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Biodeinking of Photocopier Waste Paper Effluent by Fungal Cellulase under Solid State Fermentation

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Author's contribution

The sole author designed, analyzed and interpreted and prepared the manuscript.

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ABSTRACT

Aims: In this study, the purified cellulase was examined for its ability to deink the photocopier waste paper effluent and the application of enzymatic deinking improved ink removal.
Study Design: OFAT (One Factor At a Time).
Place and Duration of Study: Medical Labs Department of Unayza Community College, Qassim University, KSA between October 2012 and April 2013.
Methodology: Four fungal isolates were isolated from rice husks and screened for their production of cellulase and the most potent ones were selected. The resultant extracts were identified and used for deinking of photocopier waste paper. One isolate that showed efficient deinking and maximum cellulase production was identified morphologically and microscopically.
Results: The enzyme was successively optimized for its maximum productivity and purified by precipitation with 80% ammonium sulfate followed by chromatography on G-100 Sephadex. The purification procedure provided 2.36 folds purification with 64.78% yield recovery of cellulase. The maximum production was achieved in flasks with shaking speed of 200 rpm at pH 5.0, 30°C and incubation time of 5 days. SDS-PAGE indicated that the molecular weight of the purified cellulase was 71.6 KDa. Results also revealed that, the ink color was removed gradually by increasing the reaction time between cellulase and the effluent and the maximum color removal was achieved after 9 hours.
Conclusion: This study suggested that toxic pollutants like waste paper effluents (concentrated in

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industrial wastes and contaminated sites) can potentially be eliminated by low cost bioremediation systems using microbial cultures. The knowledge about the optimum environmental factors or conditions could help to employ biological approaches efficiently to clean up the water discharged from waste paper recycling industry. *Mucor hiemalis* cellulase effectively deinked wastepaper effluents.

Keywords: Cellulase; Mucor hiemalis; biodeinking; photocopier wastepaper; biotechnology.

1. INTRODUCTION

Nowadays, the life without the paper would be almost impossible. Huge quantities of paper and paper products have been used in the whole world today, in households as well as on the working places, in industry, schools and in all the domains of the public life. Paper is made from fibers of vegetable origin, mostly of wood. In order to protect world's woods, which are used as the raw material for paper production, the recycling of the used papers is necessary [1]. Recycling of wastepaper has gained momentum over the past decades due to the increase in the demand of green plants being imposed by the paper industry throughout the world [2]. Non-impact printed white office papers that include laser printed papers are difficult to deink with conventional deinking methods [3]. Because offices use more laser printers and copy machines every year, the amount of non-impact printed papers entering the recycled paper stream is increasing. Ink removal from these papers remains a major challenge. The efficiency is due primarily to the strong adherence of the toner particles to the paper surfaces [4,5]. The photocopier printers use thermosetting toners, consisting of non-dispersible synthetic polymers, as ink for printing the paper. This ink is physically bonded to the fibers because of high heat, making it difficult and expensive to remove by conventional chemical methods [6,7].

Solid state fermentation (SSF) process, using agro-wastes as a carbon source, was employed for the production of many plant cell wall degrading enzymes [8-10].

A biological process (using enzymes) had been evaluated and proven successful in deinking various types of wastepaper. One of the benefits of using enzymes in the deinking process is the minimum treatment of effluent produced; it is also less harmful to the environment [11]. The removal of the printing ink from the used paper is one of the most important processes in recycling of paper [12]. Several enzyms such as cellulases, hemicellulases, pectinases, lipases, esterases, α-amylases and lignolytic enzymes have been used for deinking of various waste papers [13].

The enzymatic treatment favors ink detachment from paper fibers without discharge of pollutants, thus contributing to environmental compatibility. The effect of cellulase in facilitating ink detachment during deinking of laser printed paper has been shown using preparations containing mixed enzymes and mono-component activities [14,15]. Most of cited studies reported the deinking of mixed office wastes consisting of photocopier papers by using commercially available enzymes.

The objective of present study was to investigate the deinking of photocopier paper effluent by using cellulase produced from some fungal isolates. The parameters for enzymatic deinking were optimized.

2. MATERIALS AND METHODS

2.1 Microorganisms

Four fungal isolates were isolated from rice husks. They were grown at 30°C on yeast starch agar (pH 7.0) of the following composition (%; w/v): starch 1.5, yeast extract 0.4, KH_2PO_4 0.23, K_2HPO_4 0.2, $MgSO_4$ $7H_2O$ 0.05, citric acid 0.057, and agar 2.0 and maintained on slants of the same medium at 4°C according to the method described by Coony and Emerson [31]. The isolates were screened for their production of cellulase and the most potent ones were selected. The resultant extracts were identified and used for deinking of photocopier waste paper. One isolate that showed efficient deinking and maximum cellulase production was identified morphologically and microscopically.

2.2 Microorganism Identification

The most potent isolate was identified in Culture Collections and Identification Unite of the Regional Center for Mycology and Biotechnology

at Al-Azhar University, Cairo, Egypt. Identification was based on current universal keys [16,17].

2.3 Substrates

Palm leaves were used as carbon source in all experiments. They were collected from farms of Unayzah city, Qassim governorate, Kingdom of Saudi Arabia. The leaves were dried and pulverized to about 40 mesh in size prior to use in the culture medium.

2.4 Solid Substrate Culturing for Enzyme Production

Solid state fermentation was carried out in Erlenmeyer flasks (250 ml) that contained ground palm leaves as carbon source (5 g) and basal medium (15 ml) of the following composition (%; w/v): yeast extract 0.4, KH_2PO_4 0.23, K_2HPO_4 0.2, $MgSO_4$ $7H_2O$ 0.05, citric acid 0.057. The components were autoclaved at 121°C for 20 min. After cooling the flasks were inoculated with spore suspension (2 ml; 10^7spores/ml), prepared from 7 days old culture grown on agar plates. The flasks were incubated for 7 days at 30°C. The extract was harvested by adding 50 ml of sodium acetate buffer (50 mM pH 5.5) to the flasks and kept at 30°C for 1 hour under shaking (150 rpm). The resulted slurry was filtered and centrifuged at 8800 x g for 10 min, and the extracts were used for enzymatic assay [18].

2.5 Enzymes Assay

Cellulase activity was conducted by dinitrosalicylic acid (DNS) method as a modification of the method by Gawande and Kamat [19] with carboxymethyl (CM) cellulose (Sigma Aldrich, USA) as the standard. One gram of CM-cellulose was dissolved with stirring in 100 mL of 50 mM sodium acetate buffer, pH 5.5 at 60°C, boiled for several minutes and continued stirring for 3 h at room temperature. To 1.8 mL of this cellulose solution, 200 µL of extract was added and incubated at 30°C for 10 min. 3 mL DNS solution (a mixing of 16 g NaOH, 10 g DNS, 300 g sodium potassium tartrate and 8 g sodium metabisulfite in 1 L water) was added to stop the reaction, and the solution was boiled for exactly 5 min and then cooled down rapidly using ice bath until room temperature. This was followed by measuring the absorbance at 540 nm. A blank for each sample in which no reaction takes place (by immediately stopping the reaction after adding the extract) was carefully conducted in order to obtain accurate data. One unit of cellulase activity was defined as the amount of glucose (µg) released per ml of enzyme solution per minute. The total extracellular protein concentration was measured by the Bradford method [20] with bovine serum albumin (BSA) as standard.

2.6 Cellulase Purification and Molecular Weight Determination

To determine the molecular weight of cellulase obtained by the method described in this study, 1 mL sample was added to 80% (w/v) ammonium sulphate solution until complete precipitation and centrifuged at 40000 x g and 4ºC during 30 min. The precipitated obtained was reconstituted with sodium acetate 10 mM (pH 5.0), after that was dialyzed using cellophane tubing having a cut-off molecular weight of 14 KDa. The buffer was altered three times (after 2, 14 and 19 h) to ensure removal of ammonium sulfate before the next purification step. Then the sample was allowed to pass through a column (Pharmacia column, 2.5 x 50 cm) of G-100 Sephadex previously equilibrated with a 0.2 M citrate-phosphate buffer (pH 5.0). The elution was made with the same buffer, for the obtained fractions and cellulase activity was evaluated. The fractions that showed cellulase activity were dialyzed and the molecular weight of the purified enzyme was determined using an electrophoresis test (SDS-PAGE). Preparation of the column and the fractionation procedure was carried out as mentioned by Scopes [21].

2.7 Wastepaper Collection

Photocopier paper (sheet size 210x297 mm, basis weight 80 g/m^2 from Xerox was used to produce photocopier waste paper after printing with Samsung black Toner (MLT-D101S) used for Samsung SCX-3405 photocopier machine. One set of "standard" printed sheets was prepared and used for all the trials performed in this study.

2.8 Printed Paper Preparation

Waste papers were shredded and soaked overnight in tap water at room temperature. Soaked papers were washed several times and were disintegrated with the help of a grinder to obtain full inked effluent.

2.9 Analysis of the Collected Effluent

The resulted effluent was scanned in a spectrophotometer from 200 nm to 800 nm to ascertain the wavelength and the maximum

absorbance was observed at 465 nm and the rate of deinking was monitored at this wavelength. The deinking process was carried out according to the methodology of Prabu and Udayasoorian [22]. The effluent sample was centrifuged at 16000 x g for 30 min to remove all the suspended matter and the resulted supernatant was tested in combination with different concentrations of the purified diluted enzyme. The pH of the supernatant was adjusted to 5 and then used for the measurement of absorbance at 465 nm against distilled water as blank (at room temperature). The residual color (RC) percentage [23] was measured using the following equation:

$$RC\ (\%) = \frac{A_1}{A_0} * 100$$

Where, RC is the percentage of residual color, A_1 is the absorbance of reaction mixture (effluent in the presence of enzyme) and A_0 is the absorbance of reaction mixture in the absence of enzyme. Optimization (incubation time and different enzyme doses) of the deinking process was conducted.

2.10 Statistical Method

The significance of differences between each test variable (three replicates were done for each test) was determined using one way ANOVA analysis (Sigmaplot version 12.0).

3. RESULTS AND DISCUSSION

The present study demonstrated that an improved level of deinking was achieved by using cellulase produced from *Mucor hiemalis*. The substantial reduction in residual ink concentration clearly showed the effective role of cellulase in deinking of waste paper effluent. In the last decades, control of the water pollution has gained great importance. Disposition of ink effluents and dyes into water resources composes an important part of the pollution. Even a small amount of such effluents in these resources is not desired. Thus, because of ecological reasons, the process to remove ink from waste paper effluents gains utmost importance. Biological deinking by fungi was effected by different factors such as pH, shaking speed, temperature etc. Fungi are considered efficient degraders because of their ability to produce large variety of extracellular enzymes [24].

3.1 Isolates Identification and Screening of their Cellulases Productivity

The results revealed that four fungal isolates (F1, F2, F3 and F4) had variable potentials to produce cellulase where the most potent one was F2 (Fig. 1). It was followed in descending order by F1 and F4. On the other hand, F3 Isolate was the least cellulase producing isolate. According to the identification procedure of the most potent fungal isolate, it was found to be *Mucor hiemalis* (Fig. 2).

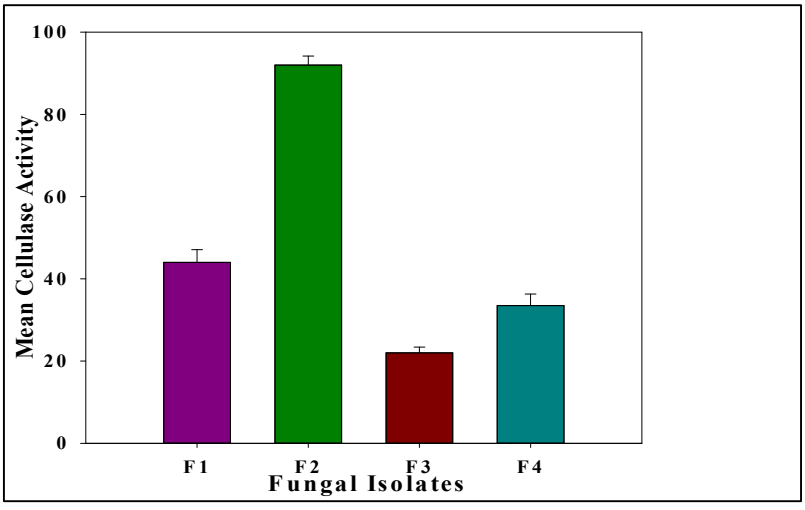

Fig. 1. Different fungal Isolates and their cellulase activities

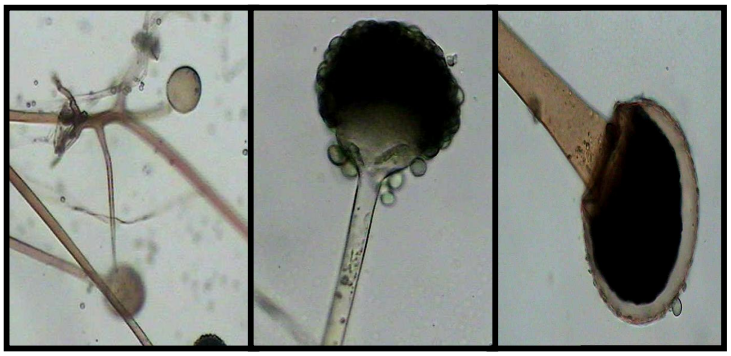

Fig. 2. Morphological identification of *Mucor hiemalis*

3.2 Optimization of Cellulase Productivity

For the optimization of cellulose produced by *Mucor hiemalis*, different parameters (pH, temperature, incubation time and shaking speed) were studied.

3.2.1 pH values

By determining the optimization of cellulase productivity, Different ranges of pH values (between 3 and 11) were studied. Acidic medium (pH 5) was the optimum environment for the production of the resulted enzyme. Cellulase activity was the highest (98 U/ml) at pH 5 (Fig. 3), however mild to moderate activities were observed bellow and above this value. The differences in the mean values among the pH values are statistically significant (P = <0.001). The present study indicated that acidic pH supported fungal activity to produce cellulase. Acidic medium may enhance the production of cellulase by *Mucor heimalis*.Effect of acidic medium on cellulase production by fungi supports the findings of Lynd et al. [25] who reported that Carboxy methyl cellulase activity exhibit a pH optimum of approximately 4, while the pH optimum of β-glucosidase was between pH 5-6.

3.2.2 Temperature

Based on increasing of cellulase activity, 30°C was considered to be the best temperature (Fig. 4) for the production of *Mucor hiemalis* cellulase. Increasing temperatures above 30°C had inhibiting effect on fungus productivity. The differences in the mean values among the treatment groups are not statistically significant (P = 0.341) this may exclude the possibility that the difference is due to random sampling variability. Similarly, *Mucor plumbeus* also was found to grow rapidly and produce cellulase at 25-30°C [26].

3.2.3 Incubation time

Results in Fig. 5 revealed that, there was a gradual increase in cellulase activity from day 2 to 5 with a maximum activity at day 5 followed by an obvious decrease in activity started after the fifth day of incubation (there was a significant difference where P< 0.001). This is in accordance with the cellulase produced by *Trichoderma* sp. where the maximum enzyme production was obtained on the 5th day [27].

3.2.4 Shaking speed

By increasing the shaking speed the activity of cellulase increased accordingly (there is no significant difference P = 0.094). However, the activity was slightly better at 200 rpm than 150 rpm (Fig. 6).

3.3 Purification of Cellulase Produced by *Mucor hiemalis*

Crude cellulase from *Mucor hiemalis* was chosen for conducting a stepwise purification strategy. By ammonium sulfate precipitation method, specific activity increased upon adding 80% ammonium sulfate and increased further after dialyzed. Recovery of 88% was obtained as shown in Table 1. It can be obvious also from the table that crude cellulase was purified 2.36 times from a specific activity of 39.16 to 92.38 U/mg. The table also shows that the activity of the purified cellulase was reduced by less than 40 % of the crude enzyme activity, i.e. from 954 to 618 U/ml. This resulted in a high purification yield of 64.78 %.

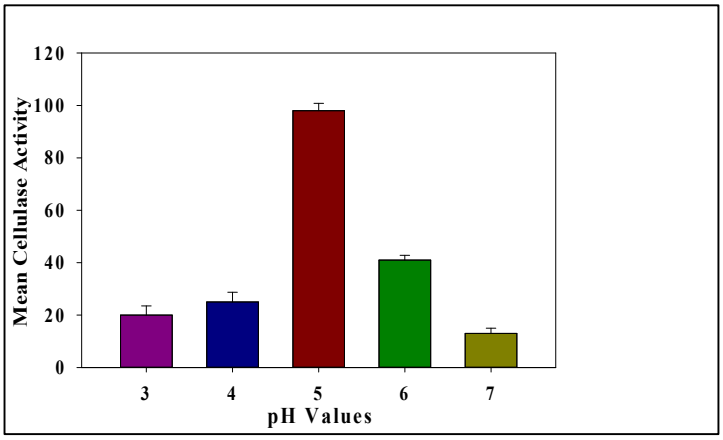

Fig. 3. The effect of different pH values on the productivity of cellulase produced by *Mucor hiemalis*

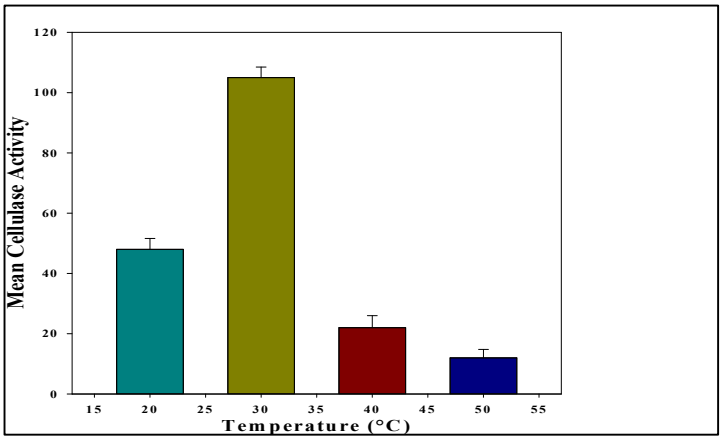

Fig. 4. The effect of different temperature degrees on the productivity of cellulase produced by *Mucor hiemalis*

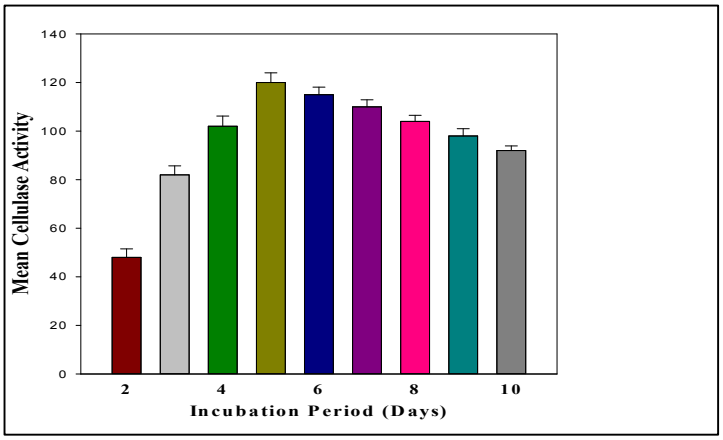

Fig. 5. The effect of different incubation times on the productivity of cellulase produced by *Mucor hiemalis*

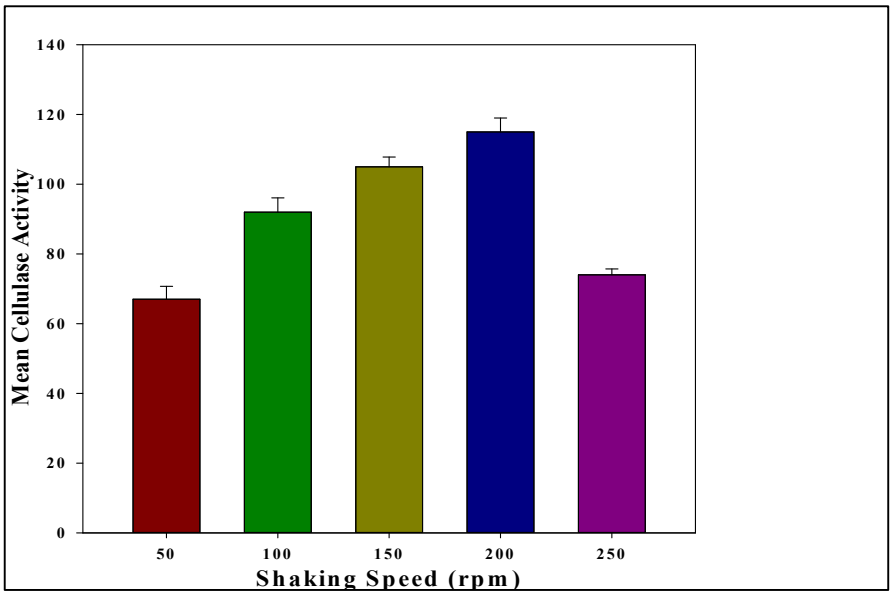

Fig. 6. The effect of different Shaking speed on the productivity of cellulase produced by *Mucor hiemalis*

Table 1. Purification steps of cellulase produced by *Mucor hiemalis*

Purification steps	Cellulase activity (U/ml)	Protein content (mg)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude cellulase	954	24.36	39.16	1.0	100
$(NH_4)_2SO_4$ precipitation	876	15.72	55.73	1.40	91.82
G-100 Sephadex	618	6.69	92.38	2.36	64.78

Result obtained using SDS-PAGE technique for the determination of cellulase molecular weight is shown in Fig. 7. The result revealed that, the molecular weight of purified cellulase was determined to be 71.6 KDa.

3.4 Cellulasede inkability of Waste Paper Effluent

Results recorded in Table 2 revealed that, the ink color removed gradually by increasing the time of reaction between cellulase and the effluent ($P < 0.001$). The maximum color removal was achieved at 9 hours of incubation where, the purified cellulase showed an obvious ability to deink the photocopier wastepaper effluent in an acidic medium (there is a significant difference $P < 0.001$). This result gives the hope for using this enzyme as an ideal source for the elimination of pollutants (i.e. agro-wastes and inks) from an environment. The obtained results are in accordance with the results of Prasad et al. [14] and Jeffries et al. [15]. They mentioned that cellulase facilitated ink detachment during deinking of laser printed paper using preparations containing mixed enzymes and mono-component activities.

By studying the effect of different doses of purified cellulase on the effluent resulted from photocopier wastepaper, the maximum deinkability ($P < 0.001$) was achieved at 400 µ/ml (Fig. 8). Enzymatic deinking methods represent a new approach to convert these recycled papers into quality products [28,29]. Toners are not only associated with cellulose fibers but also with white pigments, filters, and coating components, such as calcium carbonate. Under acidic conditions, the dissolution of the removed calcium carbonate coatings can be improved. It was observed that low pH also decreases the particle sizes of the toner, which subsequently helped the removal of the toner from the surfaces of the paper fibers [30].

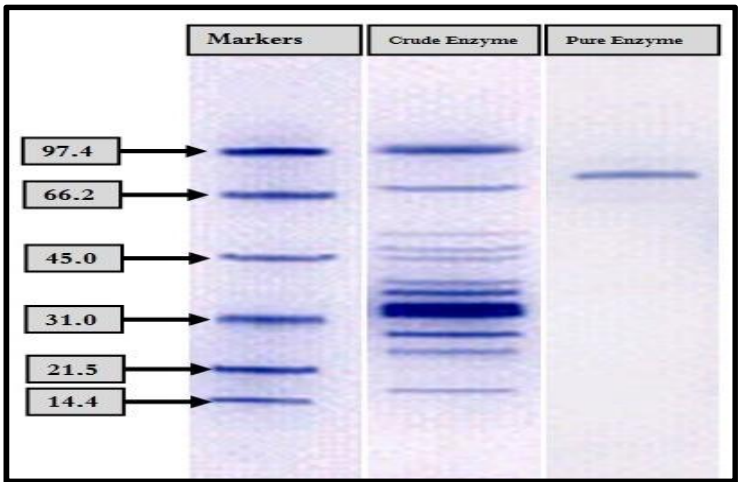

Fig. 7. Analysis of crude (obtained after precipitation by $(NH_4)_2SO_4$) and purified cellulase (obtained after G-100 sephadex) performed by using SDS-PAGE technique

Table 2. Effect of cellulase on the residual color of ink during different times

Time (hours)	RC (%)
0	95.2
1	79.0
2	76.7
3	59.7
4	19.4
5	11.3
6	3.23
7	1.60
8	0.65
9	0.02
10	0.02

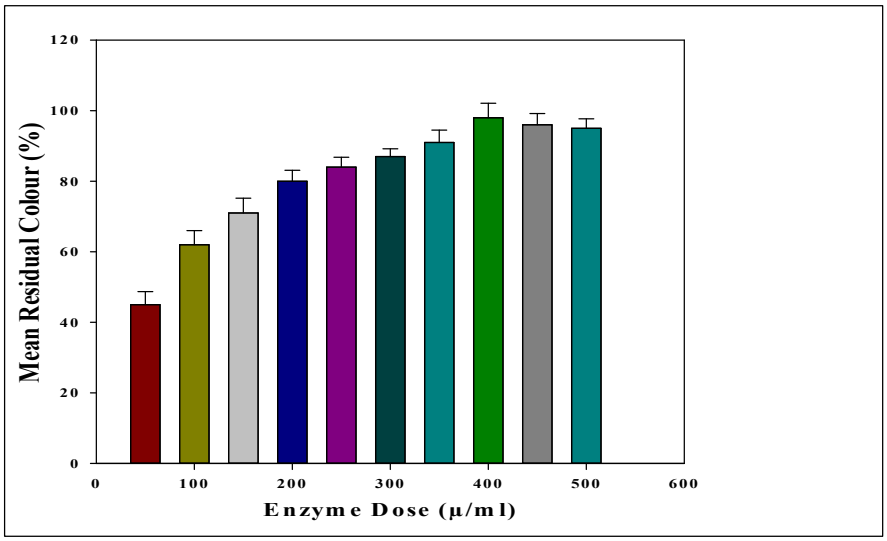

Fig. 8. Effect of different doses of cellulase on inkcolour

4. CONCLUSION

This study suggested that toxic pollutants like waste paper effluents (concentrated in industrial wastes and contaminated sites) can potentially be eliminated by low cost bioremediation systems using microbial cultures. The knowledge about the optimum environmental factors or conditions could help to employ biological approaches efficiently to clean up the water discharged from waste paper recycling industry. *Mucor hiemalis* cellulase effectively deinked wastepaper effluents. SSF technique has an important role in the using of solid agriculture wastes for the production of useful products (cellulase) that may be applied in several industries. Different parameters for maximum cellulase production have been optimized. Application of cellulase produced in the present study in the removing of ink color from photocopier waste paper showed an excellent advantage for industrial usage. The optimum removal of ink color was achieved at 9 hours (0.02 % of ink residual color) at room temperature. From the obtained results it is obviously that the application of cellulase is successful method to improve usage of photocopier waste papers in biorecycling process.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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