Immobilization Of Aspergillus Niger Cellulase in Calcium Alginate Beads for Enhanced Catalytic Stability and Industrial Applications

Jain Chetan M.[1], Dabhade Kalpana[2], Nerurkar Madhura[3]
[1,2]*Department of Life Sciences and Biotechnology, Chhatrapati Shivaji Maharaj University, Panvel 410206, Navi Mumbai, India*
[3]*Calantha Biotech Pvt. Ltd., Mumbai, India*

***Abstract*—Cellulases are among the most important industrial enzymes owing to their extensive applications in biomass conversion, paper and pulp processing, textile finishing, food technology, biofuel production and environmental biotechnology. However, the industrial utilization of free cellulase is frequently limited by poor operational stability, susceptibility to thermal and pH-induced denaturation, and the inability to recover and reuse the enzyme efficiently, resulting in increased process costs. The present study investigated the production of cellulase from Aspergillus niger using wheat bran as a substrate under solid-state fermentation (SSF) and evaluated the effect of calcium alginate entrapment on enzyme performance. Cellulolytic fungal isolates were initially screened on carboxymethyl cellulose (CMC) agar medium, and the most efficient isolate was selected for enzyme production. The crude enzyme was extracted, partially purified and immobilized by entrapment in calcium alginate beads. The catalytic properties of free and immobilized cellulase were comparatively evaluated under varying pH and temperature conditions. The free cellulase exhibited an activity of 125.66 U mL^{-1}, whereas the immobilized enzyme showed a higher activity of 143.43 U mL^{-1}, indicating enhanced catalytic efficiency following immobilization. Both enzyme preparations exhibited optimum activity at pH 5.0 and 50°C. However, the immobilized enzyme retained greater catalytic activity over a broader pH range and demonstrated superior thermal stability compared with the free enzyme. Furthermore, the immobilized cellulase effectively facilitated paper de-inking, highlighting its potential application in environmentally sustainable paper recycling processes. The findings demonstrate that calcium alginate entrapment significantly improves cellulase stability, catalytic performance and operational robustness without altering its optimum reaction conditions. The enhanced stability and reusability of immobilized cellulase suggest that calcium alginate is an efficient, economical and environmentally friendly immobilization matrix for developing reusable biocatalysts suitable for industrial biotechnology, biomass conversion and paper recycling applications.**

***Index Terms*—Aspergillus niger; Calcium alginate; Cellulase; Enzyme immobilization; Paper de-inking; Solid-state fermentation; Thermal stability.**

I. INTRODUCTION

Cellulase are a group of hydrolytic enzymes that catalyse the degradation of cellulose into soluble oligosaccharides and glucose. Owing to their ability to convert lignocellulosic biomass into value-added products, cellulases have gained significant industrial importance and are extensively employed in biofuel production, food processing, textile manufacturing, paper and pulp industries, pharmaceuticals and environmental biotechnology [6],[8]. Despite their high catalytic efficiency, the industrial application of free cellulase is often limited by poor operational stability, susceptibility to denaturation and lack of reusability, resulting in increased process costs [4].
Enzyme immobilization has emerged as an effective strategy to overcome these limitations by enhancing enzyme stability, facilitating recovery and enabling repeated use in industrial processes [2-4]. Several immobilization techniques, including adsorption, covalent binding and entrapment, have been developed for enzyme stabilization. Among these methods, entrapment within calcium alginate beads has attracted considerable attention due to its

simplicity, biocompatibility, low cost and non-toxic nature [11],[12]. In this approach, enzyme molecules are physically confined within a porous polymeric matrix that permits the diffusion of substrates and products while retaining the enzyme, thereby preserving its catalytic activity and improving its resistance to environmental stress.

Cellulase are produced by a wide range of microorganisms, including bacteria and fungi. Several bacterial genera such as Bacillus, Cellulomonas and Clostridium are known producers of cellulolytic enzymes. However, filamentous fungi are generally preferred for industrial cellulase production because of their ability to secrete large quantities of extracellular enzymes. Among them, Aspergillus niger is one of the most extensively studied and commercially exploited fungal species due to its high enzyme productivity, ease of cultivation and adaptability to diverse substrates. [7] This ubiquitous filamentous fungus is widely distributed in soil, decaying organic matter and various natural and artificial habitats, making it an important organism in industrial biotechnology and applied mycology [10].

Solid-state fermentation (SSF) has been recognized as an efficient and economical method for fungal enzyme production, particularly when agro-industrial residues are used as substrates. Wheat bran is commonly employed as a substrate for SSF because of its rich nutrient content, low cost and widespread availability. The utilization of such agricultural by-products not only reduces production costs but also contributes to sustainable waste management practices.

Immobilized enzymes have become increasingly important in industrial biotechnology because they improve process efficiency, reduce energy consumption and support environmentally sustainable manufacturing. In addition to conventional industrial applications, immobilized cellulases have shown potential in biomass conversion, paper recycling and environmental biotechnology [1-3]. Recent investigations have demonstrated that calcium alginate entrapment significantly improves enzyme stability, catalytic efficiency and operational reusability by providing a protective microenvironment around enzyme molecules [5-9]. Considering the growing demand for stable, reusable and cost-effective biocatalysts, the present study aimed to produce cellulase from Aspergillus niger using wheat bran under solid-state fermentation and to evaluate the effect of calcium alginate entrapment on enzyme performance. The study involved screening cellulase-producing fungal isolates, optimizing enzyme production, extracting and partially purifying cellulase and immobilizing the enzyme within calcium alginate beads. The catalytic properties of free and immobilized cellulase were subsequently assessed under different pH and temperature conditions, and their potential industrial application was investigated through paper de-inking studies. The findings are expected to contribute to the development of robust cellulase-based biocatalytic systems for industrial and environmental biotechnology applications.

II MATERIALS AND METHODS

2.1 Screening of Cellulase-Producing Fungi

Aspergillus niger was screened for cellulase production using carboxymethyl cellulose (CMC) agar medium. The fungal isolate was aseptically inoculated onto sterile CMC agar plates and incubated under appropriate growth conditions. Following incubation, the plates were stained to visualize cellulose hydrolysis. The formation of a distinct clear zone surrounding the fungal colony confirmed extracellular cellulase production and cellulolytic activity. The isolate exhibiting the highest cellulolytic potential was selected for subsequent enzyme production studies.

2.2 Production of Cellulase by Solid-State Fermentation

Cellulase production was carried out by solid-state fermentation (SSF) using wheat bran as the fermentation substrate. Wheat bran was selected because of its high nutritional value, low cost and widespread availability as an agro-industrial by-product. The substrate was moistened with a suitable nutrient solution, sterilized and inoculated with the selected Aspergillus niger culture under aseptic conditions. Fermentation was conducted under controlled environmental conditions for the required incubation period to maximize cellulase production. At the end of fermentation, the fermented substrate was processed for enzyme extraction.

2.3 Extraction and Partial Purification of Cellulase

The crude cellulase enzyme was extracted from the fermented substrate using an appropriate extraction buffer followed by filtration to remove solid residues. The filtrate was subsequently centrifuged to eliminate suspended particles and obtain a clear enzyme extract. Partial purification of cellulase was carried out by ammonium sulphate precipitation. The precipitated protein fraction was recovered by centrifugation, dissolved in an appropriate buffer and used for immobilization and further characterization studies.

2.4 Determination of Cellulase Activity

Cellulase activity was determined using the carboxymethyl cellulose (CMC)-3,5-dinitrosalicylic acid (DNS) assay following the method described by Wood and Bhat (1988). The reaction mixture containing enzyme solution and CMC substrate was incubated under standard assay conditions. The reaction was terminated by the addition of DNS reagent followed by boiling for colour development. After cooling to room temperature, absorbance was measured at 540 nm using a UV–Visible spectrophotometer. Cellulase activity was calculated using a glucose standard curve. One unit (U) of cellulase activity was defined as the amount of enzyme required to release 1 µmol of reducing sugar per minute under the assay conditions.

2.5 Immobilization of Cellulase in Calcium Alginate Beads

Cellulase immobilization was carried out by entrapment in calcium alginate beads. A predetermined volume of partially purified cellulase solution was thoroughly mixed with sodium alginate solution to obtain a homogeneous mixture. The enzyme–alginate mixture was added dropwise into calcium chloride solution under gentle stirring to facilitate bead formation through ionic gelation. The resulting calcium alginate beads were allowed to harden completely, washed repeatedly with distilled water to remove loosely bound enzyme molecules and stored under refrigerated conditions until further use. Calcium alginate provided a protective polymeric matrix that enhanced enzyme stability while maintaining catalytic activity.

2.6 Characterization of Free and Immobilized Cellulase

The catalytic properties of free and immobilized cellulase were comparatively evaluated under different pH and temperature conditions. Enzyme activity was measured over a pH range of 3.0–11.0 using appropriate buffer systems. Thermal stability was evaluated by determining enzyme activity at temperatures ranging from 30°C to 70°C. Relative enzyme activity was calculated by considering the maximum observed activity as 100%, and the performances of free and immobilized cellulase were compared to evaluate the influence of calcium alginate entrapment on enzyme stability and catalytic efficiency.

2.7 Application of Immobilized Cellulase in Paper De-inking

The industrial applicability of immobilized cellulase was investigated through enzymatic paper de-inking. Printed paper samples were treated with immobilized cellulase under optimized experimental conditions. The efficiency of ink removal was assessed qualitatively by comparing enzyme-treated samples with untreated controls. Improvement in paper appearance following enzymatic treatment was used to evaluate the effectiveness of immobilized cellulase as an environmentally friendly alternative to conventional chemical de-inking processes.

III. RESULTS AND DISCUSSION

Screening and Production of Cellulase

Screening of Aspergillus niger on carboxymethyl cellulose (CMC) agar medium confirmed its cellulolytic potential through the formation of distinct clear hydrolysis zones around the fungal colonies, indicating cellulose degradation by extracellular cellulase enzymes (Figure 1; Table 1). The observed hydrolysis zone demonstrated the ability of the isolate to efficiently utilize cellulose as a carbon source and justified its selection for further enzyme production studies. Wheat bran proved to be an effective substrate for cellulase production under solid-state fermentation conditions (Figure 2). The abundance of cellulose, hemicellulose, proteins, and other nutrients in wheat bran likely supported fungal growth and enzyme synthesis, making it a suitable low-cost substrate for industrial enzyme production.

Effect of Immobilization on Cellulase Activity

The cellulase produced by Aspergillus niger was extracted, partially purified, and immobilized through entrapment in calcium alginate beads. The activity of free cellulase was recorded as 125.66 U mL^{-1}, whereas the immobilized enzyme exhibited a higher activity of 143.43 U mL^{-1} (Table 2). (Figure 3 and 4) The increase in enzyme activity following immobilization suggests that calcium alginate provided a favourable microenvironment that preserved the native conformation of the enzyme and minimized structural alterations during catalytic reactions. (Figure 5) The porous nature of calcium alginate beads may have facilitated substrate diffusion while simultaneously protecting the enzyme from inactivation. Similar improvements in cellulase activity following alginate entrapment have been reported in previous studies, where immobilization enhanced catalytic efficiency and operational stability.

Effect of pH on Enzyme Activity

The influence of pH on cellulase activity was investigated over a pH range of 3–11. Both free and immobilized cellulase exhibited maximum activity at pH 5, indicating that the enzyme is optimally active under acidic conditions (Figures 6 and 7; Table 3). However, significant differences were observed at higher pH values. While the activity of free cellulase declined sharply beyond the optimum pH, the immobilized enzyme retained a considerably higher proportion of its activity at pH 7 and pH 9. This enhanced pH tolerance may be attributed to the protective effect of the calcium alginate matrix, which creates a stable microenvironment around the enzyme molecules and reduces conformational changes induced by extreme pH conditions. The improved pH stability of immobilized cellulase is advantageous for industrial applications, where fluctuations in pH are common and can negatively affect enzyme performance.

Effect of Temperature on Enzyme Activity

The effect of temperature on enzyme activity was evaluated within the range of 30–70°C. Both free and immobilized cellulase demonstrated maximum activity at 50°C, indicating that immobilization did not alter the optimum temperature of the enzyme (Table 4). At temperatures above 50°C, a gradual decline in enzyme activity was observed for both preparations. However, immobilized cellulase retained significantly higher activity than the free enzyme at elevated temperatures (Figures 8 and 9). The enhanced thermal stability can be attributed to the physical confinement of enzyme molecules within the calcium alginate matrix, which restricts structural unfolding and protects the enzyme against heat-induced denaturation. Thermal stability is a critical parameter in industrial bioprocesses, as higher operating temperatures often improve reaction rates and reduce the risk of microbial contamination. Therefore, the superior thermal tolerance exhibited by immobilized cellulase enhances its practical utility in large-scale applications.

Application of Immobilized Cellulase in Paper De-Inking

The applicability of immobilized cellulase was further assessed through paper de-inking studies. Treatment of printed paper samples with immobilized cellulase resulted in noticeable ink removal and improved paper appearance when compared with untreated controls. The enzymatic degradation of cellulose fibres at the ink–paper interface facilitated the release of ink particles, thereby enhancing the de-inking process. The successful application of immobilized cellulase in paper recycling demonstrates its potential as an environmentally friendly alternative to conventional chemical de-inking methods. (Figure 10). The use of enzyme-based de-inking not only reduces chemical consumption but also minimizes environmental pollution associated with paper recycling industries.

The present study demonstrated that immobilization of cellulase in calcium alginate beads significantly improved enzyme performance without adversely affecting catalytic activity. Enhanced enzyme activity, broader pH tolerance, improved thermal stability, and successful application in paper de-inking collectively indicate that calcium alginate entrapment is an effective strategy for developing robust and reusable cellulase biocatalysts. Immobilized cellulase produced from Aspergillus niger represents a promising candidate for industrial applications in biomass processing, paper recycling, textile treatment, and other biotechnological processes requiring stable and efficient cellulolytic activity.

IV. CONCLUSION

The present study successfully demonstrated the production of cellulase from Aspergillus niger using wheat bran as a substrate under solid-state fermentation and its subsequent immobilization through entrapment in calcium alginate beads. The immobilized cellulase exhibited enhanced catalytic activity compared to the free enzyme, indicating that calcium alginate provided a favourable microenvironment for enzyme stabilization and functionality. Furthermore, immobilization significantly improved the enzyme's tolerance to variations in pH and temperature, resulting in greater operational stability under conditions relevant to industrial processes. The immobilized enzyme retained higher activity over a broader pH range and demonstrated superior thermal stability, highlighting its suitability for applications requiring prolonged enzyme performance. The successful utilization of immobilized cellulase in paper de-inking studies further confirmed its industrial applicability and potential as an environmentally sustainable alternative to conventional chemical treatments. Overall, the findings suggest that calcium alginate entrapment is an effective and economical approach for enhancing cellulase performance, thereby increasing its potential for application in paper recycling, biomass conversion, textile processing, and other biotechnological industries. Future studies focusing on enzyme reusability, storage stability, and large-scale process optimization may further facilitate the commercial exploitation of immobilized cellulase systems.

ACKNOWLEDGMENTS

The authors express their sincere gratitude to the Department of Life Sciences and Biotechnology, Chhatrapati Shivaji Maharaj University, Panvel, Navi Mumbai, India, for providing laboratory facilities and continuous academic support throughout this research. The authors also acknowledge Calantha Biotech Pvt. Ltd., Mumbai, for providing technical guidance and support during the experimental work.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest regarding the publication of this manuscript.

REFERENCES

[1] A. Basso and S. Serban, “Industrial applications of immobilized enzymes—A review,” *Molecular Catalysis*, vol. 479, Art. no. 110607, 2019.

[2] B. M. Brena and F. Batista-Viera, “Immobilization of enzymes: A literature survey,” in *Immobilization of Enzymes and Cells*, J. M. Guisan, Ed. Totowa, NJ, USA: Humana Press, 2006, pp. 15–30.

[3] J. Chapman, A. E. Ismail, and C. Z. Dinu, “Industrial applications of enzymes: Recent advances, techniques and outlooks,” *Catalysts*, vol. 8, Art. no. 238, 2018.

[4] S. Datta, L. R. Christena, and Y. R. S. Rajaram, “Enzyme immobilization: An overview on techniques and support materials,” *3 Biotech*, vol. 3, pp. 1–9, 2013.

[5] R. Guo, X. Zheng, Y. Wang, Y. Yang, Y. Ma, D. Zou, and Y. Liu, “Optimization of cellulase immobilization with sodium alginate-polyethylene for enhancement of enzymatic hydrolysis of microcrystalline cellulose using response surface methodology,” *Applied Biochemistry and Biotechnology*, vol. 193, pp. 2043–2060, 2021.

[6] G. Immanuel, R. Dhanusha, P. Prema, and A. Palavesam, “Effect of different growth parameters on endoglucanase enzyme activity by bacteria isolated from coir retting effluents of estuarine environment,” *International Journal of Environmental Science and Technology*, vol. 3, pp. 25–34, 2006.

[7] E. S. Kassim, “Cellulase enzyme from *Aspergillus niger*,” *Microbiology and Immunology*, vol. 26, pp. 449–454, 1982.

[8] S. Mohammadi, H. Tarrahimofrad, S. Arjmand, J. Zamani, K. Haghbeen, and S. Aminzadeh, “Expression, characterization and activity optimization of a novel cellulase from the thermophilic bacterium *Cohnella* sp. A01,” *Scientific Reports*, vol. 12, Art. no. 10301, 2022.

[9] K. N. Rajnish, M. S. Samuel, A. J. John, S. Datta, N. Chandrasekar, R. Balaji, and E. Selvarajan, “Immobilization of cellulase enzymes on nano and micro-materials for breakdown of cellulose for biofuel production—A narrative review,” *International Journal of*

Biological Macromolecules, vol. 182, pp. 1793–1802, 2021.

[10] R. A. Samson, C. M. Visagie, J. Houbraken, S. B. Hong, V. Hubka, C. H. W. Klaassen, G. Perrone, K. A. Seifert, A. Susca, J. B. Tanney, and J. Varga, "Phylogeny, identification and nomenclature of the genus *Aspergillus*," *Studies in Mycology*, vol. 78, pp. 141–173, 2014.

[11] S. Talekar and S. Chavare, "Optimization of immobilization of α-amylase in alginate gel and its comparative biochemical studies with free α-amylase," *Recent Research in Science and Technology*, vol. 4, pp. 1–5, 2012.

[12] T. Q. Viet, N. P. Minh, and D. T. Dao, "Immobilization of cellulase enzyme in calcium alginate gel and its immobilized stability," *American Journal of Research Communication*, vol. 1, pp. 254–267, 2013.

[13] T. M. Wood and K. M. Bhat, "Methods for measuring cellulase activities," in *Methods in Enzymology*, vol. 160, W. A. Wood and S. T. Kellogg, Eds. New York, NY, USA: Academic Press, 1988, pp. 87–112.

Table 1. Composition of CMC Agar Medium.

Components	Quantity
Carboxymethylcellulose (CMC)	0.5 g
K2HPO4	0.1 g
MgSO4	0.05 g
KCl	0.1 g
Yeast Extract	0.05 g
NaCl	0.1 g
Glucose	0.1 g
Agar Agar	1.8 g
D/W	50 ml

Table 2. Production parameters and preliminary cellulase activity.

Parameters	RESULTS
Microorganism Used	Aspergillus niger
Substrate used	Wheat bran
pH	5
Incubation Time	72-96 hrs
Extraction	Using Distilled water followed by Centrifugation at 4000 rpm
Activity Estimation	Spectrophotometer at 540 nm using Filter Paper Unit Assay
Free Cellulase Activity	125.66 U mL^{-1}
Immobilized Cellulase Activity	143.43 U mL^{-1}

Table 3. Effect of pH on free Cellulase and Immobilized cellulase Activity

pH Values	Free Cellulase	Immobilized Cellulase activity
3	102.64	108.6
5	125.37	143.43
7	112.29	133.51
9	101.43	132.56
11	98.56	99.49

Table 4. Effect of Temperature on free Cellulase and Immobilized Cellulase Activity

Sr. no	Temperature	Free cellulase Activity	Immobilized cellulase activity
1	30°C	96.74	108.65
2	40°C	117.53	115.86
3	50°C	125.69	125.89
4	60°C	106.33	111.46
5	70°C	98.16	98.72

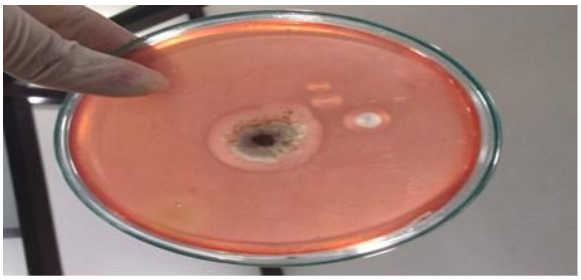

Figure 1. Screening of Aspergillus niger on carboxymethyl cellulose (CMC) agar showing cellulolytic activity.

(a) (b)

Figure 2. Wheat bran substrate during solid-state fermentation: (a) before fungal growth and (b) after growth of Aspergillus niger.

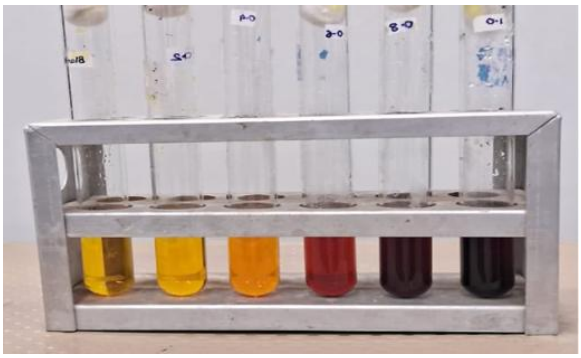

Figure 3. Standard glucose calibration curve used for cellulase activity assay.

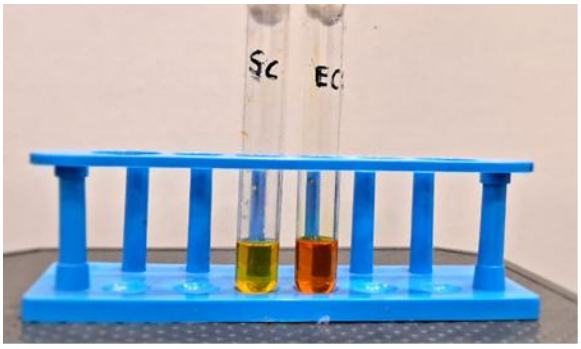

Figure 4. Cellulase activity assay showing enzyme activity of free cellulase.

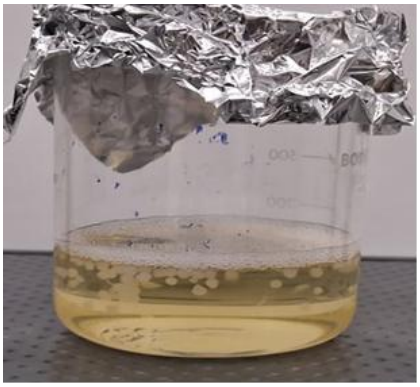

Figure 5. Calcium alginate-immobilized cellulase beads.

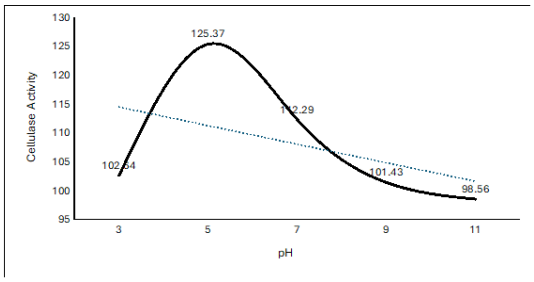

Figure 6. Effect of pH on free cellulase activity

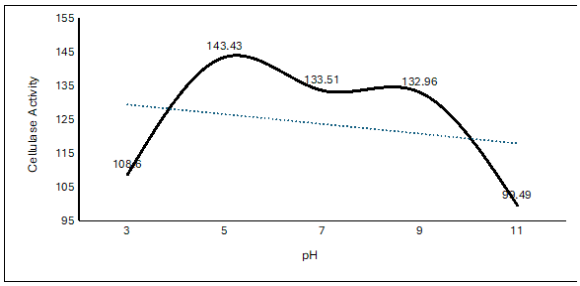

Figure 7. Effect of pH on Immobilized cellulase activity

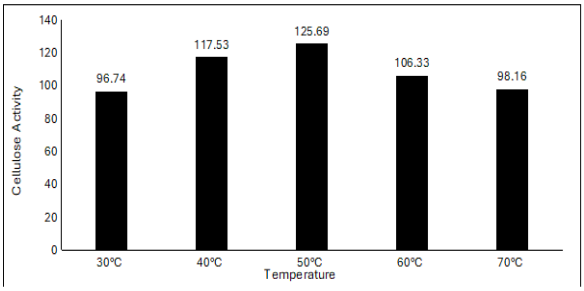

Figure 8. Effect of temperature on free cellulase activity

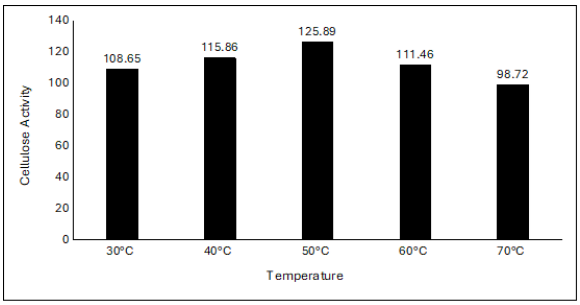

Figure 9. Effect of temperature on immobilized cellulase activity

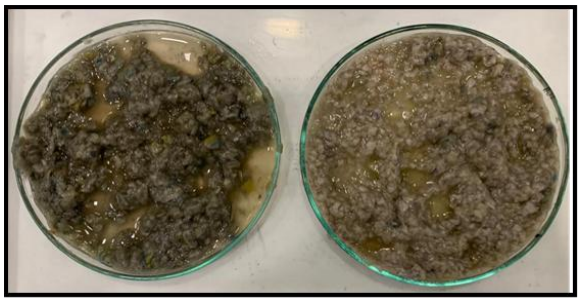

Figure 10. Enzymatic de-inking of newspaper using calcium alginate-immobilized cellulase.