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ISOLATION OF FUNGUS FROM ROOTS OF WATER *HYACINTH* AND *CELLULASE* PRODUCTION BY THE MICROORGANISM FROM CARDBOARD WASTE

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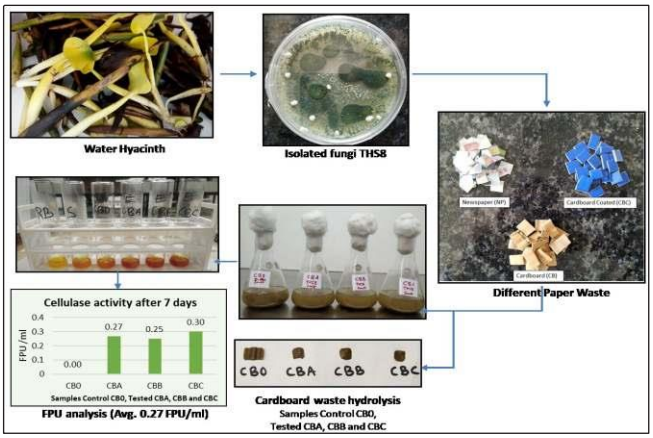
Abstract
Bioethanol production from agro-industrial waste is becoming a major research area for alternative fuel. The cellulase enzyme is the key ingredient used for the cellulosic bio-ethanol fermentation process. India is abundant in cellulosic resources that come from paper and pulp industries. Single-use newspaper and packaging cardboards constitute a significant portion of cellulosic wastes. Here in this study, an industrial enzyme cellulase was produced from single-use newspaper and cardboard wastes. A fungus was isolated (THS8) from the root of the water hyacinth collected from an abundant pond located near Badoli village, Faridabad (India). The fungus was used to do a comparative study for cellulase production by using different substrates such as pure carboxymethyl cellulose (CMC), cardboard (CB), coated cardboard (CBC) and newspaper (NP). The maximum cellulase activity 0.149 FPU (Filter paper unit)/ml was estimated in cardboard (20g/l) grown in MW (Mandel Weber) medium. Likewise, a total of 60% degradation of the cardboard was observed after 7 days of incubation at 30°C with 0.27 FPU/ml optimized at CB 20g/l. This demonstrates the feasibility of using cardboard as a less expensive feedstock, which is usually dumped after unpacking items in households and industries, to produce cellulase enzyme by the isolated microorganism.

Keywords
Cardboard, Water hyacinth, *Trichoderma*, Cellulase, FPU.

'Graphical Abstract'



1. Introduction

Lignocellulosic biomass is a kind of renewable resource present in abundant quantities in India. Rice straw, wheat straw, sugar cane bagasse, etc. are different types of lignocellulosic biomasses. The burning of such biomass is a major concern due to resulting air pollution. Bioethanol production from lignocellulosic biomass is a suitable way to process it into a useful product. Bioethanol can be blended with fuel and have advantages of less greenhouse gas emission. The whole process of bioethanol production includes pretreatment, hydrolysis, and fermentation. Improvements in the enzymatic hydrolysis can also make it more feasible for commercialization. As the cellulosic biorefineries are setting up in India, the demand of cellulase enzyme is increasing day-by-day. So, a cost-effective process can reduce the production cost using cheaper or waste cellulosic sources for cellulase production. Cellulase is a well-studied enzyme which converts cellulose to glucose by using a cocktail CBHs (Cello-bio-hydrolases) and glucosidase. Therefore, this study is focused on finding a way

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to use the cheapest cellulosic waste for cellulase production. A waste biomass can be applied as raw material to reduce waste pollution as well as production cost, which can boost the bioeconomy of the country.

Lignocellulosic biomass is a renewable, abundant and cost-effective bioresource to produce bioproducts [1]. It comes from the non-edible part of the feedstock (rice straw, wheat straw, sugarcane bagasse, cotton stalk) so its application does not affect the food supply [2]. There are many challenges of this process like poor sugar recovery, high enzyme cost, long hydrolysis time, less inhibitor tolerant microbes [3]. Lignocellulosic biomass is composed of cellulose, hemicellulose and lignin, and ash in a variable amount in different kinds of biomasses. Cellulose is a major part of biomass, which contains long chains of glucose sugar that are further utilized for fermentation into ethanol [4]. Hemicellulose is made up of a combination of sugars like xylose, arabinose, and mannose [5]. Lignin is a complex polymer that gives strength to the biomass and highly cross-linked phenols are present in it [6].

Enzymatic hydrolysis is the process of conversion of cellulose to glucose by the action of cellulase enzyme. Cellulase is made up of three main enzymes exo-β-glucanase, endo-β-glucanase and β-glucosidase [7]. Similarly, hemicellulase is composed of mostly xylanase. *Trichoderma reseei* and *Aspergillus niger*are the major micro-organisms studied and used to produce cellulase and xylanase enzyme [7]. Meanwhile, the cost of the enzyme is too high so that the improvement in the enzyme activity and in-house production strategies are used to find a better solution for a reduction in the cost of the enzyme. There are various methodologies used for enzyme activity improvement, such as optimization of pH and temperature [8], immobilization methods [9], the addition of surfactants [10], chemical or radiation mutation [11,12], mild sonication [13], and enzyme cocktail from different species [14] to reduce the time and cost of the process.

Fermentation of cellulosic biomass can be done with and after enzymatic hydrolysis process. Separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SHF), simultaneous saccharification and co-fermentation are three approaches [2] used with hydrolysis. Hydrolysis and fermentation are separately carried out in the case of SHF. SSF includes hydrolysis along with fermentation of only hexose sugar and SSCF includes the hydrolysis and fermentation of both hexose and pentose sugars [15]. Consolidated bioprocessing (CBP) is the new method in which one single micro-organism like C*lostridium* processes the cellulose directly into ethanol by the release of enzyme [16]. *Saccharomyces cerevisiae* is the primary source of microbe used to produce ethanol due to its robustness [17]. Some other thermo-tolerant yeast *Kluveromyces marxianus* is also used (mostly for the SSF) to carry out the process at high temperatures (nearly 45^{O}) [18]. *Zymomonas mobilis* is a bacterium that can metabolize sugars into ethanol [19]. *Clostridium themocellum* is the best thermophilic bacterium for the CBP process [16] but the yield in both the bacterium is lower than the yeast. Pentose fermenting micro-organismslike *Pichia stipitis*, *Pachysolentennophilus*, and *Candida shehata*are also used for xylose to ethanol production. *Pichia stipitis* is preferred because it does not produce any byproduct, rather has lower ethanol and pH tolerance [20].

Co-fermentation of hexose and pentose is carried out by metabolically engineering the strain. Metabolic engineering of the *Saccharomyces cerevisiae* for xylose to ethanol production can be achieved by the transfer of xylose reductase enzyme-encoding (XR) gene and xylose dehydrogenase enzyme-encoding (XDH) genes into *Saccharomyces cerevisiae* [21]. There is a co-factor imbalance issue due to which xylitol is produced instead of ethanol. While in the case of xylose isomerase enzyme coding (XI) gene does not have any cofactor imbalance issue and only ethanol is produced [22]. So, SSCF is the advanced technology focused on the bioethanol production from lignocellulosic biomass. The purpose of the present study was to find a novel isolate from a non-agricultural area filled with water hyacinth and to produce cellulase enzyme from different kinds of wastes.

2. Material and Methods

2.1 Sample collection

The water hyacinth sample was selected for isolation of micro-organism because of softwood biomass and high-water content. It was suggested as the degrading stem and root can be a source of cellulose-degrading fungi or other micro-organisms. The water hyacinth sample was collected from Badoli village, Faridabad (India).

2.2 Isolation of cellulose enzyme-producing microbes

Water hyacinth wet root liquid and the solid portion samples were plated on the PDA (potato dextrose agar) and CMC agar (1% carboxymethyl cellulose agar medium) respectively and incubated at 37°C for 4 days. The screening was done by Congo-red zone test [23].

2.3 Cellulase enzyme production and activity assay

The cellulase enzyme production medium was MW (Mandel Weber) medium [24]. Initially, isolate S8 was tested for cellulase enzyme activity on the MW medium with CMC 1.5 % as agar plate and as broth. After that comparative study was done between all isolates for cellulase enzyme activity. All the strains SL1 to SL6 (from a liquid sample) and S8 (from the solid root) were grown in the MW agar medium at pH 5 and kept as the slant. 1ml of the autoclave distilled water was

poured into each slant sample, mixed well and 1ml of the spore solution was inoculated in 50 ml of the MW production medium containing 1% CMC. After 72 hours, the sample was centrifuged at 8000 rpm for 5 minutes and the supernatant was filtered by a 0.22-micron filter. Then, filtrate was used for the filter paper assay. FPU assay was done by the Gosh FPA assay protocol [25].

2.4. Saccharification of cellulose by cellulase crude enzyme extract

Five gm CMC was added to two 50 ml flasks and autoclaved. After autoclaving and cooling of dried cellulose, 20 ml of the crude enzyme filtrate from the S8 culture was added in the sample (CMCase). Control samples contained20 ml autoclaved water to maintain the moisture up to 80%. Components were mixed well and incubated at 50°C for 48 hours. After 48 hours, the CMCase sample became liquefied as compared to control.

2.5 Cellulase enzyme production in different paper waste

Four kinds of substrates were taken such as newspaper (NP), cardboard (CB), dye coated cardboard (CBC), carboxymethylcellulose (CMC) for the cellulase enzyme production. 20 gm/l of each substrate in 50 ml fermentation medium was inoculated with spore suspension (1ml) of the isolate THS8 with the spore count of 5.32×10^6. Thesamples were incubated at 30ºC at 120 rpm for 120 hours. After 120 hours, samples were extracted, centrifuged and the supernatant was taken as a crude enzyme sample. FPU assay was done by standard Gosh FPA assay protocol for cellulase enzyme activity.

2.6 Cardboard substrate utilization

2% of cardboard was mixed with 50 ml MW medium. Sterilization was done by autoclaving and 1ml of the spore suspension was inoculated and incubated at 30°C for 7 days. After 7 days, the treated biomass was centrifuged, and the crude enzyme extract was taken out. Then, the crude enzyme was tested for FPU analysis per ml of the enzyme sample. The remaining solid substrate was also tested for the degradation and compared with the control sample.

2.7 Substrate concentration optimization for cellulase production from cardboard

The cardboard waste substrate was used from 5g/l to 50g/l concentration (5g/l, 10 g/l, 20 g/l, 30 g/l, 40 g/l and 50 g/l) in MW medium prepared in duplicates for test against control. The samples were sterilized by autoclaving and spore suspension was prepared for the inoculation. The 5.40×10^6 spores per ml were inoculated as 1 ml suspension in each test sample and1ml water in control. Then, it was kept for incubation at 30°C for 7 days. After incubation, the crude enzyme was tested for the highest cellulase activity and maximum substrate utilization. Cellulase kinetic activity with respect to enzyme to substrate concentration was applied similar to that used for the growth and consumption of substrate by microbes. Enzyme-specific activity depicted as μ^E (similar to the specific activity of microbes), a maximum enzyme-specific activity depicted as μ_{max}^E (similar to μ_{max} of microbes) and substrate saturation constant depicted as K_s^E (similar to Ks for microbes) was calculated by using Michaelis Menten equation [26], where superscript E indicates enzyme.

2.8 Characterization of isolate TSH8

THS8 isolate was grown on PDA plate for 4-5 days at 30°C. After the development of full mycelium morphological characterization was conducted. Then, a spore suspension was prepared from the plate containing nearly 5.5×10^9 spores per ml of the suspension. Then, conidial spores were examined under an inverted microscope (EVOS XL imaging system, ThermoFisher).

3. Result and Discussion

3.1 Isolation of cellulolytic microorganism

New isolates with the ability to convert cellulose were screened.7 different fungal isolates viz., THSL 1, 2,3,4,5,6 and THS8 were obtained by plating the collected sample on 1% CMC agar plate. THS8 was isolated from the solid root area sample. Out of which, the S8 isolate was morphologically similar to the fungus *Trichoderma sp*. A total of 6 liquid isolates SL1 to SL6 and solid sample isolate S8 were selected, which showed cellulose degradation as presented in Figure 1. The potential cellulase producing 7 microbes were screened on Congo red medium containing carboxymethyl cellulose as a substrate with the MW medium. Hendricks *et al*. also used a similar method for identifying cellulase producing bacterial strains from the soil through Congo-red dye depletion screening [27].After screening with Congo red dye and substrate hydrolysis (Figure 2), the S8 was selected as a potential cellulase producing fungal candidate. As per morphology and growth of the isolate,S8 showed similar characteristics with cellulose-degrading fungus *Trichoderma sp.* and was selected for further analysis [28]. Congo red test also showed zone on the S8 plate as shown in Figure 2B.

3.2 Screening based on cellulase activity

Hydrolysis of cellulose can be visualized on the plate as yellow rings and glucose release from THS8 broth in Figure 3B was determined by DNS test. The filter paper unit (FPU) was also calculated as shown in Table 1 [26,29]. It was observed that sample THS8 showed fungal growth in clumps formation in which these were grown as fungal pellets and did not fully mix with the medium. When allowed to settle, these easily settled as shown in Figure 4. The isolate S8 has an advantage for purification of cellulase which may omit the centrifugation step. The FPU assay results are shown in Table 2 and THS8 showed maximum activity.

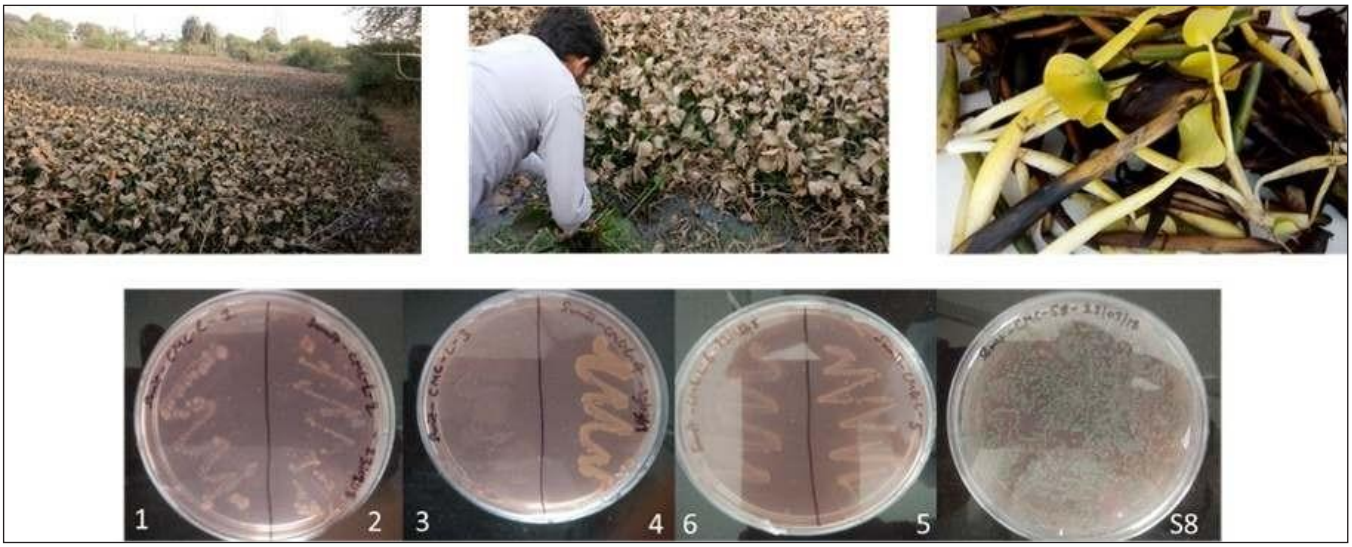

Figure 1: Sample collection of water hyacinth and isolated samples SL1 to SL6 and S8 grown of CMC agar plate with Congo red dye.

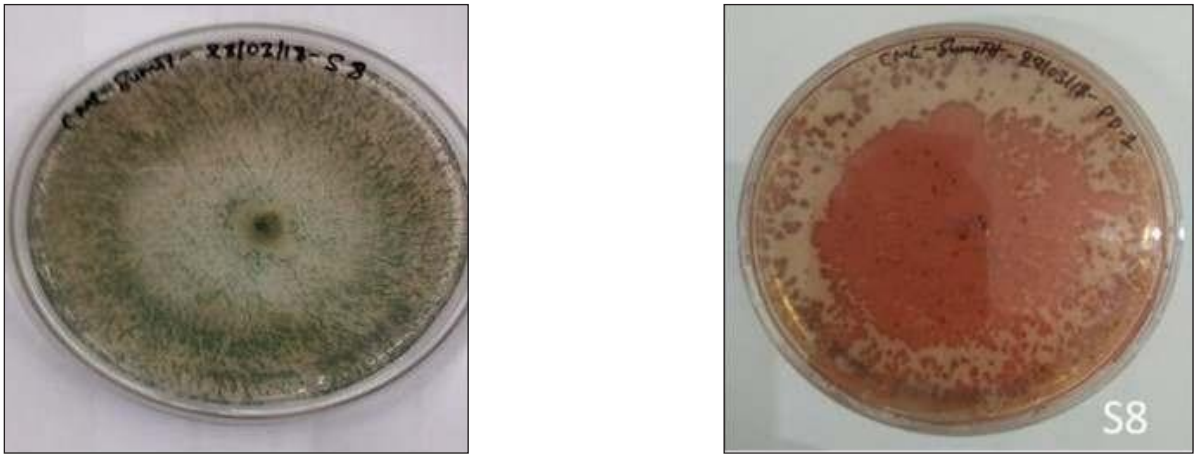

Figure 2: Congo red test showed a clear zone of degraded cellulose by isolate S8.

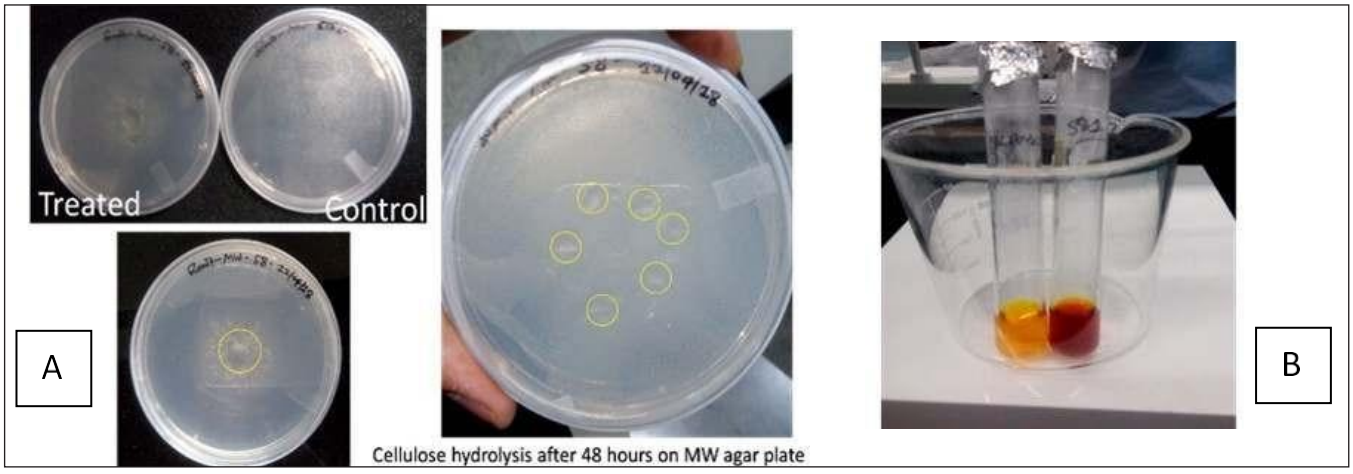

Figure 3: **(A) Clear zones as a yellow ring of hydrolyzed cellulose1% with MW agar plates (B) release of sugar from cellulose observed by DNS method.**

Table 1: FPU and glucose to cellulose conversion.

Parameters	Values
CMC Broth cellulose at Zero-day	15 g/l
Glucose produced after 4 days	3.16 g/l
Cellulose consumed	2.84 g/l
FPU/ml	0.1670 unit/ml

3.3. Cellulose saccharification

The saccharification study was done on pure carboxymethyl cellulose (CMC). This was done to verify hydrolysis of pure cellulose by the cellulase enzyme produced by the isolate. 5g CMC was treated with 20ml crude enzyme against control as water. After 48 hours of incubation at 50°C, sample CMCase was liquefied as compared to control (shown in Figure 5). 23.42% hydrolysis of pure cellulose was observed with THS8 crude enzyme broth. FPU of the crude enzyme was 0.0534

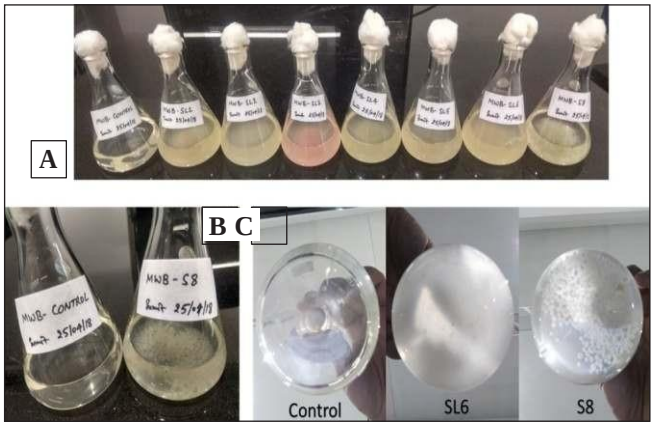

Figure 4:(A) Fungal isolates in 1%CMC broth, (B) S8 culture against control, (C) Observation of fungal pellets in S8 as compared to SL6.

Figure 6: Hydrolysis of pure cellulose by using crude enzyme.

Table 2. FPU/ml of different isolates on MW medium with 1% CMC.

Sample Name	FPU/ml
SL1	0.1150
SL2	0.1336
SL3	0.1460
SL4	0.1596
SL5	0.1534
SL6	0.1348
S8	0.1658

U/g of cellulose. Herr and Dieter used 10% cellulose suspension for hydrolysis by crude enzyme and resulted in a 40% hydrolysis. However, in this study, the suspension was 20% and hydrolysis was nearly similar to 10% cellulose suspension used by Herr and Dieter [30].

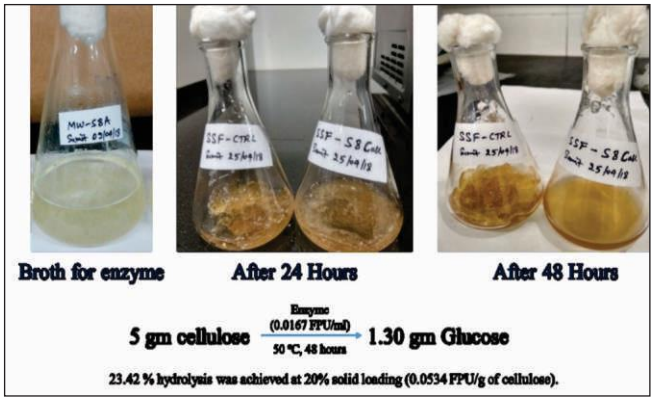

Figure 5: Hydrolysis of pure cellulose by using crude enzyme.

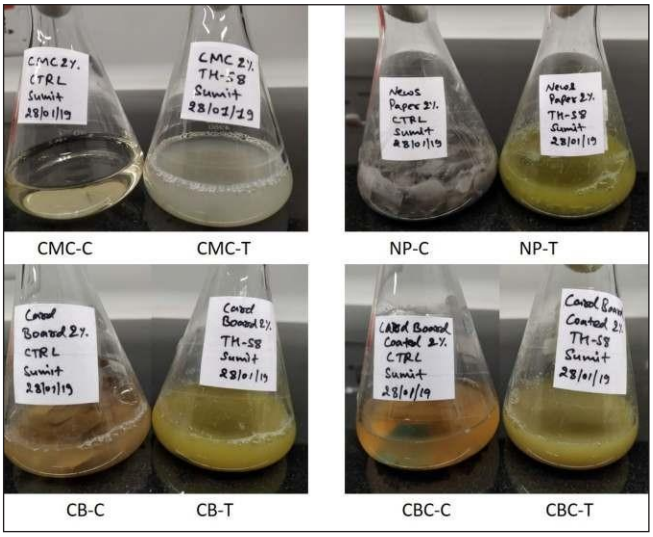

Figure 7: Comparative growth difference between control (C) and treated (T) and cellulase production in MW medium.

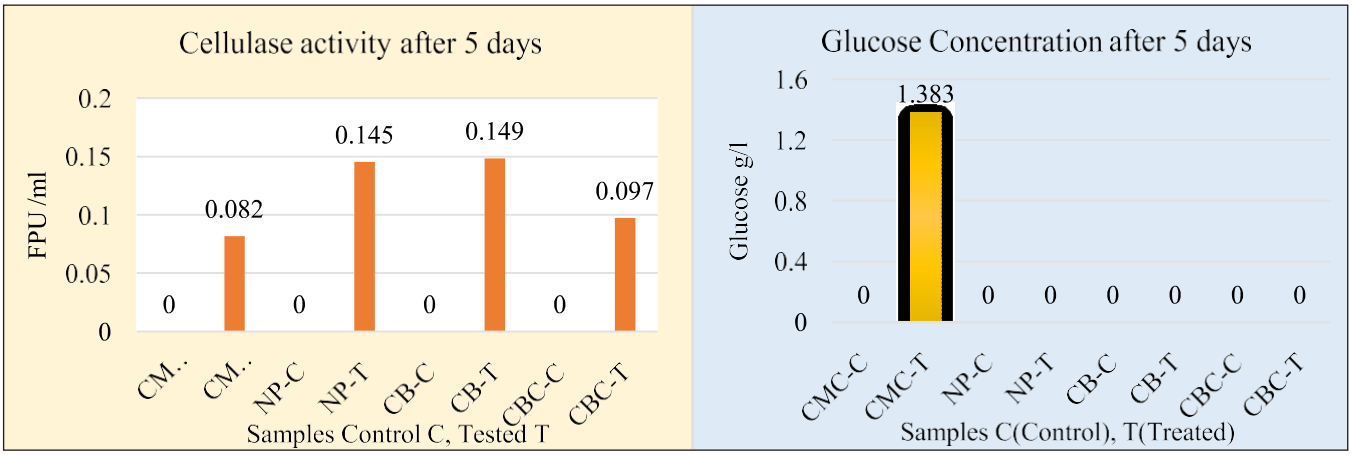

Figure 8: Left: Cellulase activity in FPU/ml in different substrates, Right: Glucose concentration after 5 days of incubation in the different samples.

3.4. Paper waste utilization

The use of the cheapest cellulose source was a major objective of this study. Therefore, 3 kinds of paper waste (Figure 6) as NP, CB, and CBC along with CMC was tested for cellulase production. Figure 7 depicts comparative growth differences in different substrates provided. The enzyme activity was found higher in NP, CB and CBC (0.145, 0.149and 0.097 FPU/ml) as compared to the CMC (0.082 FPU/ml) as shown in Figure 8(left side). The newspaper and cardboard waste samples showed nearly similar cellulase activity. Cardboard is preferable because it is free from ink and dye and mostly dumped as waste while newspapers are used for other purposes too. The consumption of sugar and its release was also estimated. It was found that sugar was left in CMC-T (Treated) as 1.383 g/l, which can cause the feedback inhibition for the production of the enzyme in the CMC-T sample (Figure 8 right side). Ahlam *et al.*, also showed the utilization of cardboard waste nearly 25 gm/l of the substrate with 2.95 FPU/ml, which is quite higher than the observation done in this study. Here, direct cardboard was used as a substrate, while Ahlam *et al.*, used 1% H_2SO_4 for pre-treatment first, then further for enzymatic hydrolysis [31]. Similarly, newspaper, filter paper and other substrates were also tested for cellulose hydrolysis [32]. Here in this study, maximum enzyme activity was found with cardboard substrate i.e. 0.149 FPU/ml.

3.5. Cardboard utilization for cellulase production

It was found that the cardboard is a good substrate for enzyme production, as it showed maximum enzyme activity. So, the experiment was done in triplicates (CBA, CBB, and CBC) with cardboard used as substrate (Figure 9 left side). Here, 2% of cardboard was suspended in MW medium treated with THS8 for 7 days at 30ºC measured as the average maximum of 0.27 FPU/ml(Figure 9 right side). There was around 60% degradation of substrate observed with respect to remaining solid wet weight as shown in Figure 10.

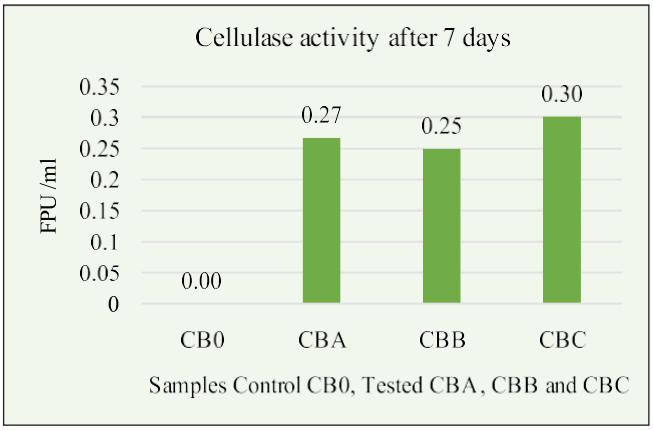

Figure 9: Left: Growth of the S8 in cardboard medium after 7 days, Right: Cellulase enzyme activity measured in cardboard medium cultures in triplicate: CB0 is controlled, CBA, CBB, and CBC are the grown cultures.

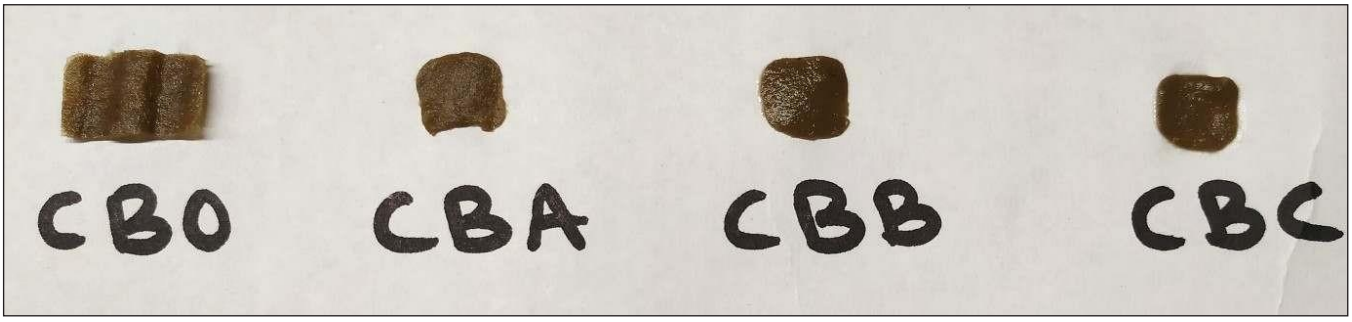

Figure 10: Degradation of the pure cardboard substrate: First left control (CB0) and the other three are samples in triplicate treated with the enzyme.

3.6. Solid-state fermentation with cardboard

Growth of mycelium on the cardboard waste as solid support was observed with nearly 85% moisture developed by the spore suspension on the substrate. Solid-state fermentation was previously used for cellulase production by using *Trichoderma reesei* QMY-1 with FPU as 17 U/ml [33]. The purpose of this study was to determine whether it is feasible to grow the fungus on solid cardboard or not. After 7 days of static incubation, the growth was observed on the medium and visually compared against control. Figure 11 clearly showed very high growth of isolate THS8 as green-colored patches on the wet cardboard.

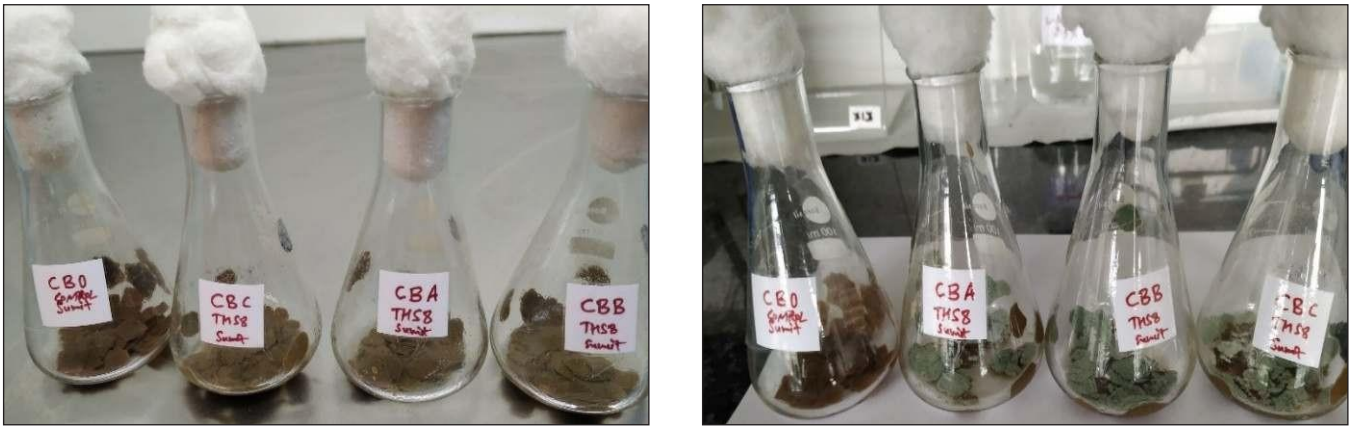

Figure 11: The solid-state fermentation of the isolate S8 on waste cardboard (Left: at zero-day Right: after 7 days).

3.7. Substrate concentration optimization for cellulase and kinetic modeling

Usually, with the increase in the substrate, there should be an increase in product yield. However, in the case of cellulases, feedback inhibition due to the product was observed. When the product is produced in a higher amount, the feedback inhibition occurs. A similar case was found in this study during substrate optimization of cardboard concentration. However, an increase in the activity was observed from day 2 to day 6. While enzyme production was not increased much due to feedback inhibition by glucose concentration, it was found higher in more concentrated substrate samples. There was a substantial increase in glucose concentration after 6 days as 0.10, 0.10, 0.11, 0.12, 0.19, 0.56 g/l in 5, 10, 20, 30,40 and 50 g/l samples respectively. Maximum enzyme activity was found to be 0.117 FPU/ml in 50g/l samples, but it was reduced due to higher glucose feedback inhibition (Figure 12). Meanwhile, inhibition was lower and nearly similar up to 30g/l sample. The μ^{E}_{max} and K^{E}_{s} were calculated as 14.93 min^{-1} and 0.022 respectively by using slope and intercept values of Figure 13. Similarly, other kinetic parameters like K_m and Vmax were measured by Shafique et. al. by using *Trichoderma viride* FCBP-142 with 0.6 and 8.33 values respectively [34].

3.8. Morphological identification

During the initial 2 days of inoculation, the mycelium grown as white clouds then, after maturity, the color change to dark green (Figure 14A), which showed the similarity with *Trichoderma hazaricum* [35]. However, spores shown under the microscope are oval (Figure 14B), nearly 6.250 µm in length and 3.125 µm in width as measured with the scale bar after zooming at 8×from an image captured at40x objective. Conidial spores count was ~5.42×10^{6} per ml.

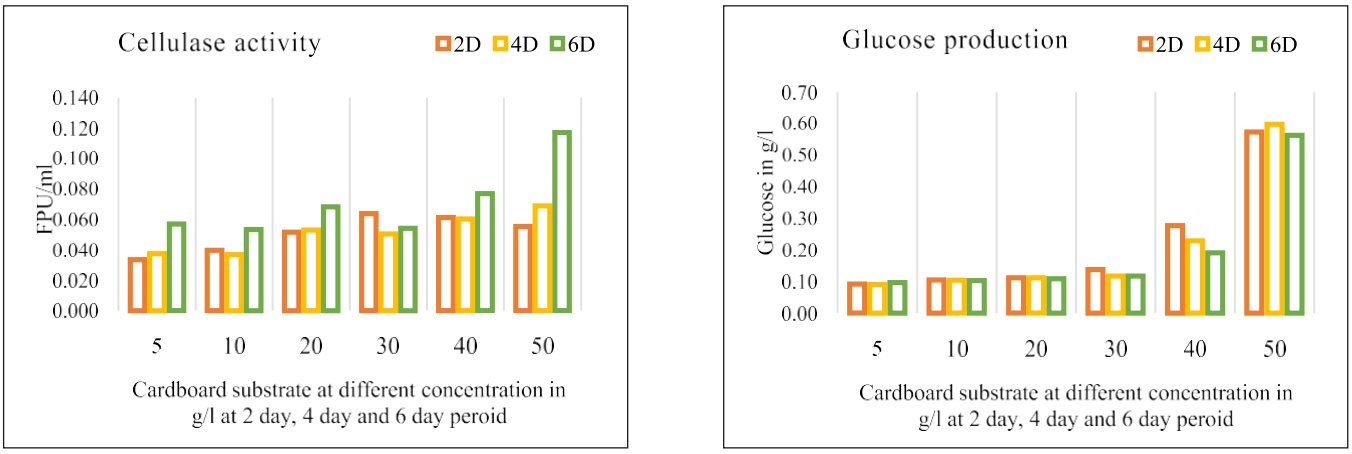

Figure 12: Left: Cellulase activity in FPU/ml in different concentrations of cardboard substrate Right: The glucose concentration of samples grown till 6 days of incubation with different concentration of substrate in g/l

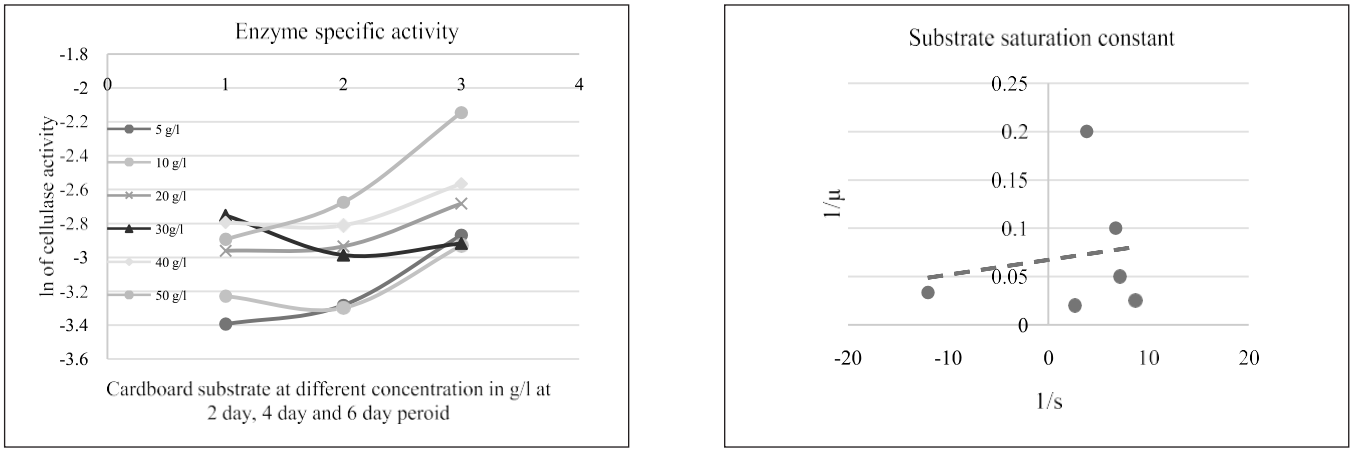

Figure 13: Left:Cellulase enzyme-specific activity measured as µ against each substrate over the time 2-day, 4 days and 6 days. Right: Substrate saturation constant KsE value determination by plotting 1/µ against 1/s values.

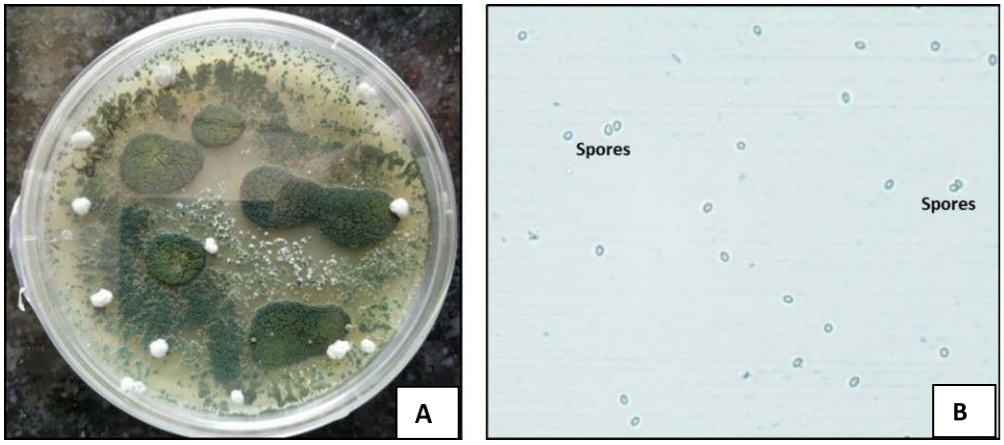

Figure 14: (A) Morphological identification of cellulase producing isolated fungi THS8. (B) microscopic image of conidial spores.

4. Conclusion

Application of paper waste for cellulase production at an industrial scale can be promising for reducing the cost of the enzyme as well as for waste management. This study signifies the use of cardboard waste as pure cellulosic feedstock replacement, which can contribute a cost-effective approach

with higher cellulase enzyme production as compared to pure carboxymethyl cellulose. According to the results, isolated THS8 fungal strain was the best cellulase producing strain. The strain was capable of degrading nearly 60% of the cardboard waste during the study period and showed maximum cellulase activity of 0.27 FPU/ml, which is a good start for its large-scale production after further process optimization. In conclusion, the cardboard waste is a very good potential source of cellulose substrate for cellulases production, irrespective of the dyes or ink it contains. It is more valuable because it can reduce the whole production cost and the process becomes simpler.

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