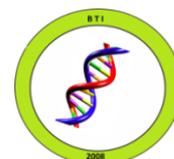




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Research Article

STUDY OF DEINKING OF OFFICE WASTE PAPER USING CULTURE FILTRATE OF *ASPERGILLUS FUMIGATUS* FRESENIUS (MTCC 3070)

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ABSTRACT

In the paper industry the waste paper recycling is considered environment friendly approach but during recycling process the removal of ink becomes very important processes which determine the quality of recycled paper. Chemical and enzymatic methods are used for deinking process but deinking using enzymes is more efficient and environment friendly. In this present study culture extract of *Aspergillus fumigatus* Fresenius was used for deinking of waste paper (newspaper, office non-printed white paper). Growth conditions for growth of strain were studied. Strain was grown at 40°C and culture filtrate was prepared. Ink released from newspaper and office waste paper by pure enzymes and filtrate was analyzed as indicator of deinking. Deinking process was conducted using this culture filtrate. For comparative study the deinking using cellulase and xylanase enzymes was also performed. Including this sugar released by the action of these enzymes was also studied.

Keywords: Deinking, paper recycling, endoglucanases, cellulose, *Aspergillus fumigatus* Fresenius.

INTRODUCTION

The fast depletion of forest resources and its impact on the environment has forced the paper industry to turn to the use of waste papers and recycling of waste paper. In the paper industry recycling of waste paper has significantly increased during last 20 years. Recycling of waste paper has now become a crucial and environmentally benign source of new fiber for paper. Generally, the industrial process for paper recycling involves re-pulping, screening, cleaning, washing, and flotation (Pala *et al.*, 2006; Zhang *et al.*, 2006). The conventional recycling of waste papers uses chemical methods (Sodium hydroxide, sodium silicate, hydrogen peroxide, chelating agents and surfactants), which are not only costly but also involves multistep treatment processes, and cause hazardous effect to the environment (Shrinath *et al.*, 1991). Normally the ink used in paper printing contains the polar pigments or dyes which are bound to paper surface normally with the cellulose fibers of paper. During deinking process ink formulations used in papers are removed chemical or enzymatic treatments and removed ink particles are separated by washing and floatation (Ibarra *et al.*, 2012). Chemical treatments used to remove ink are also not promising but use of enzymes (biological deinking) has significantly improved ink detachment from waste papers. The biological deinking is proposed as an alternative deinking method.

Pulp is made up of plant cell wall components like lignin, hemicellulose and cellulose and thus enzymes catalyzing degradation of these compounds may be used for deinking from paper. Hemicellulases, cellulases, endoglucanases

and xylanases are reported enzymes for deinking (Call, 1992; Xu *et al.*, 2009) which interact with cellulose fibrils and involved in hydrolysis of bonding between fiber and ink and perform ink removal from paper. With this process small fibers are also removed from the surface of the paper with toner/ink particles and thus facilitate easy floatation of ink particles during floatation process. But the enzymatic degradation of cellulose also affects strength of paper fibers (Virk *et al.*, 2013). Floating ink particles on the surface are removed by skimming. It can be understood that in this overall cellulose degradation process, the role of these enzymes is to break the cellulose fibers at several positions (Fig.1). Due to this action the ink particles are released from the fibers of wastepaper which are also loosened during floatation in the presence of surfactants. It can be suggested that short fiber forming activity of cellulase also removes residual fibers from the toner surface (Vyas and Lachke, 2003). The enzymes are specific, safe and easy to control. Therefore, cellulases, xylanases are good option that can prove value alternative in order to reduce use of toxic chemicals. Microbial cellulases (Jeffries *et al.*, 1994; Vyas and Lachke, 2003), hemicellulases (Morkbak and Zimmermann, 1998), have are reported to increase the brightness of the pulp. Xylanase action has been reported to enhance the strength and bioleaching of pulp (Prasad *et al.*, 1993; Viikari *et al.*, 1994).

Endoglucanase produced by *A. fumigatus Fresenius* that lack cellulose binding domain (CBD) showed significant deinking with improved brightness, tensile strength and tear index of recycled paper waste (Soni *et al.*, 2008). Researchers have

used many microorganisms to produce enzymes like glycanases, cellulase, xylanase, lipase etc. (Okada, 1985; Marques *et al.*, 2003).

The present study aimed to study deinking of waste paper (newspaper, office non-printed white paper) using *A. fumigatus Fresenius* filtrate. Including this, role of commercially available enzymes like cellulase and xylanase was also studied in deinking process. In this article, the laboratory deinking procedure is shown having four stages; sample preparation, pre-washing, pulp treatment and fiber/ink particles separation. In this study two goals were envisaged: (a) the enzymatic detachment of the ink particles from the paper surface; (b) separation and removal of the ink particles.

MATERIALS AND METHODS

The chemicals used in this study were of analytical grade and procured from HiMedia (Mumbai, India), SRL (Mumbai, India) and Merck (Germany). All culture media were procured from HiMedia (Mumbai, India). *A. fumigatus Fresenius* is a disease causing strain and thus standard precautions were followed during this study.

Inoculum and growth of culture

The lyophilized culture of *A. fumigatus Fresenius* was procured from Microbial type culture collection (MTCC 3070), Institute of Microbial technology, Chandigarh (India). Culture vial was broken and with flame sterilized loop and the culture was transferred onto the agar plates by streaking and was kept in an incubator at 30°C for visible growth.

Recommended culture medium used for revival was Czapek Yeast Extract Agar (CYA) growth medium. The composition of this medium were

dipotassium hydrogen phosphate 1.0 g/L, yeast extract 5.0 g/L, Sucrose 30.0 g/L, sodium nitrate 0.30 g/L, potassium chloride 0.05 g/L, magnesium sulfate heptahydrate 0.05 g/L, ferrous sulfate heptahydrate 0.001 g/L, Zinc Sulfate 0.001g/L, copper sulfate 0.0005g/L and Agar 15.0 g/L in double distilled water. Broth medium was prepared by removing agar from this medium.

For further study, sterilized broth medium was inoculated by *A. fumigatus Fresenius* in laminar air flow by transferring the loop full of strain grown on agar plates. After inoculation it was kept for incubation at 40°C in an incubator shaker at 220rpm.

Parameter optimization

Growth of fungus *A. fumigatus Fresenius* was optimized in terms of pH and temperature by analyzing biomass formation. For this optimization, growth of *A. fumigatus Fresenius* was studied at different pH i.e. 6, 7 and 8 of culture medium and at different incubation temperatures i.e. 25°C, 30°C, 40°C and 45°C.

Broth culturing for enzyme production

The broth medium was prepared in shake flask for culture filtrate preparation. It was inoculated and then incubated at 40°C at 220rpm in an incubator shaker. After few days, the fungal colonies were visible in the form of pellets. The number and size of the pellets increased as incubation period increased.

Thereafter, the enzyme was harvested by adding 50mL of sodium citrate buffer (50mM pH 6.0) to the shake flasks and kept at 40°C for 1 h under mild shaking. The resultant slurry was filtered through a muslin cloth and culture filtrate was kept to 4°C for further experiments.

Analytical procedures

Protein content in culture filtrate was estimated by Lowry's method using bovine serum albumin (BSA) as standard (Lowry *et al.*, 1951). Enzymatic activity of cellulase, xylanase and culture filtrate activity was assayed by measuring the amount of released sugar by dinitrosalicylic acid (DNS) method (Miller, 1959).

Procedure of deinking of paper

Deinking sequences of experiments were performed in the laboratory. Prior to the enzymatic treatment, newspaper and office non-printed waste (30g each) was shredded and pulped by soaking in tap water at 4% (w/v) consistency under shaking (100 rpm) at 37°C for overnight. The soaked papers were washed and disintegrated into soft pulp. Pulp was dried at 50°C and treated with buffer, pH 6.0 (10% consistency). This pulp was divided and fractions were treated with 100 CMCase units of cellulase as well as xylanase. In another sets of experiments, pulp was treated with culture filtrate.

Analysis of deinking

Removal of ink during pulp preparation, enzymatic and filtrate treatment and floatation was analyzed by estimating the amount of released sugars and ink from the filtrate. The released sugar was analyzed and compared with standard solution. But to analyze release of ink, spectrophotometric absorption of ink was performed. Because standard for ink solution could not be prepared hence spectrum of absorbance of solution having released ink was analyzed for different dilutions using spectrophotometer (Labindia 3000⁺, India) and absorbance at 550nm was selected. Amount of sugar content released by degradation of cellulose was also measured. For deinking

analysis, different amount of filtrate was used as sample 1 (1mL filtrate), sample 2 (2mL filtrate), sample 3 (3mL filtrate), sample 4 (4mL filtrate) and sample 5 (5mL filtrate).

RESULTS AND DISCUSSION

Revival and growth of culture

Aspergillus fumigatus strain was revived in Czapek Yeast Extract culture medium and fungal growth was observed on agar plates. It was observed that *Aspergillus fumigatus* grows very slow on culture medium and after 120 h of incubation fungal growth was visible. Morphologically heads were black or dark colored resulting in black or dark brown colonies (Frisvad and Samson, 1990; Maheshwari *et al.*, 2000). Colonies started white to pale yellow but quickly form jet-black conidia (Fig. 2) due to maturation stage. Similar kind of study has been reported by Frisvad and Samson in 1990.

Inoculum in broth

Inoculation of *Aspergillus fumigatus* was performed into broth medium and pellets or mycelial suspensions were visible after 144 hrs of incubation (Fig.3). Production of microbial product is growth associated and maximum biomass can be obtained by optimization of culture conditions. Researchers have reported the importance of physiochemical conditions playing key role in obtaining maximum biomass (Dubey *et al.*, 2011; Kumar and Dubey 2015; Kumar and Dubey, 2016). In the present study experiments were performed to obtain maximum biomass by studying the growth of *Aspergillus fumigatus* at different pH (6, 7 and 8) and different incubation temperatures (25°C, 30°C, 40°C and 45°C). It was observed that at pH 7.0 and incubation temperature 40°C,

maximum growth (biomass) was obtained and thus further study was conducted at optimized conditions. The similar results of getting higher biomass by optimizing conditions have been supported by researchers in other microbes (Dubey *et al.*, 2008; Yadav *et al.*, 2016).

Filtrate and processing

Maheshwari *et al.* (2000) reported the physiology of enzymes in thermophilic fungi. These fungi are recognized for their growth at temperature above 20°C and are source of various enzymes like lipase, protease, xylanase and cellulase. Xylanase and cellulase are well recognized for their role in paper recycling (Elegir *et al.*, 2000). In this experiment the culture filtrate was processed to get mixture of proteins (possibly cellulase and xylanase) secreted by *Aspergillus fumigatus* during growth in culture medium. The presence of proteins was determined by measuring their concentration by Lowry's method. Lowry's assay provided the concentration of proteins present in the filtrate at 7.2 µg/mL which supports the assumptions of previous researchers about the production of proteins by fungi (Maheshwari *et al.*, 2000; Marques *et al.*, 2003). The biochemical activity of filtrate was checked by deinking of paper using enzymes present in this crude filtrate.

Analysis of deinking

Basic idea behind deinking of waste paper using cellulase and xylanase was that these enzyme act on surface of paper and release sugar by enzymatic action on cellulose. By this action, sugar and ink are released thus amount of sugar and ink released during each step of deinking procedure of the newspaper and the office non-printed white paper, released ink was measured (Fig. 4, Table 1). After all the experimental steps, dried

pulp of all samples looked similar but analytical observations of released sugar content and absorbance of solution having ink at 550nm were different for each samples.

As per data presented in table 1, it was observed that some deinking process took place in buffer without any added enzyme or culture filtrate at different time of incubation. Including this floatation also removed ink without enzymatic assistance in buffer but sugar was not released. It is possibly due to that part of ink was removed due to mechanical action that took place during incubation and change in surface tension of solution on addition of buffer. The deinking without enzyme was very less than enzyme assisted deinking. The ink was also removed by addition of filtrate at different dilutions. And increasing amount of filtrate produced increases amount of ink (absorbance varies from 0.292 to 0.591). These experiments supported the assumptions of researchers that cellulase and xylanase play pivotal role in deinking of waste paper (Jefferies *et al.*, 1994; Elegir *et al.*, 2000; Marques *et al.*, 2003).

Deinking procedure during the incubation time was studied in terms of ink removal with enzymatic treatment. It was found that in all the samples, release of ink was increased with increase in incubation duration. It was also observed that after floatation, more ink was released from paper and it may be assumed that enzymatic treatment removed the fibers from paper surface biochemically but some ink fibers remain attached physically to the paper and floatation caused mechanical detachment of ink particles from paper. Thus more ink is released from paper after floatation (Table 1). It was found that after overnight incubation

in cellulose (100CMCase units), absorbance at 550nm of solution with ink was found at 0.191 and after floatation this absorbance was found at 0.277. In case of xylanases (100CMCase) after overnight incubation absorbance at 550nm was 0.293 and after floatation it was 0.335. In case of filtrate, it was observed that absorbance of ink in sample 1 after overnight treatment was 0.251 and after floatation it was 0.292. In sample 5, absorbance of ink after overnight incubation was 0.389 and after floatation it was 0.591. It is clearly indicated that more culture filtrate released more ink from waste paper.

The analysis of released sugar from waste paper by action of pure enzyme treatment and culture filtrate treatment revealed that sugar released from paper during overnight enzymatic treatment was varying in each experiment. Combination of cellulase and xylanase produced more amount of sugar than cellulase or xylanase individually. These observations supported the assumption about the importance of enzymes on deinking of the waste paper (Jefferies *et al.*, 1994; Elegir *et al.*, 2000; Marques *et al.*, 2003), including this increasing amount of filtrate released enhanced the amount of sugar (Fig. 4). It was observed that cellulase (100CMCase) treatment released 39.66 $\mu\text{g/mL}$ sugar, xylanase (100CMCase) treatment released 44.11 $\mu\text{g/mL}$ sugar, while combination of cellulase and xylanase (50CMCase each) released 49.11 $\mu\text{g/mL}$ of sugar. In case of culture filtrate it was found that sample 1(1mL of filtrate) produced 34.11 $\mu\text{g/mL}$ of sugar and sample 5 (5ml of filtrate) produced 63.55 $\mu\text{g/mL}$ of sugar. Comparison of the results of sugar released by filtrate samples after overnight incubation with pure cellulose showed that filtrate contains 32.04 CMCase units/mL

equivalents to cellulase enzyme. Hence it is clear that filtrate contains cellulose and xylanase like enzymes which are having paper degrading capability which is useful in deinking and also the presence of sugar may directly be associated with removal of ink.

It is clearly observed that culture filtrate is capable to remove ink from waste paper (newspaper, office non-printed white paper). These experiments supported the assumption that culture filtrate of *Aspergillus fumigatus* contains cellulase and xylanase like enzymes (Vyas and Lachke, 2003; Soni *et al.*, 2008) which are involved in deinking of waste paper (Woodward *et al.*, 1994; Marques *et al.*, 2003; Puneet *et al.*, 2010) and help in recycling of waste paper. Side by side crude extract is able to release ink and sugar like pure enzymes and this filtrate may be used in future for deinking process at large scale and it is cheaper than pure enzymes.

CONCLUSION

Enzymatic deinking procedure is capable to remove ink in much environmentally friendly way (Eom and Ow, 1990). Efficient enzymatic procedures for ink removal from waste paper are reported by many research groups (Woodward *et al.*, 1994; Elegir *et al.*, 2000; Zang *et al.*, 2008; Puneet *et al.*, 2010) and role of cellulase and other enzymes in enzymatic deinking was demonstrated. The present research was focused to analyse the application of cellulases produced by an indigenous thermotolerant fungus *A. fumigatus*, for bio-deinking of waste paper. A thermotolerant fungus *A. fumigatus* Fresenius was used to produce cellulase that was apparently devoid of CBD (Soni

et al., 2008) was taken up for further studies (Maheshwari *et al.*, 2000).

In this study, two parameters; released ink and produced reducing sugar content (CMCase units) were considered as the potential of the enzyme and filtrate extracts for bio-deinking of newspaper waste and office non-printed paper. The optimal pH and temperature were observed to be 7.0 and 40°C, respectively. Although *A. fumigatus* grew well at 50°C but optimum temperature for cellulase production was found to be 40°C. Cellulase purified from *A. fumigatus* Fresenius exhibited higher thermostability under acidic pH conditions, and is significant in view of their potential industrial applications.

The use of cellulase enzymes may represent an alternative deinking method to conventional chemical deinking method of newspaper and office non-printed paper. We found that distilled water also helped in removing ink to some extent in newspaper pulp. It was found that cellulase and xylanase and filtrate of *A. fumigatus* Fresenius also exhibited deinking activity which was comparable to the enzymes reported for deinking process.

In this study culture filtrate produced from *A. fumigatus* demonstrated deinking capability comparable to the known concentration of pure enzymes.

FUTURE PROSPECTS

Since green plants serves as the basic raw material for the growing paper manufacturing industry which leads to the environmental imbalance or the deforestation of trees, so paper making is not an environment-friendly option. To assuage the stress from the environment, recycling of used paper is an alternative which is been exercised. Researchers are

keen to develop a process in which they can reuse the used papers by removing inks from them. Present study proposed use of enzymes for deinking of waste paper as potent alternative to conventional (chemical) deinking process. In this study, culture filtrate of *A. fumigatus* Fresenius showed remarkable capability of enzymatic deinking but more study is required to find out type and stability of different enzymes present in culture filtrate. Side by side reaction kinetics of enzymatic reactions, optimum conditions and involvement of factors is also required.

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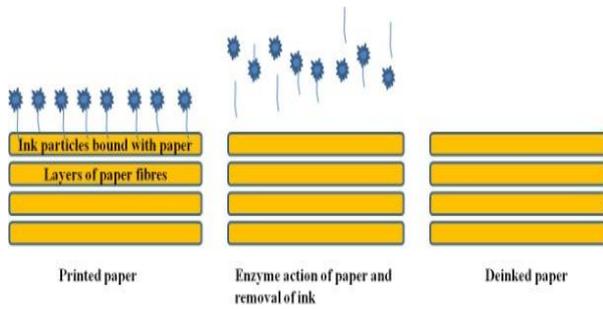


Fig. 1. Diagrammatic representation of enzymatic deinking of paper.

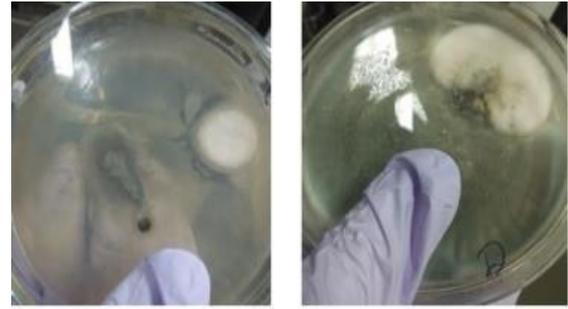


Fig. 2. Growth of *Aspergillus fumigatus* on semisolid culture medium in Petri Plate.



Fig. 3. Growth of *Aspergillus fumigatus* on broth medium in shake flask.

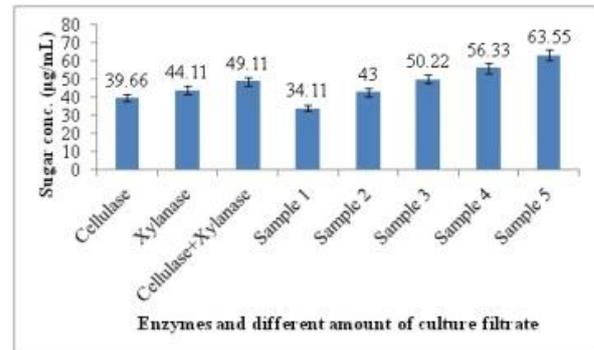


Fig. 4. Sugar content (µg/mL) released during catalytic action of enzymes and culture filtrate with waste paper. All the experiments were carried out in triplicates and the results are represented as mean values.

Table 1. Analysis of presence of ink in newspaper pulp solution

Sr. No.	Sample	Absorbance at 550nm				
		At 0 hours	At 2 hours	At 4 hours	Overnight incubation	After Flootation
1	Blank (no enzyme)	0.005	0.009	0.013	0.029	0.042
2	Cellulase	0.007	0.109	0.107	0.191	0.277
3	Xylanase	0.008	0.208	0.261	0.293	0.335
4	Cellulase+Xylanase	0.008	0.183	0.234	0.250	0.387
5	Sample 1	0.007	0.107	0.114	0.251	0.292
6	Sample 2	0.007	0.108	0.146	0.276	0.298
7	Sample 3	0.008	0.115	0.167	0.316	0.380
8	Sample 4	0.009	0.286	0.283	0.342	0.421
9	Sample 5	0.009	0.299	0.301	0.389	0.591

Experiments were carried in triplicate and each data point is mean value

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