Thermostable cellulase produced by trichoderma (*Hypocrea estonica)*

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Abstract

The present work studied thermostable cellulase extracted from 12 marine strains of *Trichoderma*. Among the strains, *Hypocrea estonica* showed the maximum activity. The cellulase activity was enhanced through the optimization of the batch fermentation. The variables sawdust (1-10.0 $g.l^{-1}$), $(NH_4)_2SO_4$ (1-6 $g.l^{-1}$) $MgSO_4.7H_2O$ (1-3 $g.l^{-1}$), KH_2PO_4 (1-3 $g.l^{-1}$), temperature (20-80ºC) and pH (4-9) were used for the selection of the important factors for the cellulase enzyme production by statistical model Plackett-Burman. Optimized conditions for the enhanced cellulase production were found as 7.69 $g.l^{-1}$ of sawdust, 3.59 $g.l^{-1}$ of $(NH_4)_2SO_4$, under 49°C temperature at pH of 8.8 and these conditions. The thermo stability was assessed by Arrhenius relation, Michaelis constant (Km) and maximal velocity (Vmax). Thermal deactivation (R^2) values showed 87-97% stability of cellulase enzyme produced by *H. estonica*. The energy of thermal deactivation of the cellulase enzyme was found to be 29.08 Kcal/mol revealing the thermostability of enzyme.

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Introduction

Microorganisms in particular fungi are capable of digesting the lignocellulosic biomass through cellulolytic enzymes, which in turn, are used in the productions of bioethanol, biomedicine, pharmaceuticals, food and agriculture biproducts. A linear polymer of D-glucose units linked by 1, 4-β-D-glucosidic bonds is the main constituent of wood and the most abundant renewable biomass on earth (De-Silva et al., 1997; Siva Bbat *et al.*, 1997). Fungal enzymes have the ability to digest the lignocellulosic waste materials into fermentable sugars and ethanol (Depaula *et al.*, 1999; Hongdong Liao et al., 2010; Ibrahim *et al.*, 2011). The cellulolytic enzymes are produced by various types of fungi including *Phanerochaete chrysosporium, Trametes versicolor, Pleurotus pulmonarius, Pycnoporus sanguienus, Neurospora crassa* and *Coriolus versicolor*. Among them, spore forming fungi are the most efficient producers which secrete ligninolytic enzymes in nature. However, only few reports are available on the production of the enzymes by marine derived microorganisms (Raghukumar *et al.*, 1999; Bugni and Ireland, 2004; Adsul *et al.*, 2004; Kathiresan and Manivannan, 2006). In general, marine-derived fungi have greater potential than their terrestrial counterparts due to their adaptation to harsh environment conditions such as high pressure, low temperature, low nutrient and high salinity. In addition, seagrasses and mangroves produce lignocellulosic waste materials and hence fungi are believed to play a critical role in degrading the wastes in these ecosystems (Newell, 1996; Raghukumar *et al.*, 1999; Kathiresan and Bingham, 2001; Kathiresan and Manivannan, 2006; Kathiresan *et al.*, 2011a; Saravanakumar *et al.*, 2013).

The rate of lignocellulosic hydrolysis varies based on both reaction conditions and enzyme activity (Aguado *et al.*, 1995; Artur Cavaco-Paulo, 1998). The best conditions for catalytic activity on the degradation of the lignocellulosic waste material and production of the mono-sugars depend on pH, temperature and substrate or product inhibition, and these are prerequisites to obtain useful design equations and optimizations. The present work was designed in two steps: (1) optimization of the cellulase production by *Trichoderma* species using response surface methodology; and, (2) kinetic attempts that addressed mostly initial rate of kinetics and also considered the presence of substrate (lignocellulosic waste material) and product (mono-sugars) in integrated kinetic models. For industrial purposes, it is desirable that integrated kinetic model is able to describe accurately the conversion as a function of time, from time zero to nearly complete hydrolysis of the substrate lignocellulose.

Materials and Methods

Microorganisms and maintenance

Twelve strains of *Trichoderma* viz., *T. asperellum* (TSK1), *T. arundinaceum* (TSK2), *T. brevicompactum* (TSK3), *T. ghanense* (TSK4), *T. aggressivum* (TSK5), *H. viridescens* (TSK6), *T. hamatum* (TSK7), *T. harzianum/H. lixii* (TSK8), *T. atroviride* (TSK9), *T. koningii* (TSK10), *Trichoderma estonicum /H. estonica* (SKS1), and *T. viride/ H. rufa* (SKS2) were isolated by using selective medium (Askew and Laing, 1993) from mangrove biotope of Pichavaram, situated in south east coast of India. The stock cultures for the strains were maintained at 4°C on potato dextrose agar slants. The strains were screened for the production of cellulase enzyme.

Primary screening cellulase assay

Trichoderma (*Hypocrea*) species were cultured on yeast extract peptone agar medium (yeast extract 0.1 g, peptone 0.5 g, agar 16 g and 50% seawater 1000 ml) supplemented with 0.5% Na- carboxymethyl

cellulose (CMC). After incubation, the plates were flooded with 0.2% aqueous Congo red and destained with 1M NaCl for 15 minutes. The clear zone surrounding the colony indicated the cellulase activity (Kathiresan *et al.*, 2011b). Among the 12 strains, a potential strain for the cellulase production was selected, identified as *Hypocrea estonica* SKS1 (JQ611722) based on DNA sequencing and phylogeny and then the strain has been deposited in NCBI.

Substrate

Sawdust was obtained from local wood mills, sieved through a 1.5 mm sieve for maintaining uniform particle size, and washed through distilled water for removing the impurities from sawdust and finally was dried at 60°C overnight (Kathiresan *et al.*, 2011).

Cellulase enzyme production by Hypocrea estonica

Spore suspension of *Hypocrea estonica* SKS1 (JQ611722) was used as microbial inoculum for fermentation. It was prepared by culturing the fungus in potato dextrose agar slant cultures at 30°C for 7 days and spore suspension was washed through tween-80 water (0.02% V/V), then the suspension was assessed for final spore count as $2.3X10^3$ CFU/ml. Then 10 ml of spore suspension was inoculated into a 250 ml Erlenmeyer flask containing 100 ml glucose pre-cultured medium (%, w/v) (glucose 3; yeast extract 0.8; K_2HPO_4 0.4; $MgSO_47H_2O$ 0.2; pH 7.0) and was cultured at 30°C, 200 rpm for 48 h in a rotary shaking incubator to prepare the mycelial suspension. At last, mycelial suspension was inoculated into a 250 ml Erlenmeyer flask containing the medium (Sawdust 10.0 $g.l^{-1}$, $(NH_4)_2SO_4$ 6 $g.l^{-1}$, NaCl 5 $g.l^{-1}$, KH_2PO_4 3 $g.l^{-1}$, $MgSO_4.7H_2O$ 3 $g.l^{-1}$) with inoculum size of 6.0% (v/w) and was cultured at 30°C for 120 h to produce cellulase.

Determination of cellulase enzyme activity

Five ml of the fermented residue was suspended in 150 ml distilled water and shaken at 120 rpm for 2 h. Then the filtrate was centrifuged (10000 rpm) using high speed centrifuge for 15 min and the supernatant was used for enzyme assay.

Endoglucanase (carboxymethyl cellulase, CMCase) activity was assayed in a reaction mixture (2.0 ml) containing 0.2 ml of the enzymes solution and 1.8 ml of 1% (w/v) carboxymethyl cellulose (CM-cellulose) solution prepared in sodium acetate buffer (50 mM, pH 5.0). The reaction mixture was incubated at 50°C for 30 min and the reducing sugar liberated in the reaction mixture was measured by dinitrosalicylic acid (DNS) method (Miller, 1959). The reaction was stopped by adding 1.0 ml of 1.0 M sodium carbonate solution and the colour that developed as a result of p-nitrophenol liberation was measured at 405 nm. One unit (U) of enzyme activity was defined as the amount of enzyme required to liberate one mole glucose per minute under the assay conditions. The enzyme activity is expressed as $U.g^{-1}$ (units per gram of dry weight substrate).

Selection of the significant factors for cellulase production using Plackett-Burman design

For screening the important medium components, Plakett-Burman design (2-level factorial) (Plackett and Burman, 1946) was used. The variables chosen for this experiment were sawdust (1-10.0 $g.l^{-1}$), $(NH_4)_2SO_4$ (1-6 $g.l^{-1}$) $MgSO_4.7H_2O$ (1-3 $g.l^{-1}$), KH_2PO_4 (1-3 $g.l^{-1}$), temperature (20-80ºC) and pH (4-9). The experimental design for the screening of the variables is given in Table 1. All the variables were denoted as numerical factors and investigated at two widely spaced intervals designated as -1 (low level) and +1 (high level). The effect of individual parameters on cellulase production was calculated by the following Equation.

$$E = (\Sigma M_+ - \Sigma M_-)/N \quad \ldots\ldots\ldots\ldots (1)$$

Where, E is the effect of parameter under study and M (+) and M (-) are responses of cellulase activities in trials, and N is the total number of trials.

Optimization of cellulase enzyme production by H. estonica using central composite design (CCD)

Formulation of the medium was determined for the optimum levels of significant variables for cellulase production. For this, the Response Surface Method (RSM) with a model of CCD was adopted. The important significant factors for the cellulase enzyme production by *H. estonica* were predicted by Plackett-Burman (2-level factorial) such as sawdust ($g.l^{-1}$), $(NH_4)_2SO_4$ ($g.l^{-1}$), temperature (ºC) and pH (Table 2). Each of these factors was assessed at five coded levels (-2, -1, 0, +1 and +2) and in this statistical model totally 30 experimental runs were carried out. In the whole experiment, minimum and maximum actual values of the production medium were used and are presented in Table 3.

Enzymatic hydrolysis of lignocellulose (sawdust) by using H. estonica

Enzymatic hydrolysis reaction was performed by mixing 1 ml of the crude cellulase solution with 3.5 ml of 1.0% (w/v) different concentrations of sawdust. The reaction mixture was incubated at 55°C for 60 min and the sample was withdrawn at 15 intervals for analysis of reducing sugar (i.e. glucose). The amount of reducing sugar in the supernatant was measured using the 3,5-dinitrosalicyclic acid (DNS) method (Miller, 1959). One unit of enzyme activity is expressed as the amount of enzyme that could liberate 1 ml mole of reducing sugar asglucose per min under the conditions (Equation .2)

$$E+S=ES= P+RE \quad \text{----------} (2)$$

Where E- *H. estonica* derived cellulase, S-Substrate as sawdust and RE-residual enzyme. For the assumption 1U/protein cellulase was ability to digest the sawdust and produce 100% of reducing sugar. After the analysis if we got 87% of reducing sugar, the remaining glucose activity assessed was treated as the residual cellulase enzyme.

Activation of the H. estonica derived enzyme cellulase

The specific initial enzyme activity was assessed under the statistically optimized temperature to calculate the energy activation (Equation .3):

$$V= V_0 . \exp (–Ea/RT) \text{ ----------} (3)$$

where: *E*a = Energy of Activation (cal/mol); *R*= Universal law gas constant (1.987 cal/gmol K); *T*= Absolute temperature (K); V_0= Arrhenius pre-exponential constant; and *V*= Initial rate of reaction (µmol of glucose/(min/mg enzyme).

Thermal stability of the cellulase enzyme produced by H. estonica

The cellulase enzyme was produced under the statistical optimized condition by *H. estonica* and it was partially purified and their thermal stability was analyzed under different temperatures (40–80°C). The enzyme was incubated at the specified temperature and pH, in a 0.2% (w/v) solution of sawdust and samples were collected each 20 min to measure the residual enzymatic activity thermally denatured the enzyme is of the first order and obeys the Arrhenius relation

$$Kd= Kd^0 \exp (–Ed/RT) \text{ ----------}(4)$$

$$Ar= A_0 \exp (–Kd\ t) \text{ ----------}(5)$$

where: A_0 = Initial enzymatic activity (U/mg of protein); *A*r= Residual enzymatic activity (U/mg of protein); *E*d= Energy of Thermal Deactivation (cal/mol); Kd^0= Deactivation constant (h^{-1}); and *t*= time (h). It is of interest to calculate also the enzyme half-life ($t_{1/2}$), i.e., the time period necessary for the residual enzymatic activity to decrease to half of this initial value. If the enzyme thermal denaturation follows Equation .5, then there is

an inverse relation between its half-life and the deactivation constant:

$t_{1/2}$= ln 0.5/(–K_d)= 0.693/K_d---------(6)

Initial Rate of Hydrolysis and Substrate Inhibition Test

The effect of substrate concentration on the rate of hydrolysis was studied under statistically optimized conditions at 49°C, pH 8.8, using sawdust solutions from 0.4–20 g.l^{-1} (1.2–58.5 m*M*). From the data the kinetic parameters *Km*, *Ks*, and *V*max were obtained as described in the item for kinetic modeling.

Effect of the cellulase on reducing sugar conversion

Target of the modeling hydrolysis of sawdust, measuring product inhibition and observing the enzyme thermal deactivation for long periods of reaction, hydrolysis was followed up to nearly 100% sawdust conversion, in the absence of exogenously added product (glucose) at the start of the test. An orbital shaker fitted with a 500 ml Erlenmeyer flask was used for the conversion tests. Each flask contained 250 ml of the reaction mixtures of enzyme and substrate. Sampling intervals (15-60 min) were used at the beginning of the reaction and they were increased with reaction time. At the end of the whole reaction period, the residual enzymatic activity was measured.

Determination of Michaelis constant (Km) and maximal velocity (Vmax) of the crude cellulases for sawdust hydrolysis

Michaelis constant (Km) and maximal velocity (Vmax) were determined by adding 1.0 ml of the crude cellulase solution to 3.5 ml of sawdust solution with different concentrations. Sawdust solutions were prepared with acetate buffer (200 mM, pH 4.4) and the concentrations of sawdust in the reaction mixture were adjusted to 2.0, 4.0, 6.0, 8.0, 10.0 and 12.0 µg.ml^{-1}. Then, reaction mixtures were incubated at 55°C for 15 min. The amount of reducing sugar as glucose in the supernatant was measured using modified 3, 5-dinitrosalicyclic acid (DNS) method (Miller, 1959). Values of Km and Vmax were calculated from double-reciprocal plots according to the method of Lineweaver and Burk (Calsavara *et al.*, 1999**;** Zhang and Sang, 2011). Km is expressed as sawdust concentration in the reaction mixture (µg.ml^{-1}) and Vmax is expressed as the amount of liberated reducing sugar as glucose per minute (µg.min^{-1}) during sawdust hydrolysis by 1.0 ml of the crude cellulases.

Results

Cellulase enzyme production by *Hypocrea* species was tested under solid state fermentation. All the 12 strains showed enzyme production. Among them, *Hypocrea estonica* displayed the highest enzyme activity as evident by the maximum zone formed around the colonies (Fig. 1). This species was selected for further statistical optimization studies to maximize the production of cellulase.

Screening of important factors for cellulase production using Plackett-Burman design

An experiment was conducted to select the important factors for the cellulase production by *H. estonica*. For this, a statistical design of Plackett-Burman (2-level factorial) of 16 runs was used The experimental and predicted responses are presented in the Table 1. The model F value of 6.29 implied that the model was significant. The values of Prob<0.01 indicated that the model terms were significant (Table 2). Based on the positive coefficient and significant effects, the important factors, such as sawdust (g.l^{-1}), $(NH_4)_2SO_4$ (g.l^{-1}), temperature (ºC) and pH (Table 2) were selected for the cellulase enzyme production by *H. estonica.*

Optimization of significant factors for cellulase enzyme production by H. estonica using CCD

The 30 runs of central composite design matrix (CCD) of response surface

methodology (RSM) experiments were conducted for the optimization study. The effects of four independent variables sawdust ($g.l^{-1}$), $(NH_4)_2SO_4$ ($g.l^{-1}$), temperature (ºC) and pH are shown in Table 3 along with the mean experimental and predicted values. The regression analysis of the optimization study indicated that the model terms, X_1, X_2, X_3, X_4, X_1X_2, X_1X_3, X_1X_4, X_2X_3, X_2X_4, X_3X_4, X_1^2, X_2^2, X_3^2 and X_4^2 were significant (Table 4) ($P<0.05$). These results indicated that the sawdust ($g.l^{-1}$), $(NH_4)_2SO_4$ ($g.l^{-1}$), temperature (ºC) and pH and also their interactions had the direct association with cellulase production and the lack of fit (0.00) also suggested that the obtained experimental data were of a good fit with the model.

The regression Equation coefficients were calculated and the data were fit to a second-order polynomial Equation. The response, cellulase production (Y) by *H. estonica* can be expressed in terms of the following regression Equation:

$$Y \text{ Cellulase enzyme } (IU.ml^{-1}) = 56.86+2.79X_1+0.42X_2-0.93X_3+8.16X_4-0.71X_1^2-3.81X_2^2-6.31X_3^2-5.26X_4^2+0.96X_1X_2-1.46X_1X_3+2.27X_1X_4-0.71X_2X_3-0.26X_2X_4-1.26X_3X_4 \quad (7)$$

Whereas: X_1 is sawdust ($g.l^{-1}$), X_2 = $(NH_4)_2SO_4$ ($g.l^{-1}$), X_3 = temperature (ºC) and X_4 = pH.

The regression Equation obtained from the ANOVA showed that the R^2 (multiple correlation coefficient) was 0.99 (a value >0.80 indicates fitness of the model). This is an estimate of the fraction of overall variation in the data accounted by the model and thus the model is capable of explaining 99% of the variation in response. The 'adjusted R^2' is 0.998 and the 'predicted R^2' is 0.995, which indicated that the model fitness was good.

A plot of the standard errors of responsible factors (sawdust and $(NH_4)_2SO_4$) in cellulase production by *H. estonica* is shown in Fig. 2a. The shape of the low level of standard error plot exhibited circular contours and symmetrical shape around the centric and this was used to generate the standard error plot for design evaluation. The standard error value was fit with model and determined the standard error value of 0.42. It was the best value for the acceptable RSM statistical model. Further confirmation of the model fitness on *H. estonica* cellulase production was plotted with experimental values and predicted values of the model (Fig. 2b). This proved that model was fit with the normal probability percentage and standardized residuals values (Fig. 2c). A perturbation plot was used for the assessing the optimized condition for the cellulase production (Fig. 2d).

In order to determine the optimal levels of each variable for maximum cellulase production, pictorial 3D response surface plots were constructed by plotting the response (cellulase production) on the Z-axis against any two independent variables, while maintaining other variables at their central levels (Figs. 3a-f). Based on the 3D surface plot and perturbation plot the statistical optimized condition was predicted for the augmentation of the cellulase production. Statistically optimized conditions for the enhanced cellulase production were found as 7.69 $g.l^{-1}$ of sawdust, 3.59 $g.l^{-1}$ of $(NH_4)_2SO_4$, under 49°C temperature at pH of 8.8. Further the statistical optimized conditions for the cellulase production were confirmed experimentally. Under the optimal culture conditions, the enzyme activity was 3.5 fold higher than that in normal conditions.

Thermal stability of the cellulase enzyme derived from H. estonica

The thermal stability of the cellulase enzyme was attributed with different ranges of the temperature (20-80°C) with intervals of the 10°C incubated up to 140 min with the intervals of 20 min sampling collected for the analysis of the residual enzyme activity.

Thermal deactivation R^2 values showed 97-87% stability of cellulase enzyme produced by *H. estonica* under the 20-30°C, and 63-81% of enzyme was stable under the temperature of 40-60°C and enzyme was denatured under the temperature of 70 and 80°C (Fig. 4 & Table 5). Table 5 showed the calculated values of enzyme deactivation.

Residual activity of enzymes under the temperatures of 20-80°C, was applied to Equation 5 giving the result for the deactivation constant (K_d) as shown in the Table 5. These values (K_d) were applied to Equation 4 as presented in fig. 5 which gave the energy of thermal deactivation of the cellulase enzyme as 29.08 Kcal/mol.

$K_d=2.5233.10^{52}$ exp [-29.08/Rt], r=0.93-----(8)

Michaelis constant (Km) and maximal velocity (Vmax) of crude cellulases for ligninocellulose (sawdust) hydrolysis

According to the curve of Linewear-Burk (Fig. 6), Michaelis constant (Km) and maximum velocity (Vmax), the crude cellulases had the optimal temperature of 49°C, pH of 8.4 and exhibited Michaelis constant (Km) value of 4.2 mg/ml and maximum velocity (Vmax) of 1.21l mol glucose/min by one $IU.ml^{-1}$ of the crude cellulases.

Discussion

Trichoderma (*Hypocrea*) species of terrestrial origin are well-known for extracellular enzyme production (Zhang *et al.*, 2006; Sohail *et al.*, 2009; Zhang *et al.*, 2011). However, marine strains of *Trichoderma* (*Hypocrea*) are little known for their enzyme production (Kathiresan and Manivannan, 2006; Saravanakumar and Kathiresan, 2011). Present study was undertaken on the potential of marine *Trichoderma* species on cellulase enzyme production and to test thermal stability of the enzyme by using the thermal integrated kinetics and conversion of the reducing sugars from sawdust. Many earlier workers have studied the mixture of sugarcane bagasse and wheat bran as substrates (Qin *et al.*, 2010), corn (Panagiotou *et al.*, 2003), wheat bran (Vu *et al.*, 2011), wheat straw (Kachlishvili *et al.*, 2005) and rice straw (Jatinder *et al.*, 2006a). However, only few studies are available on the sawdust as substrate for fungal cellulase production (Kathiresan *et al.*, 2011a&b). In the present study, all 12 strains of *Hypocrea* exhibited cellulase production on sawdust substrate. In particular, *H. estonica* was potent to exhibit the highest enzyme production and hence studied further for optimal conditions for maximium production of the enzyme.

Cellulase activity varied with species and substrate used. In the present study, cellulase activity of *H. estonica* was found to be 56.86 $IU.g^{-1}$ when sawdust was used as substrate. This is similar to that of *Trichoderma reesei* Rut C-30 with activity of 56.6 $IU.g^{-1}$ at the substrate of pulp and wheat bran (Oberoi *et al.*, 2010). These values are greater than that of *Trichoderma koningii* AS3.4262 on the substrate of vinegar waste and wheat bran with activity of 30.6 $IU.g^{-1}$ (Liu and Yang, 2007). Some strains of *Trichoderma* have been reported for high activity of cellulases. For example, the activity was 115.8 $IU.g^{-1}$on rice bran by *Trichoderma reesei* QM9414 and 77.15 $IU.g^{-1}$ on sugarcane straw by *T. citrinoviride* (Guerra *et al.*, 2006).

Important factors for enzyme production were detected by using two level factorial designs. The factors selected were sawdust ($g.l^{-1}$), $(NH_4)_2SO_4$ ($g.l^{-1}$), temperature (°C) and pH based on the positive coefficient and significant effects (Table 2). The present study observed that the cellulase production varied with sawdust concentration, in accordance with the previous works (Kalogeris *et al.*, 2003; Brijwani *et al.*, 2010), which have reported that lignocellosic, cellulose materials and hemicellulose of the substrate as a whole is a good inducer of cellulolytic enzyme

system, and that the carbon and nitrogen ratio is crucial for the growth of microorganisms and for producing specific products under solid state fermentation.

The present study identified the optimal conditions for maximum cellulase production as 7.69 $g.l^{-1}$ sawdust, and 3.59 $g.l^{-1}$ of $(NH_4)_2 SO_4$, under temperature of 49°C and at pH of 8.8. The enzyme production of *Trichoderma* was higher than that previously reported by other workers. As compared to the previous reports, the present study showed higher cellulase production under the alkaline pH and this might be attributed to the marine origin of the strains (Zhang and Sang, 2011). Similarly alkaline pretreatment of lignocellulosic feedstock causes swelling, leading to decreased polymerization, increased internal surface area, disruption of lignin structure, and separation of structural linkages between lignin and carbohydrates and also to produce the mono-sugars (Zheng *et al.*, 2009). The optimal temperature was found as high as 49°C, as is also reported previously for *Trichoderma* (Sun *et al.* 2009). Regarding thermal deactivation of cellulase enzyme, this enzyme is relatively less stable (Ed = 29.08 Kcal/mol) than amyloglucosidase (Ed= 50.6kcal/mol), or invertase (Ed= 64.9kcal/mol) and cellulase (81.6kcal.mol^{-1}; Calsavara *et al.*, 1999).

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Table.1. Selection of significant parameters influencing cellulase production and predicted and actual cellulase activity by using Plackett-Burman (2-level factorial) experimental design

(A) Sawdust ($g.l^{-1}$)	(B) $(NH4)_2$ ($g.l^{-1}$)	(C) $MgSO_4$ ($g.l^{-1}$)	(D) KH_2PO_4	(E) Temperature (^oC)	(F) pH	Cellulase activity ($IU.ml^{-1}$)	
						Experimental	Predicted
1	1	1	1	20	4	22.5	24.9
10	1	1	1	80	4	42.5	40.0
1	5	1	1	80	9	31.3	21.6
10	5	1	1	20	9	32.3	41.6
1	1	3	1	80	9	12.5	15.6
10	1	3	1	20	9	36.3	35.6
1	5	3	1	20	4	23.2	25.8
10	5	3	1	80	4	45.5	40.9
1	1	1	3	20	9	22.2	18.7
10	1	1	3	80	9	32.2	33.8
1	5	1	3	80	4	20.2	24.0
10	5	1	3	20	4	45.2	44.0
1	1	3	3	80	4	13.0	18.0
10	1	3	3	20	4	43.3	37.9
1	5	3	3	20	9	23.2	19.6
10	5	3	3	80	9	31.3	34.7

Table 2. Statistical parameters for selected the linear polynomial model using Plackett-Burman design

Term	Coefficient	SE Coefficient	F value	Probability
Constant	29.79	1.53	6.29	0.01
(A) Sawdust ($g.l^{-1}$)	8.78	1.53	32.80	0.00
(B) $(NH_4)_2$ ($g.l^{-1}$)	1.74	1.53	1.28	0.29
(C) $MgSO_4$ ($g.l^{-1}$)	-1.27	1.53	0.68	0.43
(D) KH_2PO_4 ($g.l^{-1}$)	-0.97	1.53	0.40	0.54
(E) Temperature (^oC)	1.22	1.53	0.64	0.45
(F) pH	-0.49	1.81	-0.65	0.03

Table 3. Central composite design matrix for the experimental design and predicted responses for cellulase activity (IU/ml)

Run order	(A) Sawdust ($g.l^{-1}$)	(B) $(NH_4)_2SO_4$ ($g.l^{-1}$)	(C) Temperature (ºC)	(D) pH	Cellulase activity ($IU.ml^{-1}$)	
					Experimental	Predicted
1.0	10.0	5.0	80.0	4.0	29.0	28.9
2.0	5.5	7.0	50.0	6.5	42.6	42.4
3.0	10.0	5.0	20.0	9.0	55.5	55.4
4.0	5.5	3.0	50.0	11.5	52.3	52.2
5.0	5.5	3.0	-10.0	6.5	33.1	33.5
6.0	5.5	3.0	50.0	6.5	56.9	56.9
7.0	5.5	3.0	110.0	6.5	29.9	29.8
8.0	1.0	5.0	20.0	9.0	40.7	40.5
9.0	10.0	1.0	20.0	9.0	52.0	51.7
10.0	1.0	1.0	20.0	4.0	27.0	25.8
11.0	1.0	5.0	80.0	9.0	37.7	37.6
12.0	5.5	3.0	50.0	6.5	56.9	56.9
13.0	10.0	5.0	20.0	4.0	32.8	32.5
14.0	5.5	3.0	50.0	1.5	19.2	19.5
15.0	10.0	1.0	80.0	9.0	46.0	45.9
16.0	5.5	3.0	50.0	6.5	56.9	56.9
17.0	1.0	5.0	80.0	4.0	29.1	28.8
18.0	1.0	5.0	20.0	4.0	26.3	26.7
19.0	10.0	1.0	20.0	4.0	27.5	27.9
20.0	14.5	3.0	50.0	6.5	43.5	43.4
21.0	10.0	1.0	80.0	4.0	27.3	27.0
22.0	1.0	1.0	80.0	9.0	40.8	40.6
23.0	5.5	3.0	50.0	6.5	56.9	56.9
24.0	-3.5	3.0	50.0	6.5	31.9	32.2
25.0	1.0	1.0	80.0	4.0	30.5	30.8
26.0	10.0	5.0	80.0	9.0	46.0	46.7
27.0	5.5	3.0	50.0	6.5	56.9	56.9
28.0	1.0	1.0	20.0	9.0	40.3	40.6
29.0	5.5	-1.0	50.0	6.5	40.4	40.8
30.0	5.5	3.0	50.0	6.5	56.9	56.9

Table 4. Analysis of variance table (ANOVA) for response surface methodology of main effects and interacting effects of parameters in quadratic model for cellulase production

Source	Sum of squares	Df	Mean Square	F Value	p-value Prob > F
Model	4025.214	14	287.5153	1418.078	< 0.0001
(A) Sawdust ($g.l^{-1}$)	186.4187	1	186.4187	919.4512	< 0.0001
(B) $(NH_4)_2SO_4$ ($g.l^{-1}$)	4.257437	1	4.257437	20.99845	0.0004
(C) Temperature (ºC)	20.55659	1	20.55659	101.3889	< 0.0001
(D) PH	1598.19	1	1598.19	7882.566	< 0.0001
AB	14.65294	1	14.65294	72.27097	< 0.0001
AC	33.89672	1	33.89672	167.1848	< 0.0001
AD	82.31777	1	82.31777	406.0063	< 0.0001
BC	7.992405	1	7.992405	39.42	< 0.0001
BD	1.044656	1	1.044656	5.152435	0.0384
CD	25.32189	1	25.32189	124.8922	< 0.0001
A^2	622.5222	1	622.5222	3070.393	< 0.0001
B^2	399.0018	1	399.0018	1967.95	< 0.0001
C^2	1090.903	1	1090.903	5380.533	< 0.0001
D^2	757.8862	1	757.8862	3738.033	< 0.0001
Residual	3.04125	15	0.20275		
Lack of Fit	3.04125	10	0.304125		
Pure Error	0	5	0		
Cor Total	4028.255	29			

Statistically significant *** (P<0.0001), *(P< 0.05), NS Non-significant

Table 5. Thermal deactivation constant values (K_d) derived for cellulase from *H. estonica* at different temperatures

Temperature (°C)			Experimental
	K_d (h^{-1}) (Equation .4)	R^2	$t_{1/2}$
20	0.01	0.87	69.3
30	0.016	0.94	43.3125
40	0.021	0.81	33
50	0.03	0.66	23.1
60	0.036	0.63	19.25
70	0.05	0.56	13.86
80	0.04	0.46	17.325

Figure. 1. Cellulase production by different *Trichoderma* species: (TSK1-TSK10) and ((SKS1), and (SKS2)

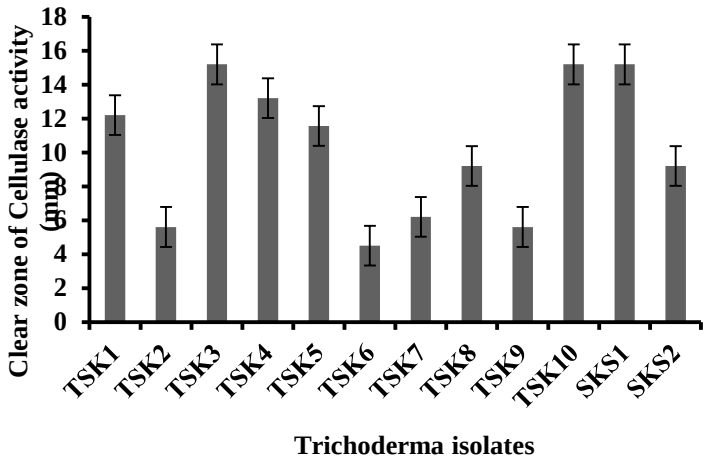
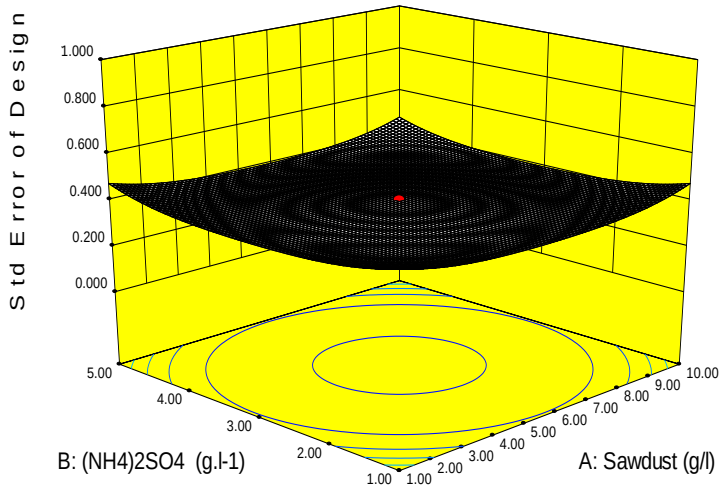
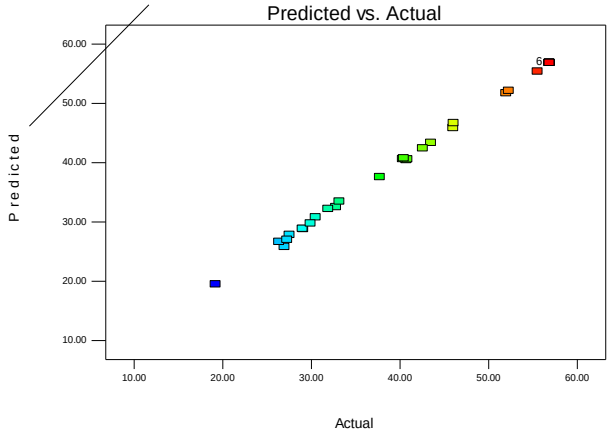


Figure. 2. (a) Three-dimensional standard error plot (b) Predicted and actual experimental response (c) Normal plot for the residuals and normal percentage of probability for the response of predicted and experimental values, and (d) Perturbation plots for cellulase production

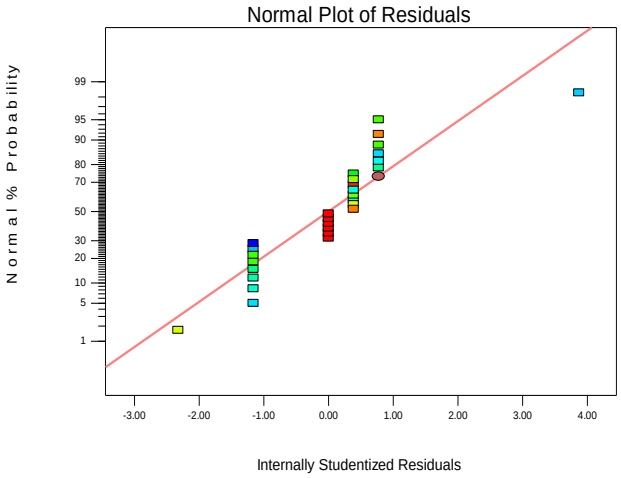
(a)



(b)


Predicted vs. Actual
Predicted
Actual
60.00
50.00
40.00
30.00
20.00
10.00
10.00
20.00
30.00
40.00
50.00
60.00
6

(c)


Normal Plot of Residuals
Normal % Probability
Internally Studentized Residuals
99
95
90
80
70
50
30
20
10
5
1
-3.00
-2.00
-1.00
0.00
1.00
2.00
3.00
4.00

(d)

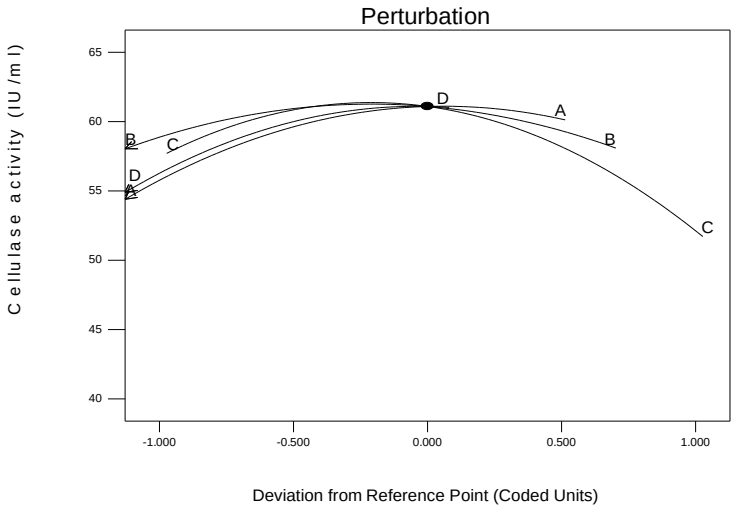
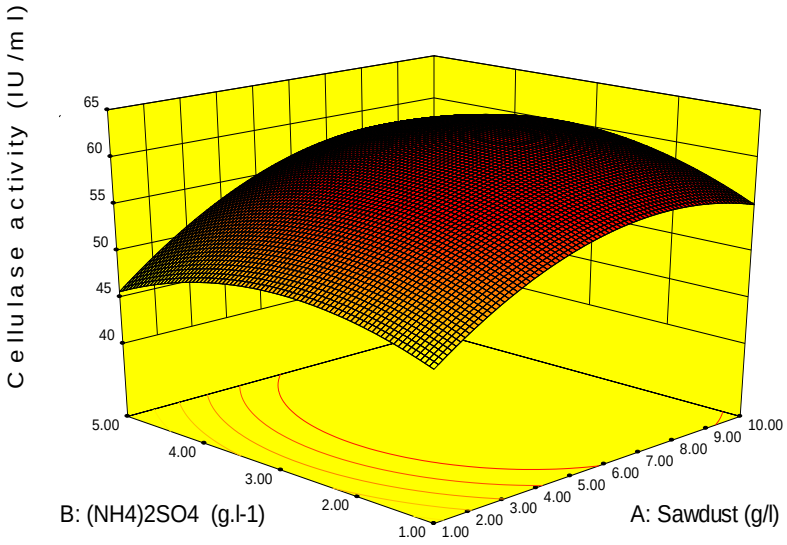
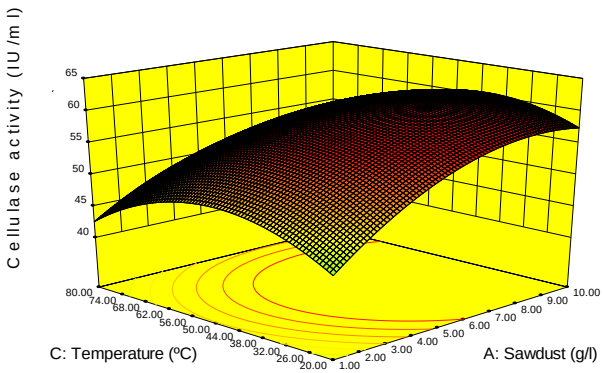


Figure. 3. Three-dimensional response surface plot for the (a) Effect of $(NH_4)_2SO_4$ and sawdust, (b) Effect of temperature and sawdust, (c) Effect of pH and sawdust, (d) Effect of temperature and $(NH_4)_2SO_4$, (e) Effect of pH and $(NH_4)_2SO_4$, and (f) Effect of pH and temperature on enzyme production by *H. estonica*

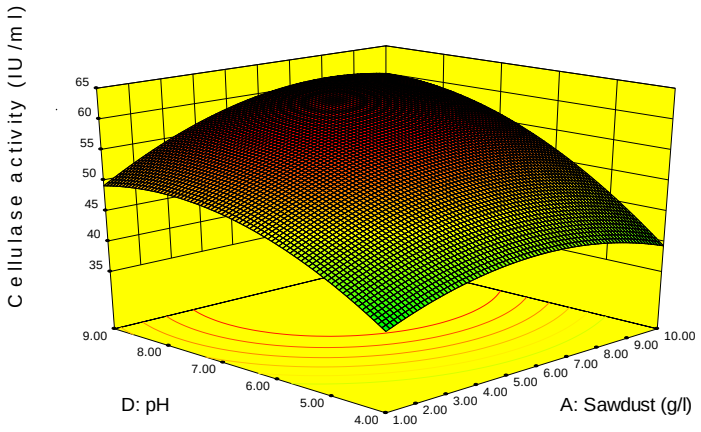
(a)



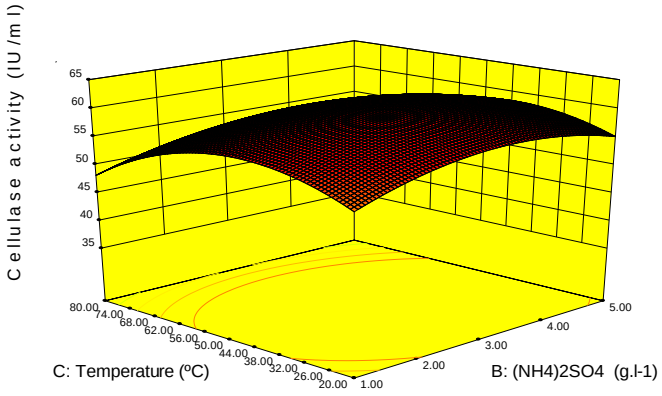
(b)


Cellulase activity (IU/ml)
65
60
55
50
45
40
80.00
74.00
68.00
62.00
56.00
50.00
44.00
38.00
32.00
26.00
20.00
C: Temperature (ºC)
1.00
2.00
3.00
4.00
5.00
6.00
7.00
8.00
9.00
10.00
A: Sawdust (g/l)

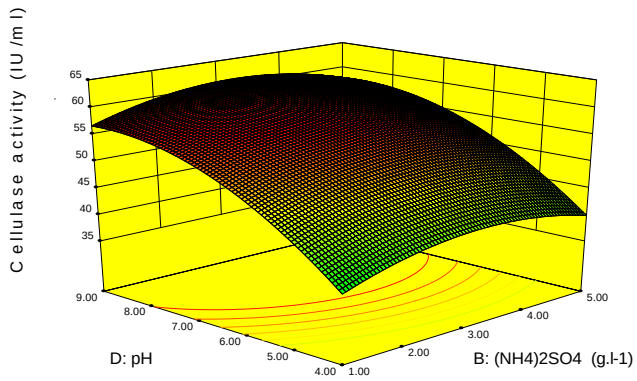
(c)


Cellulase activity (IU/ml)
65
60
55
50
45
40
35
9.00
8.00
7.00
6.00
5.00
4.00
D: pH
1.00
2.00
3.00
4.00
5.00
6.00
7.00
8.00
9.00
10.00
A: Sawdust (g/l)

(d)


Cellulase activity (IU/ml)
65
60
55
50
45
40
35
80.00
74.00
68.00
62.00
56.00
50.00
44.00
38.00
32.00
26.00
20.00
C: Temperature (ºC)
1.00
2.00
3.00
4.00
5.00
B: (NH4)2SO4 (g.l-1)

(e)



(f)

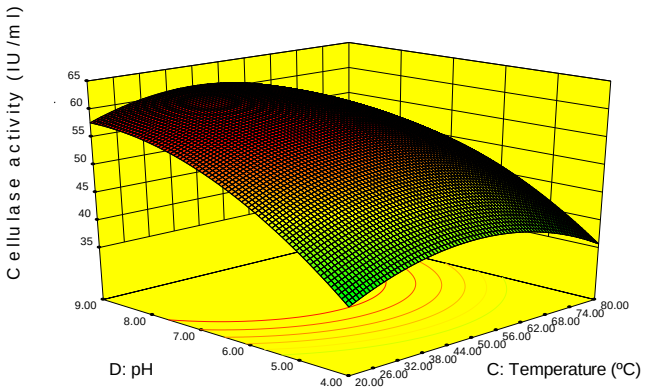


Figure. 4. Residual cellulase activity incubated under different temperatures

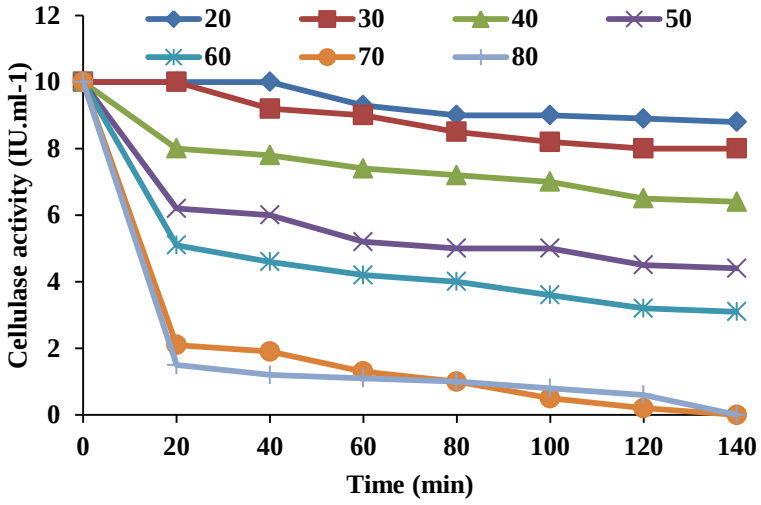


Figure. 5. Arrhenius plot for the energy of thermal deactivation of cellulase

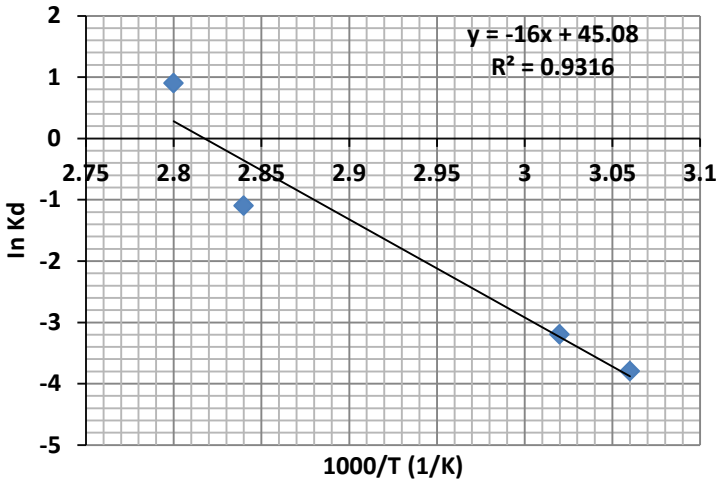


Figure. 6. Curve of Linewear-Burk of the crude cellulases for cellulase (sawdust) hydrolysis