Supporting Information

Cellulase Immobilization on to Magnetic Halloysite Nanotubes: Enhanced Enzyme Activity and Stability with High Cellulose Saccharification

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Materials Required

Halloysite nanotubes (HNTs), ferric chloride hexahydrate ($FeCl_3.6H_2O$), ferrous chloride tetrahydrate ($FeCl_2.4H_2O$), 1-butyl-3-methylimidazolium chloride ([bmim][Cl] ≥98.0% purity), (3-aminopropyl)triethoxysilane (APTES), sodium carboxymethyl cellulose (CMC, Mol. wt. 90,000) and glucose assay kit (GAGO20) were purchased from Sigma Aldrich, USA. Bovine serum albumin (BSA), and glutaraldehyde were obtained from Hi Media Laboratories Pvt. Ltd. (Mumbai, India). Other chemicals, Coomassie Brilliant Blue G-250, 3, 5-dinitrosalicylic acid (DNS), potassium sodium tartrate and ammonia solution ($NH_3.H_2O$) were procured from Loba-Chemie Pvt. Ltd, India. All reagents were of analytical grade and used without any additional purification. All aqueous preparations were carried out in deionized water.

Free cellulase (dried powder) from *Aspergillus niger* (Sigma Aldrich, USA, code: 22178) was procured and used without any further purification. As per the manufacturer's specifications, free cellulase is a complex consortium of three enzymes endo-glucanase, cellobiohydrolase, and β-glucosidase. Through a multi-step hydrolytic reaction, endoglucanases and cellobiohydrolases synergistically initiate the hydrolysis of cellulose chains into short intermediates i.e., short cellulooligosaccharides and cellobiose, which eventually get transformed into glucose units by the action of β-glucosidase enzyme.

Characterization

The structural morphology of MHNTs was studied using field emission-scanning electron microscopy (FE-SEM, Hitachi SU8010, Japan) and high resolution transmission electron microscopy (HR-TEM, Jeol JEM-2100, Japan). The sample crystallinity was evaluated using XRD analysis (X-ray diffractometer, PANalytical X-pert Pro, The Netherlands) with Cu Kα radiation (λ = 1.5406 Å) over a scanning range (2θ) from 10° to 70°. The magnetic properties of

the samples were analyzed by a magnetometer (7407 Lake Shore Cryotronics Inc., USA) at room temperature with an applied field between −10,000 and 10,000 Oe. The interaction between functional groups over MHNTs before and after enzyme immobilization was determined using FT-IR spectroscopy (Cary 630 FTIR, Agilent Technologies, USA). X-ray photoelectron spectroscopy (XPS, ESCALAB, Thermo Fischer Scientific, USA) was done to analyze the elemental surface composition of the nanobiocatalyst. Thermal degradation properties of nanobiocatalyst were assessed using thermogravimetric analysis (TGA, EXSTAR TG/DTA 6300, Seiko Instruments Inc., Japan) by placing the samples (~10 mg) in a temperature resistant crucible and heated from 25-800°C at a heating rate of 10°C min^{-1} under nitrogen atmosphere. For determining % enzyme loading and other enzyme assays, UV-Vis spectrophotometer (UV-2600, Shimadzu Corp., Japan) was used.

Statistical Optimization using RSM

Various process parameters *i.e.*, catalyst load, solution pH, crosslinker conc., enzyme conc. and immobilization time were studied separately so as to get an idea of parameters and their range having the most significance effect on enzyme loading. The factor levels were coded as -1 (low), 0 (central point or middle), and 1 (high).The factors and their levels studied for immobilization of cellulase onto magnetic matrix are given in Table S1.

Second-order polynomials were used to represent the experimental data to obtain the best-fit regression equations. The cubic model was found to be aliased and could not be used for modelling of the experimental data. A second order polynomial equation was used to express enzyme loading (%) as a function of studied variables:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad \text{(S1)}$$

Where Y= predicted response variable, X_i and X_j = independent variables which affect Y. The regression coefficient was defined as β_0 for the intercept, β_i for linear, β_{ii} for quadratic and β_{ij} for the cross product terms.

Optimization process was carried out considering enzyme loading (%) as the 'response' using RSM (Figure S1). The impact of loaded enzyme on corresponding activity was studied separately for better understanding of the complex interaction of densely packed enzyme and its activity. Analysis of Variance (ANOVA) was employed to examine model adequacy for responses in experimentation (Table S2). This is a statistical diagnostic tool to estimate the performance of tested experiments, by taking into account p-value, lack of fit, multiple correlation coefficients (R^2) and adjusting coefficient of determination (R^2-adj). In this case, a set of experimental plan was generated based on three levels for each factor. The model was then validated by conducting experiments at the given optimal conditions. The predicted R^2 (0.7229) and adjusting coefficient of determination (R^2-adj=0.9199) for calculated optimal conditions were in good harmony with the value of multiple correlation coefficients (R^2=0.9649), indicating that the model can predict 96.49% of the values (Table S3).

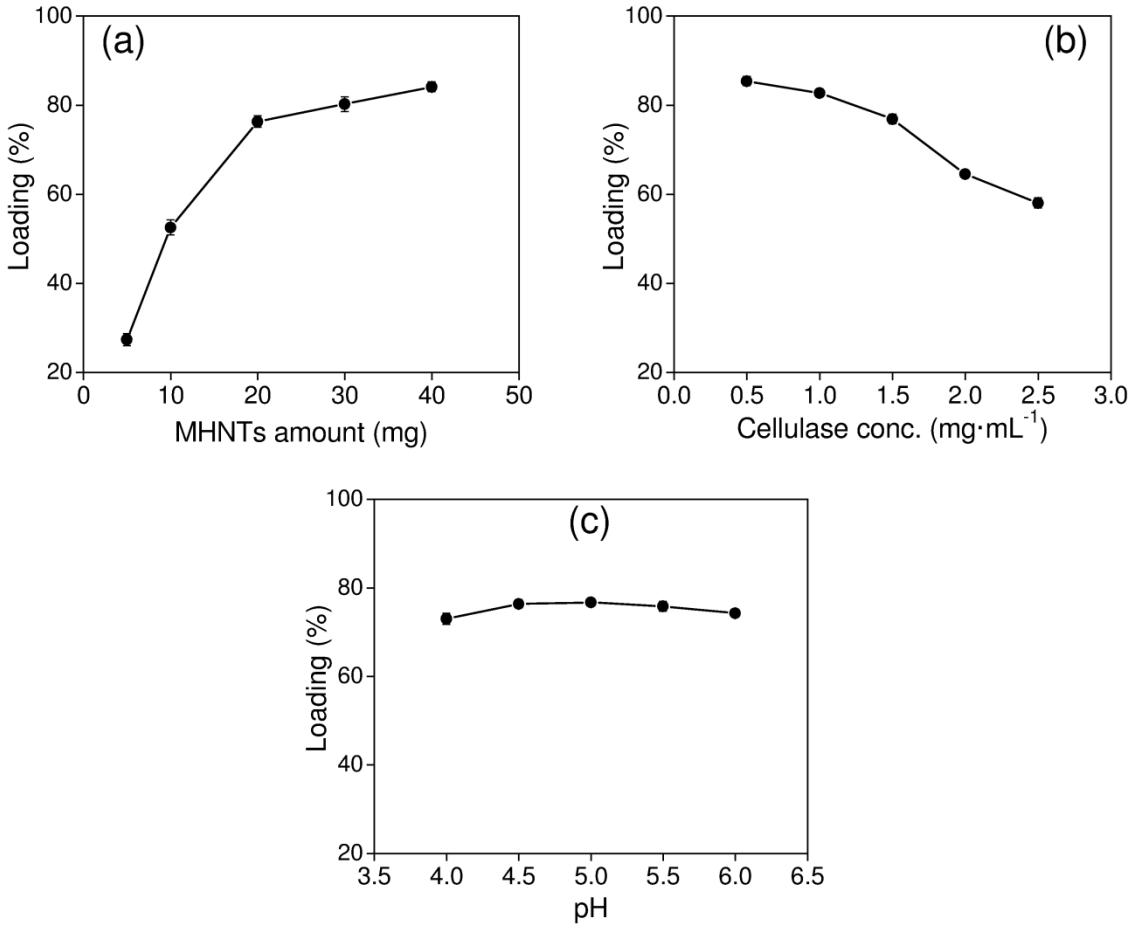

Figure S1. Single factor studies for optimizing various process parameters so as to achieve maximum % loading of cellulase on to MHNTs. The impact of (a) MHNTs matrix amount, (b) concentration of cellulase and (c) the solution pH was evaluated.

A set of experiments were conducted to verify whether a high enzyme loading (%) would cause a similar increase in its catalytic activity as well. We observed that a significant reduction in catalytic activity of the immobilized cellulase was manifested when the amount of MHNTs was increased beyond 20 mg (Figure S2). This reduction in catalytic activity might be attributed to steric hindrance posed by the immobilized enzymes due to high payload. Though such decline in catalytic activity of enzyme (enzyme units·g^{-1} MHNTs) beyond 20 mg HNTs is marginal, this can be compensated by prolonging the duration of biocatalytic reaction up to 48 h, which would result in better substrate conversion. Since it is desirable to choose a minimum amount of nano-biocatalytic system which could perform better for cellulose hydrolysis than its corresponding higher enzyme loading, a 20 mg MHNTs matrix was chosen for further optimization experiments.

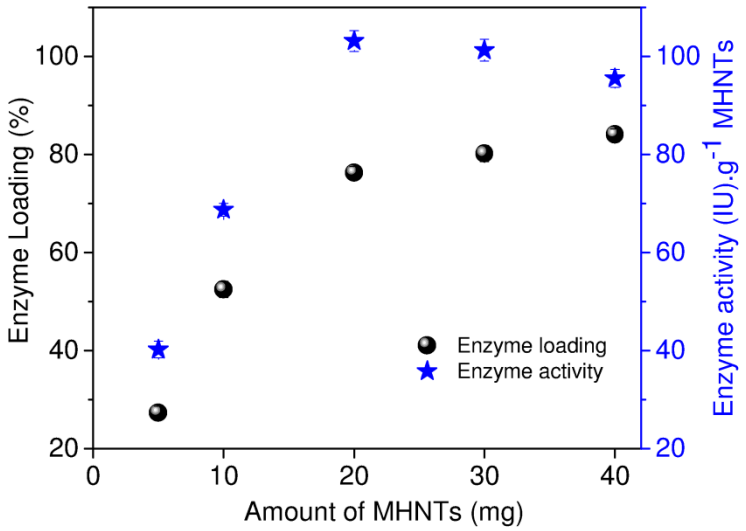

Figure S2. A correlation between enzyme loading (%) and enzyme activity (enzyme IU·g^{-1} MHNTs) on varying the amount of MHNTs matrix.

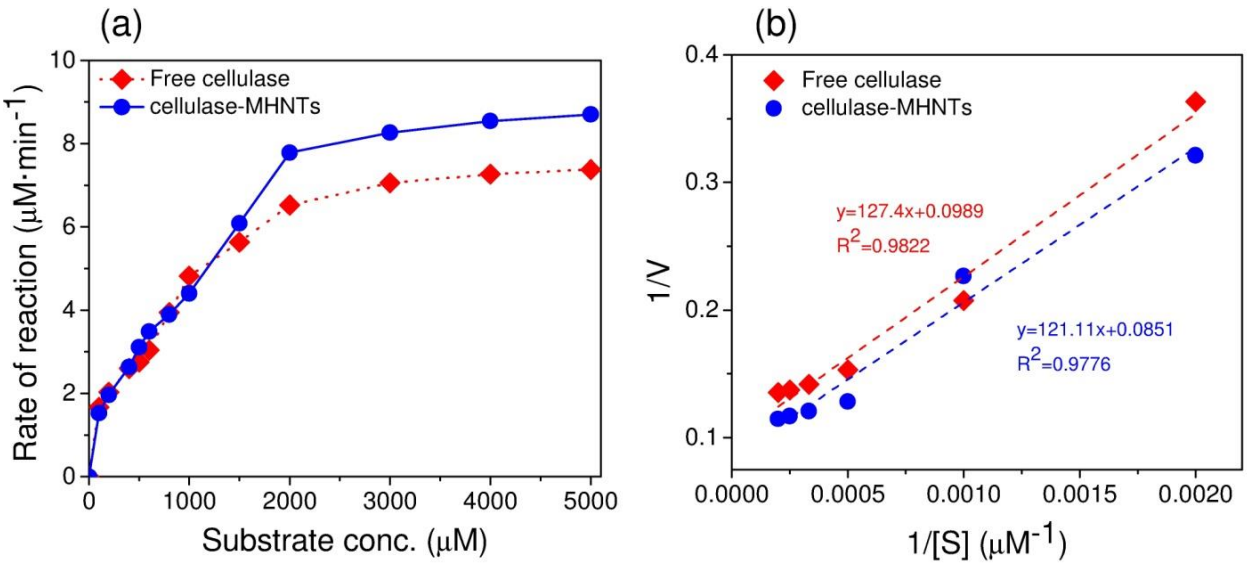

Figure S3. (a) Biocatalytic reaction kinetics of cellulase enzymes (both, free and immobilized) against broad substrate concentration (CMC, 100-5000 μM). (b) A graphical representation of double reciprocal (Lineweaver-Burk) plot to evaluate the kinetics parameters, k_M and V_{max} of free and immobilized cellulase enzymes.

Table S1. Levels and ranges of variables in Box–Behnken statistical experiment design

Independent variable	Symbol	Coded variable level		
		Low -1	Centre 0	High +1
MHNTs amount (mg)	A	20	25	30
Cellulase conc. (mg·mL^{-1})	B	1.25	1.5	1.75
Solution pH	C	4.5	5.0	5.5

Table S2. ANOVA for response surface quadratic model

Source	Sum of Square	DF	Mean Square	F Value	Prob>F	Remarks
Model	324.61	9	36.07	21.41	0.0003	significant
A	64.07	1	64.07	38.03	0.0005	
B	32.81	1	32.81	19.47	0.0031	
C	0.67	1	0.67	0.40	0.5475	
A^2	32.29	1	32.29	19.17	0.0032	
B^2	113.67	1	113.67	67.48	< 0.0001	
C^2	60.82	1	60.82	36.11	0.0005	
AB	3.19	1	3.19	1.89	0.2114	
AC	13.00	1	13.00	7.71	0.0274	
BC	5.31	1	5.31	3.15	0.1190	
Residual	11.79	7	1.68			
Lack of Fit	5.18	3	1.73	1.04	0.4640	not significant
Pure Error	6.61	4	1.65			
Cor Total	336.40	16				

*A: MHNTs amount (mg); B: cellulase conc. (mg·mL^{-1}); C: solution pH

Table S3. Coefficients of determination for response surface quadratic model

Std. Dev.	1.30	R^2	0.9649
Mean	75.21	Adj.R^2	0.9199
C.V.	1.73	Pred.R^2	0.7229
PRESS	93.20	Adeq. Precision	15.847

Table S4. Comparative details of cellulase immobilization on to nanomaterials and their features

Immobilization matrix	Substrate used	Saccharification*	Glucose yield* ($mg \cdot g^{-1}$ substrate)	Duration	Ref.
Poly(methacrylamide-co-acrylic acid)	microcrystalline cellulose	27.6 %	310	32 h	[1]
magnetic nanoparticles	Pre-treated Napier grass	37.4 %	420	24 h	[2]
magnetic-crosslinked enzyme aggregates	Pre-treated bamboo	18.7 %	210	24 h	[3]
Attapulgite/chitosan nanocomposite	Pre-treated wheat straw	6.8 %	76.6	24 h	[4]
Magnetic Poly(ionic liquid) Support	Carboxymethyl cellulose	31.1%	350	12h	[5]
magnetic halloysite nanotubes	Pre-treated sugarcane bagasse	46.5 %	521.9	48 h	This work

* For comparison, values were calculated as per the data available in above cited studies.

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