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Full Length Article

Production, Purification and Optimization of Cellulase by *Bacillus licheniformis* HI-08 Isolated from the Hindgut of Wood-feeding Termite

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Abstract

Bacteria are potential microorganisms involved in enzymatic hydrolysis of agricultural wastes by producing cellulases and other valuable chemicals. Different ionic solvents at different pH and temperature are also involved in the pretreatment of agricultural wastes to enhance the saccharification by cellulases. However, the remaining and conditioning of pretreatment buffers strongly inhibit the cellulase activity. In view of this, a symbiotic cellulolytic strain was isolated from the gut of building infesting termite *Heterotermes indicola* for the production of ionic-solvent tolerant cellulase. After screening by Congo red, strain HI-08 was identified as *Bacillus licheniformis* using 16S rDNA sequence analysis. It was accounted for maximum cellulase production on 2% sugarcane bagasse (1156 U/mL). The crude cellulase was purified by $(NH_4)_2SO_4$ precipitation, affinity and size exclusion chromatography, with 12.02 purification fold and 32.33% overall recovery. Approximately 55 kDa molecular weight of gel-purified cellulase was revealed by SDS-PAGE analysis. The optimum temperature and pH for maximum cellulase activity was determined as 45°C and 7.0, respectively. Kinetic analysis of purified cellulase showed the K_m and V_{max} be 2.24 mg/mL and 454.05 μg/mL/min, respectively. Statistical optimization using RSM indicated that sugarcane bagasse was most useful agricultural waste for cellulase production, which proved its candidature in industrial bio ventures.

Keywords: Cellulase; Agriculture wastes; *B. licheniformis*; Production; Purification; Optimization

Introduction

The demand of bio-energy has been sharply increased in the world due to high concerns about the scarcity of fossil fuels, necessity of sustainable economy and clean environment. Cellulosic biomass is the most profuse renewable biological and inexpensive resource in the world with energy content ($3-4/GJ) (Lynd *et al.,* 2008; Zhang, 2008; Zhou *et al.,* 2009). The hydrolysis of cellulosic materials can promote the local economy, environment and national energy security (Zhang, 2008). The hydrolysis process has been dependent on thermo-chemical and acid-reliant processes for decades, that have also been blamed for environmental contamination and cost intensive processes (Hamelinck *et al.,* 2005). However, the enzymatic hydrolysis is more environment friendly and low cost approach because of contemporary breakthrough in metabolic engineering, molecular genetics and enzymatic engineering in modern science (Lynd *et al.,* 2008). In addition, enzymatic hydrolysis process of biomass conversion, recovery efficiency and the cost strongly depends on fermentation competency of the enzymes and microorganisms (Piškur *et al.,* 2006). Cellulases are the enzymes that hydrolyze the cellulosic material into ethanol by breakdown of β-1, 4 linkages and subsequent fermentation. Naturally, group of cellulases, endo-1, 4 β-glucanases, exo-1, 4-β-glucanases and β-glucosidase that synergistically hydrolyze cellulose into soluble sugars and glucose (Lynd *et al.,* 2002) mediates complete hydrolysis of cellulose.

Currently, the trend of cellulase research has emphasized mostly on fungi, like basic yeast *Saccharomyces cerevisiae* in bio-ethanol production because of its unique properties of genetically modifiable, high conversion ability and alcohol tolerance (Pourramezan *et al.,* 2012). However, the upholding of the fungal mass culture and mounting concentration of aromatic compounds in industrial conversion of biomass into bio-ethanol can modify fungal biochemistry and inhibit its application (Piškur *et al.,* 2006). Therefore, isolation of an ideal cellulase-producing microorganism with high capability of biomass conversion and resistance against different inhibitors can enhance the efficiency of the hydrolysis process (Lynd *et al.,* 2008).

Some insects, like termites, dung beetles, wood roaches and crop eating pests use the lignocellulosic materials as food source with high efficiency by converting

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the cellulose into glucose (Sun and Scharf, 2010). Among these insect termites are more efficient in hydrolysis of lignocelluloses due to high adaptive diverse intestinal tract, a dual hydrolysis system enable them as highly resourceful natural bioreactors (Brune, 2014). Gut microbiota in lower termites comprises on diverse range of protozoa, bacteria, nematodes, spirochetes and fungi that are considered essential for complete cellulose digestion into glucose (Watanabe and Tokuda, 2010). Numerous studies have been conducted on isolation of flagellates and bacteria with their cellulolytic potential from lower termites species such as *Coptotermes formosanus, Reticulitermes flavipes, Hodotermopsis sjostedti, R. seperatus, Neotermes koshunensis* and *Mastotermes darwiniensis* (Tokuda *et al.*, 2007; Yuki *et al.,* 2008; Lo *et al.,* 2011; Ni and Tokuda, 2013; Sethi *et al.,* 2013). Therefore, termites are an attractive source as novel cellulolytic microorganisms and cellulases for conversion of biomass into biofuel at industrial level.

In Pakistan *Heterotermes indicola* Wasmann is considered as most destructive lower termite species to wooden structures in urban settings (Manzoor and Mir, 2010). The hindgut of *H. indicola* is enlarged than midgut and houses diverse range of protozoa and bacteria. Symbiotic cellulase system produces more complex cellulolytic enzymes. Thus, it possesses strong hydrolytic activity (40–88%) against carboxymethyl cellulose (CMC) substrate as compared to endogenous cellulase (40–85%) of total gut activity (Tokuda *et al.,* 2005). Furthermore, several intestinal bacteria have been isolated from different termite species that play an important role to degrade the cellulose fibers (Brune, 2014). It is therefore, considered that the hindgut of *H. indicola* may represent an ideal prospecting resource for identifying potential microorganisms, which can be used for cellulase production in biomass conversion application. The present study was focused on screening and identifying the potential cellulolytic bacterium from the hindgut of wood feeding termite species *H. indicola* in Pakistan. The cellulase production, purification and optimization were carried out to understand enzyme kinetics for the confirmation of cellulase candidacy in different bio-applications.

Materials and Methods

Microorganism

The workers of *H. indicola* were collected from infested storerooms in Biological Science Department of Quaid-i-Azam University, Islamabad Pakistan (33.747680°N and 73.138161°E) in monsoon season (July to September) of 2017. Approximately, 25 guts were stabbed using sterile (70% EtOH washed) forceps and needles from workers of *H. indicola* and aseptically transferred into erlenmeyer flask containing on 25 mL enrichment media and incubated at 37°C for 72 h. The enrichment media was contained on following components dissolved in dH_2O at pH 7.0: 1% CMC, 0.6% NaCl, 0.5% $(NH_4)_2SO_4$, 0.1% K_2HPO_4, 0.1% KH_2PO_4, 0.05% $MgSO_4$, 1% yeast and 1% nutrient broth. After enrichment 10 μL of culture was spot inoculated on CMC supplemented agar plates and incubated at 37°C for 24 h thrice to get pure and discrete colonies of bacteria. Among the 15 isolated strains, potential strain was selected based on the clear zone produced on 0.1% Congo red stained CMC-agar plates and subjected to further cellulase production and characterization. The strains with >1 cm hydrolysis zone were considered as significant and selected for cellulase production.

The selected strain was identified by using standard protocols of morphological and biochemical techniques described in Bergey's Manual for Determinative Bacteriology (Holt *et al.,* 1994). The molecular identification of selected strain was performed by 16S rRNA gene sequencing. In brief, DNA was extracted using phenol-chloroform method (Marmur, 1961). The PCR mixture was prepared by adding: 2 μL of genomic DNA template, 5 μL 5X PCR buffer, 4 μL 50 m*M* $MgCl_2$, 1 μL 10 m*M* dNTPs, 1 μL of each forward (UnF 5′-CCAGCAGCCGCGGTAATACG-3′) and reverse primer (UnR 5′-GGACTACCAGGGTATCTAAT-3′) (Barghouthi, 2011), 0.5 μL *Taq* polymerase enzyme and dH_2O to make a total volume of 50 μL. PCR reactions were carried out under following conditions: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 45 sec, extension at 72°C for 1.5 min and a final extension for 10 min. The hold was at 4°C and PCR product was confirmed by gel electrophoresis on 1% agarose gel and purification was carried out using GeneJET Gel (Thermo Scientific®, EU) kit. The concentration of purified DNA was estimated by nano drop (NanoDrop® ND-1000, Thermo Scientific, USA) and purified PCR product was sequenced commercially. The consensus sequences was constructed by means of MEGA6 (Tamura *et al.,* 2013).

Preparation of Crude Enzyme and Assay

The crude enzyme was obtained by transferring the 24 h old cultures to falcon tubes, sonicated for 10 min and centrifuged at 8000 rpm for 15 min. The resultant supernatant was collected as extracellular crude cellulase and cells were discarded. Cellulase activity was determined by measuring the amount of reducing sugar released from enzyme and substrate (CMC) reaction through DNSA (3, 5-dinitrosalicylic acid) method (Miller, 1959). In this method, the reaction was conducted by adding 500 μL of substrate (1% CMC; 0.1M phosphate buffer pH 7.0), 100 μL of the crude enzyme and 400 μL dH_2O in test tube. The reaction was incubated at 45°C for 30 min and stopped by adding same amount (1 mL) of DNSA solution. Then reaction was boiled in water bath for 10 min followed by cooling in ice at 5 min for stabilization. Optical density was measured using

spectrophotometer (SmartSpec™ *Plus* BIO-RAD, USA) at 546 nm. The activity of the cellulase was determined by drawing standard curve at different concentration of glucose (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 mg/mL). One unit of enzyme activity was defined as amount of enzyme required to liberate 1 μg glucose/min/mL.

Cellulase Production on Agro-wastes

The effect of five agricultural waste materials (maize bran, sugarcane bagasse, wheat bran, rice bran and rice husk) on the production activity of cellulase was determined by growing culture in cellulolytic broth media supplemented with 2% (w/v) agro-waste, 0.2% K_2HPO_4, 1% peptone, 0.03% $MgSO_4$ and 0.25% $(NH_4)_2SO_4$. Culture was incubated in rotary shakers (100 rpm) at 45°C for 36 h. The enzymatic production activity was determined by the DNSA method (Miller, 1959) as discussed in the enzyme assay section above.

Purification of Cellulases

The resultant supernatant from crude enzymes of selected strain was subjected to purification. All purification measures were carried out at 4°C. The cell free supernatant was precipitated overnight with ammonium sulphate $(NH_4)_2SO_4$ at 8000 rpm for 10 min and the pellet was recovered at 60 and 80% of saturation. The precipitated cellulase was resuspended in 0.1 M phosphate buffer pH 7.0 and dialyzed in same buffer using dialysis tube with pore size <10 kDa. The recovered cellulase was dissolved in fresh buffer, assayed for activity and protein estimation. The dialyzed cellulase was subjected to a column of ion exchange resins Roti® change (1×4 Roth®, Germany; Cat No. 6849.1) equilibrated with 0.1 M phosphate buffer (pH 7.0). The desired fractions were allowed to attach with matrix for 1 h at 4°C and eluted with a linear gradient of NaCl (0.1 to 0.4 M) at a flow rate of 0.5 mL/min. Approximately 1 mL for each fraction was collected and used for enzyme activity and protein estimation assay. Active fractions were pooled and subjected for further purification step. The pooled fractions of cellulase were further purified by gel filtration chromatography. The Sephadex G-75 column prep grade (0.7×10 cm; Luer Lock column, Sigma-Aldrich, USA; Lot#3110) was equilibrated with same buffer and eluted at flow rate of 0.5 mL/min. The gel-purified enzymes were also subjected for enzyme activity and protein estimation.

Estimation of Protein Concentrations

The estimation of protein concentration in the crude, precipitated and purified cellulases was determined by the method of Lowry *et al.* (1951) using bovine serum albumin (BSA) as standard expressed as mg/mL. The optical density was measured through spectrophotometer at 280 nm.

SDS-PAGE Analysis

The molecular weight of the cellulase in crude and purified form was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to the method of Laemmli *et al.* (1976) using 5% staking and 10% resolving gel. Gel was stained using 250G Coomassie brilliant blue and destained in de-ionized methanol acetic acid water (Sambrook and Russel, 2001). Approximate molecular weight of cellulases enzymes was estimated by running samples in SDS-Page against standard PageRuler™ pre-stained protein ladder, 10–180 kDa (Thermo-Scientific Pvt Ltd., USA).

Kinetic Parameters

The effect of substrate concentrations was investigated on the velocity of reaction of purified cellulase. The reaction was incubated with various concentrations of CMC ranging from 0.25 to 2.5 mg/mL under standard assay conditions. The Michaelis constant (K_m) and maximum velocity (V_{max}) was determined from Line weaver-Burk plots of Michaelis-Menten equation (Lineweaver and Burk, 1934).

Characterization of Purified Enzymes

The effect of temperature on enzyme activity was followed by incubating the reaction of cellulase with 0.1% CMC in 0.1 M phosphate buffer pH 7.0 at different temperatures (25, 35, 45, 55, 65, 75, 85, 95, and 105°C) under standard assay conditions. The effect of pH on the activity of cellulases was also calculated by measuring the hydrolysis of CMC in 0.1 M phosphate buffer at different pH values ranging from 3.0 to 9.0. The CMCase activity was determined at 45°C under standard test conditions (Miller, 1959). The effect of different metal ions (0.01 M) on enzyme activity was determined by incubating reaction with solutions of $CaCl_2$, $ZnSO_4$, $MgCl_2$, $CuSO_4$, $FeSO_4$, $NaCl_2$, $HgCl_2$, $AgCl_2$, Co and $NiCl_2$ at 45°C for 1 h.

Statistical Optimization using Response Surface Methodology (RSM)

Statistical optimization for cellulase production was assessed by evaluating the intensity of different factors on the production using statistical approach second order Box-Behnken Design (BBD) (Ferreira *et al.*, 2007). Based on the results of preliminary assays during cellulase characterization, most effective independent variable such as sugarcane bagasse (% w/v), temperature (°C), pH and $MgCl_2$ were selected for statistical optimization of cellulase with 3 different levels (Table 3). Experimental design was built and analyzed by subjecting the data for ANOVA, estimation of main effect and interaction and fitting the second order regression equation using the Design-Expert software 11.0 (Stat Ease Inc., Minneapolis, MN, USA) as described by Saini *et al.* (2015).

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1}^{k} \sum_{j=i+1}^{k} \beta_{ij} x_i x_j (1)$$

Where, y is response (cellulase production); β_0 is the constant value; x= independent variables effecting response; βi, βii, βij are coefficients for linear, interaction and quadratic regression respectively; and k is total number of variables. Based on results obtained from 29 experiments of BBD matrix, a quadratic non-linear polynomial regression equation (Eq. 2) was constructed that indicated the pragmatic relationship between response and selected variables in coded form.

$$Y = +1013.60 + 203.50A - 127.33B + 118.33C + 85.00D - 127.00AB + 58.50AC - 82.50AD - 1.25BC - 22.25BD - 9.75CD + 20.83A^2 - 452.67\ B^2 - 218.67\ C^2 - 64.42D^2 \quad (2)$$

Where the Y= cellulase activity (U/mL); A = Sugarcane bagasse (% w/v); B = Temperature (°C); C = pH; and D = $MgCl_2$ (m*M*).

Results

Isolation, Screening and Identification of Cellulolytic Strain

Fifteen bacterial strains were isolated from the hindgut of *H. indicola* on nutrient agar supplemented with carboxymethyl cellulose (CMC). Only seven isolates exhibited good cellulase activity that was reevaluated on CMC agar plates stained by Congo red and NaOH solutions (Fig. 1). The zones of clearance by isolates reflected their extent of cellulolytic activity. The isolates with good cellulolytic activity produced >1.0 cm zone just after 6 h of incubation at 40ºC indicating an extra cellular property of cellulases. Among seven strains, HI-08 indicating maximum diameter of clearance zone was selected for further processing (Fig. 1).

The competent cellulase producing isolate HI-08 was rod shaped *Bacillus* gram positive, motile showing high growth in aerobic and anaerobic environment with wide range of pH (5-11). It was positive in catalase, acetyl methyl carbinol, citrate and nitrate reduction test and negative in oxidase. Based on morphological and biochemical characteristics, it was identified as *Bacillus* sp. by industrial microbiology lab, Quaid-i-Azam University Islamabad, Pakistan. The analysis of 16S rRNA gene sequence of strain HI-08 indicated its 99% homology with *Bacillus licheniformis* strains and submitted as *B. licheniformis* HI-08 to Genbank [KY786028].

Cellulase Production on Agro-wastes

Bacterial strain *B. licheniformis* HI-08 showed maximum cellulase production on 2% sugarcane bagasse (1156.03 U/mL) and found it suitable substrate for highest cell growth followed by rice husk (985.09 U/mL) and rice bran (725.73 U/mL) after 36 h of incubation at 45ºC (Fig. 2).

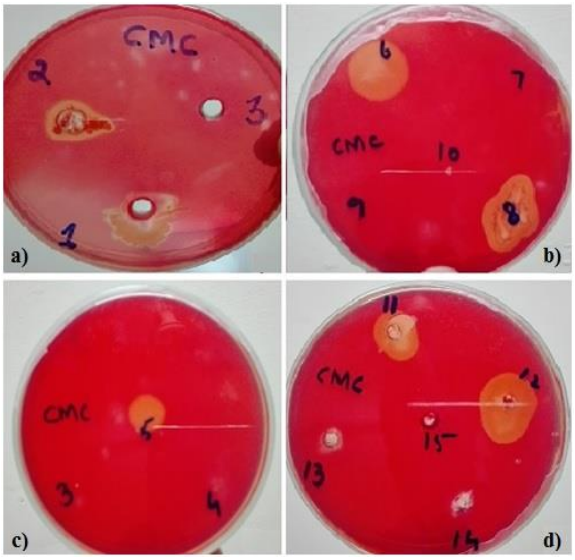

Fig. 1: The screening of cellulolytic bacteria cultures on CMC ager plates with Congo-Red assay. The zones of clearance by isolates reflected their extent of cellulolytic activity

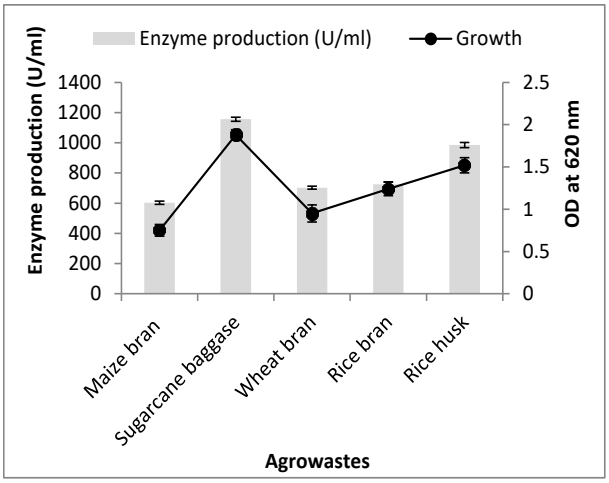

Fig. 2: The cellulase production and growth profile of *B. licheniformis* HI-08 against five different agro-wastes (carbon source) such as maize bran, sugarcane bagasse, wheat bran, rice bran and rice husk (2%). Each value indicating the mean±SD

However, the maize bran (602.33 U/mL) and wheat bran (712.56 U/mL) showed minimum cellulase production and cell growth (Fig. 2).

Purification of Crude Cellulase

The crude of extracellular cellulases with maximum activity (5598.70 U/mL) against CMC was first subjected to ammonium sulphate precipitation (salt in) dialysis (salt out) for purification. The maximum pellet and cellulase activity (4036.6 U/mL) was recovered in a fraction of 80% obtained

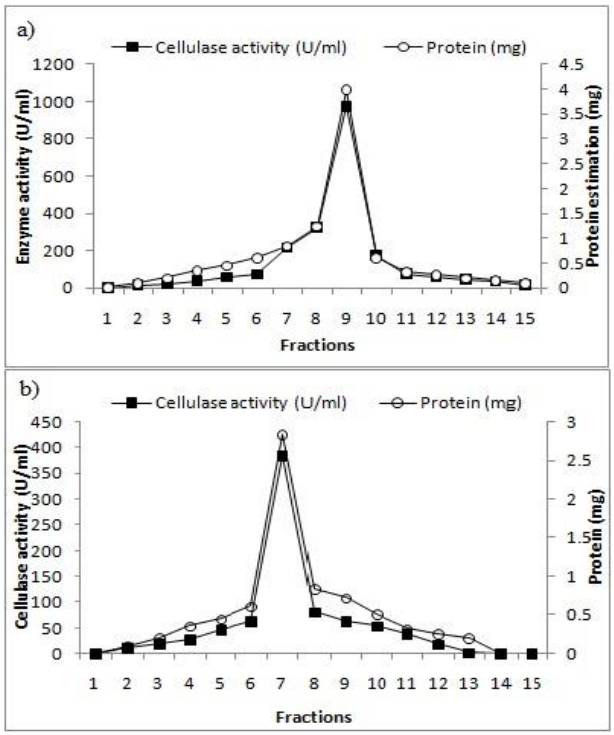

Fig. 3: Chromatographic purification profile of cellulase from *B. licheniformis* HI-08 on ion exchange column (a) and Sephadex G-75 column (b)

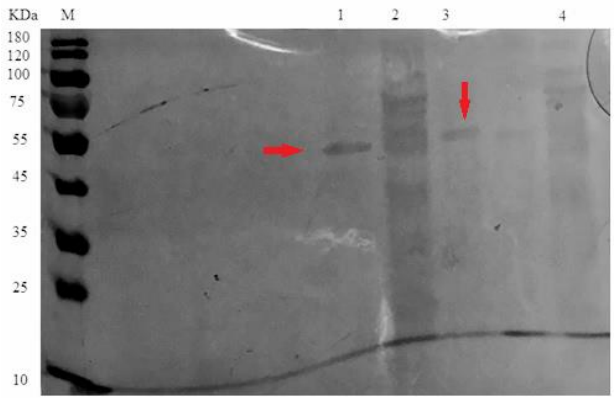

Fig. 4: SDS-PAGE analysis of cellulase from *B. licheniformis* HI-08 obtained after different purification steps; M: Markers kDa; Lane 1: arrow indicating purified cellulase band after anion chromatography; Lane2: crude supernatant; Lane 3: arrow indicating cellulase band obtained after gel-filtration Sephadex G-75; Lane 4: concentrated fraction after $NH_4(SO_4)_2$

by addition of $NH_4(SO_4)_2$. The fraction had 7.38 mg total protein and 546.88 U/mg specific activity with 72.09% recovery and 2.7 purification folds (Table 1).

No enzyme activity and protein contents were observed in collected unbound fractions. The cellulase activity and protein contents were detected in fraction No. 9 of bounded protein that was eluted by the addition of 500 m*M* NaCl (Fig. 3a). It showed high specific activity 1146 U/mg with 61% recovery (Table 1). The gel filtration Sephadex G-75 was used for further purification of active fraction obtained by ion exchange chromatography. Fig. 3b indicates the overlapping active peaks of protein contents and activity at fraction No. 7 on Sephadex G-75 column. The confirmation of cellulase purity was assessed by the presence of single band on SDS-PAGE after ion-exchange and gel filtration. The molecular weight of purified cellulase of *B. licheniformis* was approximately 55 kDa (Fig. 4).

Kinetic Analysis

The kinetics of purified cellulase with CMC exposed for HI-08 the K_m and V_{max} to be 2.28 mg mL^{-1} and 454.05 μg mL^{-1} min^{-1} respectively by Linweaver-Burk plot (Fig. 5).

Temperature and pH Optimization

Purified cellulase showed optimal activity at 45°C and turned down thereafter at varying temperature ranged from 25–105°C (Fig 6a). The optimal pH for the activity and stability of purified cellulase was determined by using different pH ranging from 3–10. Fig. 6b indicates the maximum cellulase activity and stability at pH 7.0 in 1 h of incubation.

Effect of Metal Ions on Cellulase Activity and Stability

The activity and stability of purified cellulases of strain HI-08 was enhanced in the presence of alkali metal ions such as Mg^{2+}, Ca^{2+} and Na^{+} by increasing concentration (Table 2). Maximum inhibition of cellulase activity was observed against mercury ion Hg^{+2} at both concentrations (10 and 20 m*M*) under standard assay conditions. Likewise, 20 m*M* of Zn^{2+}, Ni^{2+}, Co^{2+} and Cu^{2+} slightly inhibited the cellulase activity and stability as compared to control (Table 2).

Response Surface Methodology for Cellulase Optimization

In the preliminary assays, several variables screened out during the production and hydrolysis efficiency of the cellulase; however, the optimization of four most significant cultural and nutritional factors was carried out to enhance the cellulase production using RSM in Design Expert 11.0. Box-Behnken design was employed with experimental layout of 29 runs at three different levels (minimum (+1), medium (0), maximum (1) against selected independent variables and cellulase activity U/mL (response) as shown in Table 3. The adequacy and suitability of the second order BBD matrix for cellulase activity was scrutinized by ANOVA and Fisher's '*F*-test (Table 4). The results showed that model was found 'significant' with *F* value (25.85) and *P* value (<0.0011 at 95% confidence level). Besides, a 'non-significant' lack of fit value specified that model was good

Table 1: Purification steps indicating total activity, total protein, specific activity, purification fold and percentage yield of cellulase from *B. licheniformis* HI-08

Purification Steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude	5598.70	28.54	198.14	1	100
Precipitation $(NH_4)_2SO_4$	4036.6	7.38	546.88	2.76	72.09
Ion exchange resin	3417.52	2.98	1146.81	5.78	61.04
Sephadex G-75	1810.23	0.76	2381.88	12.02	32.33

Table 2: The activity and stability of the purified cellulase of *B. licheniformis* HI-08 against different metal ions at concentrations 10 and 20 m*M*

Metal ions	Concentrations (m*M*)	Residual activity %	
		Enzyme activity	Enzyme stability
Control	0	100	100
$CaCl_2$	10	139	117
	20	205.5	175.5
$ZnCl_2$	10	88.6	62.4
	20	56	44
$MgCl_2$	10	144.4	119
	20	215.2	180.5
$CuSO_4$	10	67.5	56.0
	20	44.5	26.9
$FeCl_3$	10	121	98.8
	20	88.6	65.5
NaCl	10	134.5	102.0
	20	182.8	144.6
$HgCl_2$	10	33**	44
	20	12**	26
$AgNO_3$	10	90	78.4
	20	126.5	108
$NiCl_2$	10	88.5	77.8
	20	67	59.6
$CoCl_2$	10	85	76.6
	20	63	55

The cellulase activity and stability in absence of metal ions was considered as control (100 %). ** indicating significant inhibition of residual activity. SD= < ± 0.05 for all values

Table 3: The Box-Bhenken design at three levels of each independent variable and response of dependent variable cellulase activity (U mL^{-1}) of *B. licheniformis* HI-08*

Run No.	Sugarcane bagasse(% w/v)	pH	Temperature (°C)	$MgCl_2$ (m*M*)	Cellulase activity (U/mL)
1	2(1)	7(0)	105(1)	10(0)	453
2	2(1)	7(0)	45(0)	5(-1)	1356
3	1(0)	10(1)	105(1)	10(0)	347
4	1(0)	7(0)	105(1)	5(-1)	388
5	1(0)	7(0)	25(-1)	20(1)	521
6	1(0)	7(0)	45(0)	10(0)	866
7	1(0)	7(0)	25(-1)	5(-1)	498
8	1(0)	10(1)	45(0)	5(-1)	702
9	0.5(-1)	7(0)	105(1)	10(0)	320
10	1(0)	10(1)	25(-1)	10(0)	680
11	2(1)	10(1)	45(0)	10(0)	1064
12	1(0)	10(1)	45(0)	20(1)	940
13	2(1)	3(-1)	45(0)	10(0)	432
14	1(0)	3(-1)	45(0)	5(-1)	390
15	0.5(-1)	10(1)	45(0)	10(0)	754
16	1(0)	7(0)	105(1)	20(1)	333
17	1(0)	3(-1)	45(0)	20(1)	667
18	1(0)	7(0)	45(0)	10(0)	986
19	0.5(-1)	7(0)	25(-1)	10(0)	453
20	1(0)	3(-1)	105(1)	10(0)	247
21	1(0)	7(0)	45(0)	10(0)	1034
22	1(0)	3(-1)	25(-1)	10(0)	675
23	2(1)	7(0)	25(-1)	10(0)	986
24	1(0)	7(0)	45(0)	10(0)	1096
25	1(0)	7(0)	45(0)	10(0)	1112
26	2(1)	7(0)	45(0)	20(1)	1465
27	0.5(-1)	7(0)	45(0)	20(1)	1040
28	0.5(-1)	3(-1)	45(0)	10(0)	356
29	0.5(-1)	7(0)	45(0)	5(-1)	889

*The values in parenthesis are indicating the three levels of independent factors in coded form

fit. The highest *F*-value (15.54) and *P*-value (< 0.05) for sugarcane bagasse indicated that individual effect of sugarcane bagasse on cellulase activity was significantly higher than other variables. However, a non-significant effect of interactions and squares among all factors was observed on the cellulase production, except the limited interaction was observed between sugarcane and temperature variables (Table 4).

Based on RSM analysis the optimal culture conditions for maximum cellulase production 393.99 U/mL were predicted as; Sugarcane bagasse 2.0% (w/v), pH 7.0, temperature 45°C and $MgCl_2$ 10 m*M*. The validation of model was conducted using model condition in 96-well plate exhibited cellulase activity of 400 U/mL that was 1.9-fold higher to cellulase production under non-optimized conditions. Besides, the validity of predictions and goodness of fit for the model can be further ensured in terms of *P*-value (< 0.05) and the coefficient of determination (R^2), predicted R2 and adjusted R^2. The value for coefficient of determination was 0.9066 that indicated the compatibility of experimental data was 90%. However, the difference among adjusted (0.7808) and predicted (0.3357) coefficients values was more than 0.2 that is indicating insignificant correlation between experimental and predicted cellulase activities.

For better perceptive the interaction between the factors and their effects on the cellulase activity (U/mL), 3D surface plots were constructed by using coefficients of quadratic polynomial equation. In this model, only factor sugarcane bagasse (A) and temperature (B) terms were significant for response (P<0.05). Fig. 7 depicts the insignificant interaction of sugarcane bagasse and temperature for the cellulase activity. By changing the concentration of sugarcane bagasse had variant effect on cellulase activity at lower (-1), middle (0) and upper (+1) level of temperature, pH and $MgCl_2$ (Fig. 7a, b and c). Similarly, at middle level 0 (45°C) of temperature the cellulase activity was highest, but pH and $MgCl_2$ had no significant effect on cellulase production at any

Table 4: Analysis of variance for quadratic model to assess the cellulase activity of *B. licheniformis* HI-08 using Response surface methodology

Source	Sum of Squares	df	Mean Square	*F*-value	*p*-value
Model	2.619E+06	14	1.871E+05	5.85	0.0011*
A-Sugarcane Bagasse	4.969E+05	1	4.969E+05	15.54	0.0015*
B-Temperature	1.946E+05	1	1.946E+05	6.08	0.0272*
C-pH	1.680E+05	1	1.680E+05	5.25	0.0379*
D-$MgCl_2$	86700.00	1	86700.00	2.71	0.1219
AB	64516.00	1	64516.00	2.02	0.1774
AC	13689.00	1	13689.00	0.4280	0.5236
AD	27225.00	1	27225.00	0.8511	0.3719
BC	6.25	1	6.25	0.0002	0.9890
BD	1980.25	1	1980.25	0.0619	0.8071
CD	380.25	1	380.25	0.0119	0.9147
A^2	2813.06	1	2813.06	0.0879	0.07712
B^2	1.329E+06	1	1.329E+06	41.55	<0.0001*
C^2	3.102E+05	1	3.102E+05	9.70	0.0076
D^2	26922.69	1	26922.69	0.8417	0.3744
Residual	4.478E+05	14	31986.50		
Lack of Fit	3.728E+05	10	37281.58	1.99	0.2652
Pure Error	74995.20	4	18748.80		
Cor Total	3.067E+06	28			

*indicating the significant (P<0.05) terms

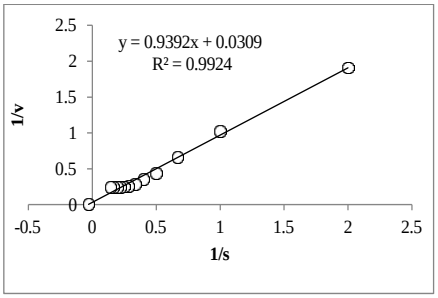

Fig. 5: Lineweaver-Bruke plot of initial velocity data of cellulase against different concentrations of CMC

concentration of temperature indicating the insignificant interaction (Fig. 7d and f).

Discussion

In earlier reports, the *B. licheniformis* has been reported from the hot springs, marine water and composite (Annamalai *et al.*, 2012; Marco *et al.*, 2017; Shajahan *et al.*, 2017). However in the present study, it was isolated from highly adaptive gut of wood-feeding termite *H. indicola* that indicates the variability and tolerance property in *B. licheniformis* for habitat selection. During screening test, HI-08 produced maximum clearance zone by degrading the congo red stain and selected for further study (Fig. 1). Gohel *et al.* (2014) reported that cellulase produces these clear zones due to degradation of polysaccharides (CMC) into monosaccharide, which have no affinity for binding towards Congo red dye.

The agricultural waste such as sugarcane bagasse was most found suitable substrate for growth and cellulase

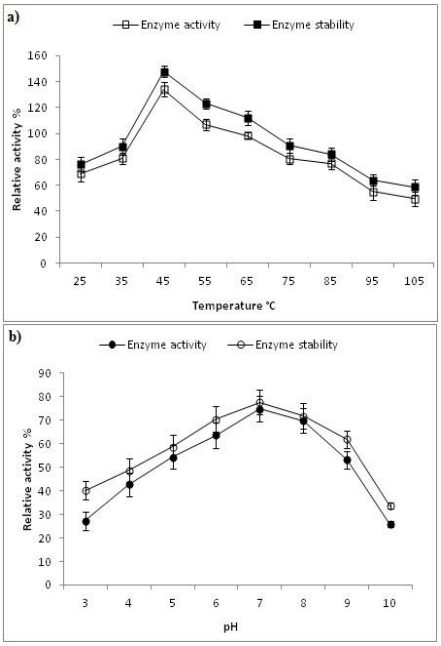

Fig. 6: Temperature (a) and pH (b) effect on purified cellulase extracted from strain HI-08 after 1 h of incubation under standard assay conditions

production of *B. licheniformis* HI-08 followed by rice husk and rice bran. Similarly, sugarcane bagasse was also reported as most suitable substrate for the *B. vallimortis* (RG-07) growth and enzyme production (Gaur and Tiwari, 2015). In another study, Sadhu *et al.* (2013) found the sugarcane bagasse as the most effective substrate for cellulase production against *Bacillus* sp. In contrast, Annamalai *et al.* (2012) reported that *B. licheniformis* AU01 isolated from marine water showed maximum cellulase production and growth on rice bran. This disparity suggests the bacterial habitat and isolation source affect the applicability of strain in enzyme production. Lee *et al.* (2008) reported that sugarcane bagasse, rice hulls and rice husk were the most suitable carbon source for the production of cellulase by increasing bacterial growth due to their inducible nature. Therefore, the cellulase production and growth profile of *B. licheniformis* HI-08 on different cellulosic wastes could be valuable in the production of bio-ethanol, as bio-stoning agent in textile industry, single cell protein and many other industrial chemicals.

The purification profile indicated that specific cellulase activity was increased by increasing purification fold of cellulases from *B. licheniformis* HI-08. The purification profile indicated 12.02 folds of purification factor and 32.33% yield with highest specific activity 2381.88 U/mg (Table 1). In contrast, cellulase obtained from hot spring (NCIM 5556) and marine water (AU01) strains of *B. licheniformis* showed 42.99 and 451.9 U/mL specific activities respectively (Annamalai *et al.*, 2012;

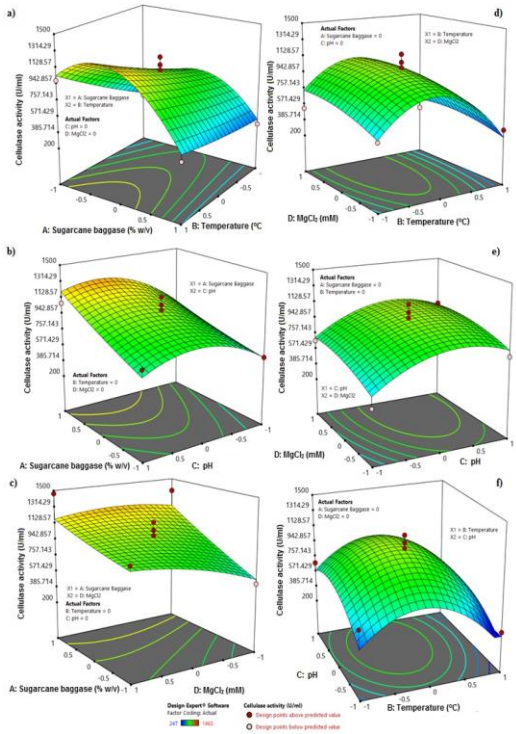

Fig. 7: 3D surface plots indicating the effects of variables and its interaction on cellulase activity. a) Sugarcane bagasse vs temperature; b) Sugarcane bagasse vs pH; c) Sugarcane bagasse vs $MgCl_2$; d) $MgCl_2$vs temperature; e) $MgCl_2$vs pH; f) pH vs temperature

Shajahan *et al.,* 2017) after ion exchange chromatography. Gaur and Tiwari (2015) reported 39.1fold purification with 28.8% yield after gel-filtration for cellulase purification from *B. vallismortis* RG-07. In another study, cellulase from *B. licheniformis* showed 8.66 fold purification and 11.5% recovery after the gel filtration that had the less hydrolysis capability from the cellulase of present study strain HI-08. Thus, the hydrolysis potential of strain isolated from termites was higher than earlier reported strains of *B. licheniformis* isolated from hot springs, marine water and composite (Annamalai *et al.,* 2012; Marco *et al.,* 2017; Shajahan *et al.,* 2017). The molecular weight of HI-08 was around 55 kDa that was less than *B. vallimortis* RG-07 (80 kDa) and approximately equal to alkaliphilic *B. licheniformis* SVD1 (55 kDa) (Annamalai *et al.,* 2012; Marco *et al.,* 2017; Shajahan *et al.,* 2017).

In the kinetic analysis, the K_m value of the cellulase obtained from HI-08 was less as compared to K_m of cellulases isolated from the *Clostridium thermocellum* Umcel5 (K_m, 16 mg/mL) (Liu *et al.,* 2009), The metagenomic study of novel C67-1 cellulase gene isolated from rumen of buffalo (K_m 37 mg/mL) (Duan *et al.,* 2009), *Salinvibrio* sp. (K_m 3.03 mg/mL) (Wang *et al.,* 2009) and *Pseudomonas fluorescents* (K_m 3.6 mg/mL) (Bakare *et al.,* 2005). The lower value of kinetic constant (K_m) illustrated that purified cellulase of *B. licheniformis* HI-08 has higher affinity of cellulase against CMC. Likewise, Azadian *et al.* (2017) reported the K_m and V_{max} of cellulase from *B. sonorensis* HSC7 0.186 mg/mL and 0.052 μmol/min, respectively. Sriariyanun *et al.* (2016) found the 0.80 mg/mL K_m and 1000 μM/min V_{max} for ionic liquid tolerant cellulase *Bacillus* sp. MSL2 isolated from rice paddy field soil.

The purified cellulase of strain HI-08 showed complete stability upto 50% over the range of 25-105°C during 30 min of incubation that coincide with the thermostability of *B. vallismortis* (Gaur and Tiwari, 2015). Similarly, thermostability for endoglucanase of *B. licheniformis* strain C108 was upto 100% isolated from the lake soil (Aygan *et al.,* 2011). Many researchers have reported the thermostability profile of cellulase from *Bacillus* sp. range from 30–100°C can retain activity up to ≈50% (Singh *et al.,* 2009). However, the cellulase from the strain HI-08 was more thermostable and can be used in extreme thermal conditions in different cellulosic applications. The similar optimum temperature was found in another strain *B. licheniformis* NCIM 5556 and AU01 isolated from hot spring and marine water respectively, in India (Annamalai *et al.,* 2012; Shajahan *et al.,* 2017). *Bacillus* strains MSL2, RG-07 and HR68 have optimum activity for cellulases at 50, 65, 70°C respectively (Robson and Chambliss, 1989; Gaur and Tiwari, 2015; Sriariyanun *et al.,* 2016).

The pH results of *B. licheniformis* HI-08 exhibited more than 50% enzyme stability over the pH range 5.0–9.0 that was higher from previously reported cellulase stability from *Bacillus* sp. (Yu and Li, 2015; Sriariyanun *et al.,* 2016). The trends of cellulase stability in the present study enhance the applicability of HI-08 for hydrolyzing the cellulosic material in broad alkaline pH range. Similarly, optimum cellulase activity was observed in alkali stable *Gracilibacillus* sp. strain SK1, *Bacillus* sp. PKM-5430 and *Pseudomonas fluorescence* at pH 7.0 (Bakare *et al.,* 2005; Lee *et al.,* 2008; Yu and Li, 2015).

The high residual activity in the presence of alkali metal such as Mg^{2+}, Ca^{2+}, and Na^{+} indicated the extreme halotolerance and alkaline nature of cellulase from *B. licheniformis* HI-08 that was similar to other cellulases of *Bacillus* strain RG-07(Gaur and Tiwari, 2015). However, other metal ions reduced the cellulase activity and stability to an inconsistent extent such as Hg^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+} and Cu^{2+}. In previous studies, the inhibition of cellulase activity due to Hg^{+2} was correlated to binding nature of mercury with thiol, carboxyl groups and tryptophan residues in the cellulase (Lusterio *et al.,* 1992). The inhibition due to transition group metal ions might be related to competition associated with exogenous cations and enzyme cations, that causing the reduction in metallo-enzyme activity (Gaur and Tiwari, 2015). High salt and alkaline nature of cellulase from HI-08 make it suitable candidate in cellulosic material processing industry because, during pretreatment of biomass different acidic

and alkali nature salts are used and create extreme salinity conditions that might be neutralized by HI-08.

A maximum cellulase activity of *B. licheniformis* HI-08 exhibited 400 U/mL during validation assay under optimization conditions using RSM design. It was higher to maximum cellulase activity (42.99 IU/mL) of *B. licheniformis* NCIM 5556 isolated from hot spring (Shajahan *et al.,* 2017). Likewise, Saini *et al.* (2015) found wheat bran 2.5% (w/v), incubation time of 8 days, ammonium sulfate 0.53% (w/v) and avicel 0.5% as predicted variable for maximum cellulase 1.26 FPU/mL from *Penicillium oxalicum* IODBF5 using response surface methodology. In another report, Yang *et al.* (2012) found that optimization technique enhanced 1.6 fold cellulase production in fungus *Penicillium* sp. CLF-S. This difference might be due to the isolation source of bacterial strains, because lower termites use symbiotic cellulases (bacterial and protozoa' cellulase) for complete and efficient wood digestion. Therefore, *B. licheniformis* HI-08 could be a potential source for cellulase production as compared to previous studies (Annamalai *et al.,* 2012; Dave *et al.,* 2015). It suggests the directly use of wild type *B. licheniformis* HI-08 for the cellulase production and biomass conversion at commercial level.

Conclusion

The cellulase of *B. licheniformis* HI-08 is unique with respect to isolation source that is a wood feeding termite. It confirmed its candidature in biomass conversion application by hydrolysis of agricultural wastes with optimal activity at temperature 45°C and pH 7.0. The retention of cellulase activity in presence of various metal ions indicated organic ionic-tolerant nature of cellulase. The optimization model using RSM enhanced the cellulase activity by 1.8 folds with optimized conditions. HI-08 is a wild type strain with lucrative cellulase titers that can be further improved using mutations and other biotechnological techniques.

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