 mg ml^{-1} 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide and sonicated for 3 min followed by cooling of the suspension to 4ºC. Following which the enzyme solution was added, sonicated for 3 min twice hourly at 4ºC and finally heated to 25ºC. The cellulase conjugated nanoparticle obtained were then separated and washed with distilled water twice and stored in PBS (pH 7.0). The efficiency of the immobilization was calculated by the binding efficiency and relative activity, the amount of enzyme bound was estimated by the bradford assay.

Optimization of pH and temperature for cellulase bound IOMNPs stability: Different pH and temperature conditions were checked for the stability of cellulase functionality after immobilization onto IOMNPs. Different pH variations (pH 3.0, 4.0, 5.0 and 6.0) were maintained using citrate buffer while phosphate buffer saline was used for pH 7.0. Temperature variations between 25ºC to 80ºC was done to check the stability of cellulose immobilized nanoparticles using water bath.

Activity preservation: Cellulase assay (FPase Assay) was performed to check for the activity of cellulase conjugated IOMNPs. They were separated after every assay and set for cellulase assay with fresh substrate to check for subsequent activity changes with each cycle (Agrawal *et al*., 2013).

Results and Discussion

Seven different production media were used to cultivate *Trichoderma viride* and checked for cellulase productivity levels. The primary focus was on high biomass build-up, its sustainability during the stationary phase and cellulase production throughout the fermentation process because stationary phase conditions favor mycelial conservation along with high cellulase productivity (Hu *et al.,* 2016). Wheat bran, microcrystalline cellulose, lactose and glucose were tested for their competence in being both a carbon source and inducer in various permutations (Fig. 2). Analyzing data obtained from cellulase production runs using different medium, several important aspects of cellulase production were observed. While PDB, the basal medium, supports good growth with a specific growth rate of 0.6 hr^{-1} and PCV of 58 at 24th hr, cellulase production was not induced due to absence of any inducer component. Hence, the media formulation was performed keeping in focus the macro-nutrient and micro-nutrient need of *Trichoderma viride* for biomass proliferation as well as cellulase production.

The primary carbon source was chosen to be a combination of simple as well as complex carbon source in order to avoid catabolite repression. Studies have reported the deleterious effects of cellulose (>3%), a most widely available complex carbon source, due to irreversible adsorption on solid substrates. In contrast, lower concentrations (1%) of cellulases acted as positive reinforcement for cellulase production and biomass growth in the later stages of cell growth after the exhaustion of simple carbon sources (Ma *et al.,* 2013). Also, in terms of complex nitrogen source, corn steep liquor (CSL) has been reported to strongly support high cellulase production in *Trichoderma reesei* which is why it was incorporated in the production media (Farid and El-Shahed, 1993; Li *et al.,* 2022).

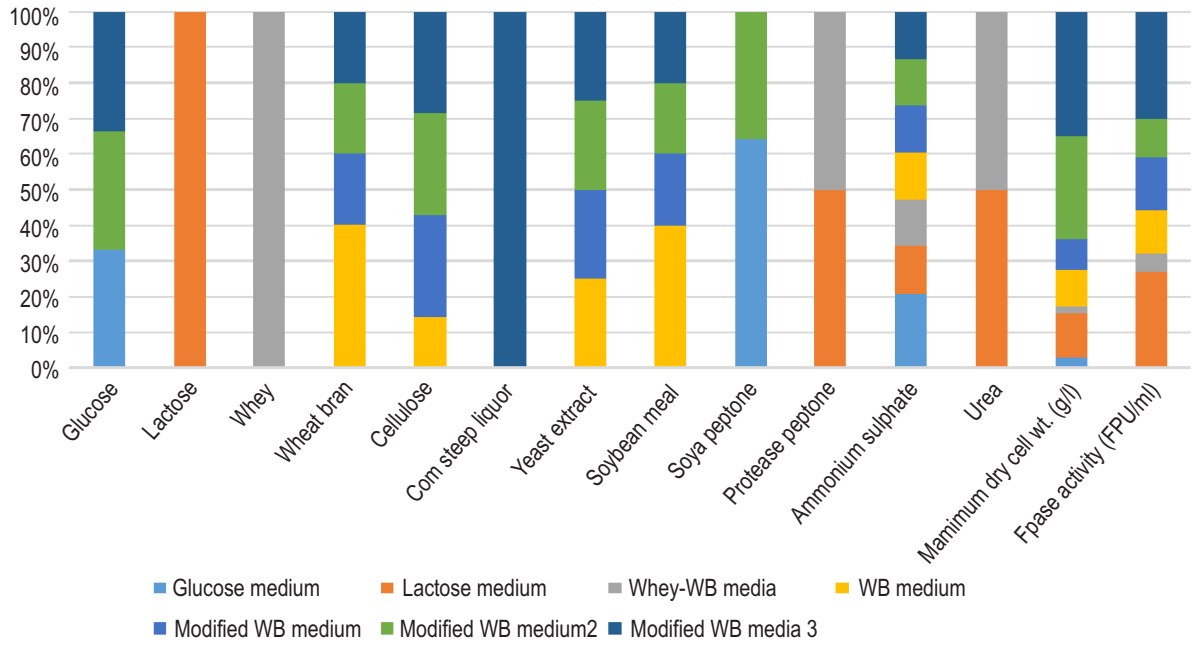

Fig. 1: Different carbon and nitrogen sources used in production medium.

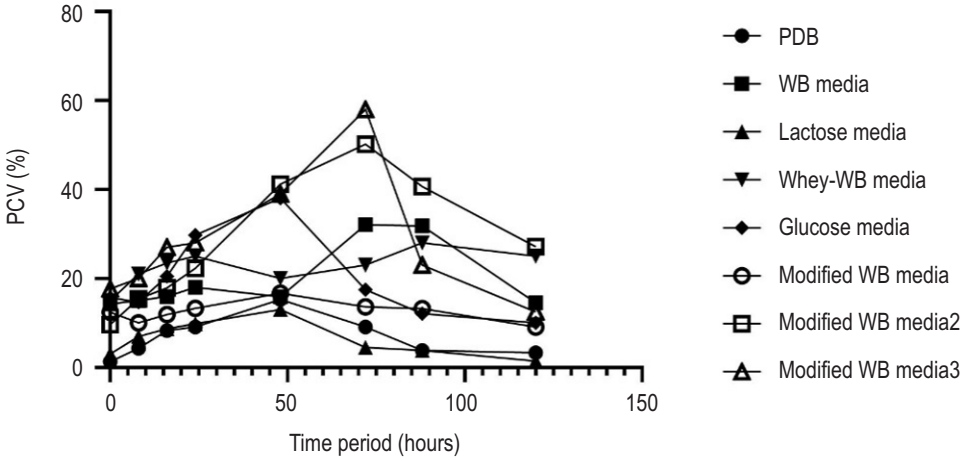

Fig. 2: Growth profile of *Trichoderma viride* in different media.

Thus, media design studies led to the formulation of a wheat bran based media incorporating wheat bran, microcrystalline cellulose, corn steep liquor and glucose for high cell density and enzyme production (Fig. 3, 4). Since the inoculum was grown in PDB containing 2% dextrose, adaptation of fungal population to production media was carefully considered before the modified wheat bran production media was formulated. Hence, 2% dextrose was included for initial biomass growth support. Yeast extract and soybean meal were added as nitrogen supplements along with cellulose, wheat bran and CSL for cellulase induction.

On further investigation, dextrose when supplemented as carbon source, led to increased fungal biomass proliferation albeit negligible cellulase production, which can be attributed to wide spread carbon catabolite repression observed in many *Trichoderma* wild strains (Amore *et al.,* 2013; Shibata *et al.,* 2021). This was avoided by incorporation of both simple as well as complex carbon sources. Such integration effectively supported early fungal growth and propagation. The complex carbon source such as wheat bran and microcrystalline cellulose, contributed heavily towards cellulase induction and cell biomass sustenance

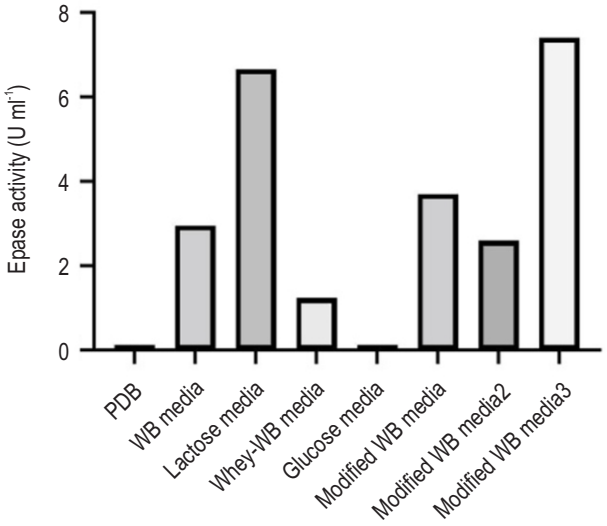

Fig. 3: Total cellulase production profile of *Trichoderma viride* in different media components.

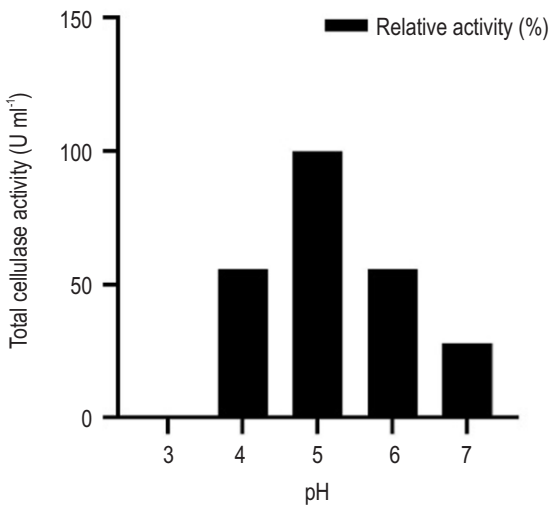

Fig. 4: Total cellulase activity at different pH values.

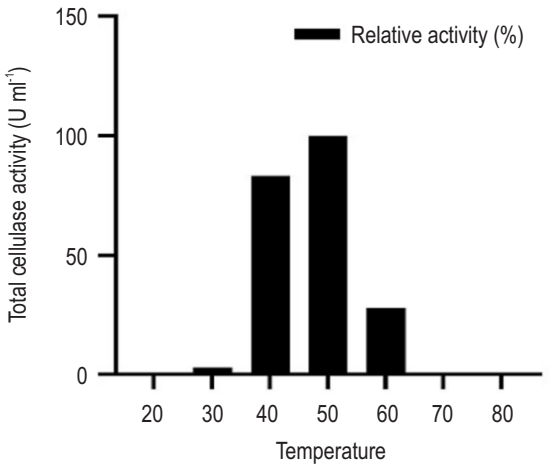

Fig. 5: Total cellulase activity at different temperature conditions.

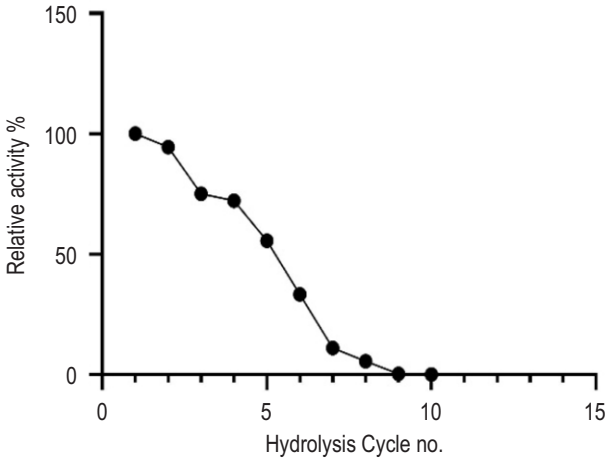

Fig. 6: Activity preservation with each hydrolysis cycle.

in the later phase of fermentation when the cells were starved of glucose, an easily utilizable carbon source.

The complex carbon and nitrogen sources in the medium took precedence during this nutrient deprived phase which induced stress in *Trichoderma viride* cells enabling them to produce cellulases in order to utilize the complex nutrients for their survival which led to the formulation of modified wheat bran medium 3. The modified wheat bran 3 medium showed promising outcomes with the inclusion of glucose as well as wheat bran and cellulose. While, corn steep liquor, ammonium sulphate and soy bean meal have prolifically supported the cellulase formation as well as cell metabolism during the production phase (Fig. 3, 4).

The submerged cellulase production from wild strain of *Trichoderma viride* was performed for 120 hrs and was stopped when the packed cell volume no longer showed an increasing trend, which may be due to carbon and nitrogen depletion, and the microscopic profiling showed abundance of emaciated mycelial fragments (Callow *et al.,* 2016). Although packed cell volume was used for quick estimation of cell growth, a more reliable method is systematic monitoring of pH throughout the fermentation process. While the media pH was fixed at 5.0 initially, as PCV increased, the pH increased steadily to 6.5, which later showed a declining trend as cellulase production started. In a batch process, this trend continued from 24th hr to 72nd hr at the end of which cellulase production declined. The maximum FPase

activity 7.4 U ml^{-1} was obtained at 72 hrs of the process.

The competence of cellulase binding to the IOMNPs was assessed using binding efficiency and relative activity calculations wherein amount of IOMNPs added was constant while the enzyme loading varied. The saturation of enzyme on the IOMNPs was assessed after which the enzyme loading: IOMNP ratio was set. This study found that lower enzyme loading led to greater enzyme binding is in agreement with the study conducted by Jordan *et al.,* 2011. The enzyme loading varied from 1 to 20 mg. The maximum binding efficiency of 83.5% was attained at 1 mg enzyme loading. Stability studies have found pH 5.0 and temperature of 50ºC to be optimal for cellulase conjugated IOMNPs (Fig. 5, 6). The loss of enzyme activity in other operating conditions (pH and temperature range) could be attributed to denaturation of one or more components of cellulases or that part of enzyme's active site is obstructed while immobilization onto IOMNPs. The activity preservation study was conducted by recycling the immobilized enzyme at the end of each 36 hr long hydrolysis reaction using Whatman's filter paper cellulose (GE Healthcare, UK) as substrate. Addition of 83.5% of crude cellulase was immobilized and after the first hydrolysis cycle about 15% activity loss was reported following which a steady decline in the activity was observed with every subsequent hydrolysis cycle. This decline in activity with every hydrolysis cycle may be linked to destabilization of the multimeric enzyme complex during the repeated interaction between the substrate and the immobilized enzyme (El-Shishtawy *et al*., 2021). Almost complete activity loss was observed at the end of 8^{th} hydrolysis cycle (Fig. 7).

In conclusion, this *Trichoderma viride* wild strain has been found to produce the highest total filter paper activity amongst its wild counterparts which could be of great significance when a more economical approach towards valorization of lignocellulosic biomass is considered. In addition, the immobilization of cellulases onto iron-oxide magnetic nanoparticle will not only contribute to increased shelf life but also enzyme stability at more than ambient conditions, thus it has great potential in various industrial domains especially, in bioethanol production, wherein the saccharification yield of lignocellulosic biomass is instrumental in determining the subsequent bioethanol yields. Furthermore, the enzyme procurement costs take up to 35% of total saccharification costs, which can be minimized when the enzymes are immobilized and recycled. Immobilization of enzymes on nanoparticles, especially magnetic iron-oxide nanoparticles confers greater ease of separation and recycle and hence, can be a powerful tool towards sustainable bioethanol production.

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Add-on Information

Authors' contribution: D. Revathi: Designed and performed the experiments including data collection and analysis; **S. Ramalingam:** Helped design and conceive the experimental idea and ultimately analyze the results.

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