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ORIGINAL PAPER

Cellulase Production from Pre-treated Pea Hulls Using *Trichoderma reesei* Under Submerged Fermentation

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Abstract

Purpose Pea hulls have a significant contribution to agricultural waste and are left unattended. It has a rich nutrient media and may have application in waste utilization. In this investigation, the potential of pea hulls for the production of cellulase has been explored.

Methods The effect of H_2O_2 concentration, agitation speed and harvesting time on cellulase production by *Trichoderma reesei* QM9414 under submerged fermentation at constant pH of 5.0 and temperature 30 ± 0.1 °C were studied.

Results The maximum filter paper (FP) cellulase activity of 0.372 ± 0.019 U/mL was obtained at 91 h incubation period and 120 rpm agitation speed. Based on the optimized results of fermentation parameters, 120 rpm agitation speed, 5% H_2O_2 concentration and 91 h harvesting time was recommended for efficient cellulase production. The effect of harvesting time on protein, reducing sugar and cellulase activity was pre-dominant. The purified cellulase enzyme specific activity was recorded as 13.8 U/mL.

Conclusions The results indicate that the production of cellulase from green pea hulls may provide a novel and economical solution for industrial waste disposal.

Keywords Cellulase · Pea hull · Submerged fermentation · *Trichoderma reesei*

List of symbols

$\beta_0, \beta_i, \beta_{ij}, \beta_{ii}$	Model coefficients
p	Number of explanatory variables (excluding constants)
y_i	Observed data
$\bar{y}_l$	Mean of observed data
$\hat{y}_l$	Predicted data
N	Sample size
$X_{i,j}$	Independent variables
Y	Dependent variable

Introduction

Increasing concern for pollution that occurs from agricultural and industrial wastes has stimulated interest in converting waste materials into commercially valuable products. Agricultural wastes are widely available in the form of crop residues (residual stalks, straw, leaves, roots, husks, sheaths, bagasse etc.) and are easily renewable. The food industry produces large volumes of wastes (both solids and liquids) which have potential for recycling and conversion into different products [1]. Agriculture waste being organic in nature is easily assimilated by microorganisms mainly fungi which make it appropriate for enzyme production. Lignocellulosic biomass such as carrot peel, onion peel, potato peel and wheat straw among others are known to be good source for microbial enzyme production. Pea hulls are another such contributor to agricultural waste, the potential of which attracts attention. Pea hulls are waste, generated after separation of pea (*Pisum sativum*) seeds and represents up to 60% of total pea. These hulls are rich in dietary fiber, twice as much as wheat bran [2] and are mostly produced by frozen food processing industries. It is a rich source of nutrition

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with 3.8% protein, 41.1% glucose, 1.7% ash, 1.0% acetic acid and 0.5% methanol in its dry matter composition [3]. Furthermore, they are lightly colored and tasteless which makes them a good source of fiber and carbon with 82.30% total dietary fiber, 62.3% cellulose and 8.2% hemicellulose [4]. Pea hulls may therefore be utilized for manufacturing of ethanol, starch, dietary fiber, enzymes and other value added products after fermentation. Since, the ultimate goal is to develop a commercial low cost technique for processing pea hull waste; submerged fermentation may be preferred over solid state fermentation. In submerged fermentation, substrate is used in a slurry form making it economical due to its lower capital and operating expenses and higher productivity [5, 6]. Industrially important enzymes have traditionally been obtained from submerged fermentation (SmF) because of the ease of handling and greater control of environmental factors such as temperature and pH. The production of cellulase can be enhanced by choosing an appropriate fungal strain complimentary to the substrate. Pea hull could be a better carbon source for cellulase enzyme using *Trichoderma reesei*. Cellulase produced by *T. reesei* hydrolyzes 91% of the cellulose in pretreated wheat straw within 24 h [7] showing good capability for enzyme production.

The use of rice straw [8, 9], rice bran [10, 11], wheat bran [12–14] olive processing residue [15], onion peel, potato peel, pineapple waste [16], sugarcane bagasse [17], sugar beet pulp [18] as a substrate for *T. reesei* has been reported in literatures. However, the use of pea hull as a sole source of carbon for this microorganism remains unexplored. The objective of this study therefore, is to examine the effect of fermenting parameters on cellulase yield from peahull using *T. reseei* under submerged fermentation. This study attempts to examine and optimize a process which could make pea production economically more viable, minimizing the problems of pollution and waste disposal in food industries.

Materials and Methods

Fungal Strain and Inoculum

Different strains have different tolerance limit for utilization of cellulose and sugar concentration as carbon source. For the present study, *T. reesei* QM9414 was used for the production of cellulase enzyme. *Trichoderma reesei* QM9414 strain was obtained from IMTECH, Chandigarh on the basis of their enzyme production properties. For experimental purposes, spore suspension was prepared by incubating the cultures on potato dextrose agar (PDA) plates at 30 °C for 5–6 days until sufficient sporulation was observed. The spores were harvested using distilled water and the count of approximately 10^8 spores/mL was used for inoculation.

Experimental Design

Agitation speed (93, 100, 110, 120, 127 rpm), H_2O_2 pretreatment concentration (3.64, 5, 7, 9, 10.36%) and harvesting time (31, 48, 72, 96, 112 h) were identified as the most important process variables as per literature review. Some parameters for the experiments were kept constant as described in Table 1. Central composite rotatable design (CCRD) was adopted for a three variable-5 level ($-a$, -1, 0, $+1$, $+a$) problem computing to a total of 20 experiments with six set of replicates. The terminal levels ($-a$ and $+a$) were calculated as $a=2^{0.25v}$, where v is the number of variables. Coded values (C_v) of levels were converted to actual values (A_v) using Eq. (1), where H and L represent A_v corresponding to $+1$ and -1. The experimental design matrix has been shown in Table 2.

$$A_v = \frac{H(1 + C_v) + L(1 - C_v)}{2} \quad (1)$$

Substrate Procurement and Pre-treatment

Pea pods were procured from the local market of Pantnagar, as per requirement and separation of peas from pea pods was done manually. Slurry was made by grinding the peahulls in a food processor (Sujata-make, 810 W motor; 500 mL capacity). No additional water was added, as sufficient amount of moisture was present in the peahulls. The sample were taken in conical flash, tightly sealed with cotton plug and placed in an autoclave for 10 min to avoid solubilization. During thermal treatment, the disruption of cell wall, cell membrane and bond hydrolization occurs by the phenomenon of blanching, resulting in greater availability of cellulosic as well as antioxidant and phenolic constituents. Under these critical situations, *T. reesei* has the ability to grow well. Literature suggests, *T. reesei* has the tolerance to grow in the phenolic environment up to a certain level [19]. After thermal treatments the slurry was cooled to 25 °C. Different

Table 1 Constant parameters selected for experimentation

Factors	Range
Type of fermentation	Submerged fermentation
Thermal pretreatment time and temperature	20 min, 121 °C
Fermentation temperature	30 °C
Fermentation time	5 days
Fermentation pH	5
Inoculum concentration	0.8×10^8 spores/mL $\approx 10^8$ spores/mL
Sample size	100 g
Grinding time	3 min

Table 2 Central composite rotatable design (CCRD) matrix with coded and actual values of process variables

Run no	Coded values			Actual values		
	X_1	X_2	X_3	Agitation speed (X_1) (rpm)	H_2O_2 conc (X_2) (%)	Harvesting time (X_3) (h)
1	− 1	− 1	− 1	100	5	48
2	1	− 1	− 1	120	5	48
3	− 1	1	− 1	100	9	48
4	1	1	− 1	120	9	48
5	− 1	− 1	1	100	5	96
6	1	− 1	1	120	5	96
7	− 1	1	1	100	9	96
8	1	1	1	120	9	96
9	− 1.682	0	0	93	7	72
10	1.682	0	0	127	7	72
11	0	− 1.682	0	110	3.64	72
12	0	1.682	0	110	10.36	72
13	0	0	− 1.682	110	7	31
14	0	0	1.682	110	7	112
15	0	0	0	110	7	72
16	0	0	0	110	7	72
17	0	0	0	110	7	72
18	0	0	0	110	7	72
19	0	0	0	110	7	72
20	0	0	0	110	7	72

concentrations of H_2O_2 (3.64, 5, 7, 9, 10.36% v/v) was then added to 100 g of slurry and taken in a 250 mL conical flask for alkaline treatment. Alkaline treatment results in the disruption of the lignin seal to increase enzyme access to holocellulose (residue after delignification), reduction of cellulose crystallinity, increase in surface area and porosity of the substrate and also the hydrolysis rate. The pH of the substrate was adjusted to 5.0 by using HCl from an initial pH of 7.23.

Estimation of Reducing Sugar

Sugar was estimated by DNS method [20]. DNS reagent was prepared and standard protocol was followed [21]. Fermented samples were withdrawn and centrifuged at 9000 rpm for 20 min. One milliliters of the centrifuged sample was taken to which 3 mL of freshly prepared DNS reagent was added. The contents were thoroughly mixed and placed in a water bath at 90 °C for 10 min. The tubes were taken out and cooled immediately and the contents of the tube were diluted if required. The absorbance was measured using a UV–Vis spectrophotometer. A reference blank was prepared containing 1 mL distilled water in place of sample extract. The concentration of sugar in the sample was estimated by computing the absorbance at 540 nm against the standard curve of glucose.

Estimation of Protein

Protein was estimated by Lowry's method and standard reagents A (copper–tartarate carbonate complex) and B (Folin–Ciocalteu reagent) were prepared [22]. The samples were withdrawn after fermentation and centrifuged at 9000 rpm for 20 min. Five milliliters of reagent A was then added to 1 mL of the supernatant collected from the centrifuged sample. The tubes were shaken vigorously and allowed to stand for 10 min following which 0.5 mL of reagent B was added and mixed well. The final solutions were incubated at room temperature under dark conditions for 30 min till the development of blue color due to the formation of heteropolymolybdenum blue complex. Absorbance measurements were taken at 660 nm against blank using UV–Vis spectrophotometer.

Determination of Enzyme Activity

Cellulase activity was measured using the filter paper activity (FPA) assay, expressed in filter paper units (FPU) as described by [23]. This method measures the release of reducing sugar produced in 60 min from a mixture of enzyme solution (1 mL) and of citrate buffer (0.05 M, pH 4.8) in the presence of 50 mg Whatman No.1 filter paper (1 × 6 cm strip) and incubated at 50 °C. Sample withdrawn after fermentation was centrifuged at 9000 rpm for 20 min. The supernatant (solution) collected

from the centrifuged sample was treated as enzyme source. The reaction was terminated by adding DNS reagent (3 mL). A reagent blank (1.5 mL citrate buffer), enzyme blank (1.0 mL citrate buffer + 1.0 mL enzyme dilution) and substrate blank (1.5 mL citrate buffer + filter-paper strip) were prepared and DNS reagent (3 mL) was added to them. All the samples were boiled for 5 min in a water bath and then cooled at room temperature for colour stabilization. The tube contents were then diluted with distilled water (15 mL), mixed thoroughly and allowed to stand for 20 min until the pulp was settled. The absorbance was recorded at 540 nm and values were quantified using the standard curve calibration after subtraction of enzyme and substrate blank. The reducing sugars released were determined using the 3, 5-dinitrosalicylic acid (DNS) assay with glucose as a standard [20].

Enzyme Purification

All procedures of cellulase purification were carried out at 4 °C. The culture supernatant was separated by centrifugation at 9500 rpm for 15 min. After obtaining maximum clarity, solid crystals of ammonium sulphate were added to the crude enzyme extract until 60% saturation and kept for over 12 h. The resulting precipitate was collected by centrifugation at 9500 rpm for 15 min. After centrifugation, the supernatant was separated and the sediments were dissolved in 0.05 M citrate buffer (pH 4.8). Ten mL solution was kept in a dialysis bag which was then sealed securely and dialyzed against citrate buffer. The buffer was changed every 4 h for a period of 12 h. The specific activity of partially purified cellulase was determined before and after dialysis. The final sample was lyophilized at a temperature of −40 °C and 0.25 mbar pressure before storage at 4 °C for further use.

Statistical Analysis

Experiments were conducted randomly. The experimental design chosen (Table 2) consisted of six replicates from which the standard deviation (SD) was computed. The same SD was assumed for all experiments due to design symmetry. Mean values for all parameters were examined for significance by analysis of variance (ANOVA). The statistical data (significant model fits, simulated graphs) was generated using Design Expert software v.9.0.6.2 (Stat-Ease) at 1, 5 and 10% level of significance. The response was expressed as a second-order polynomial as described by Eq. (2). Mathematical expressions for the coefficient of determination (R^2) and adjusted R^2 or R^2_{adj} have been shown in Eqs. (3) and (4). The use of R^2_{adj} may be justified due to non-linearity in data which may not be accurately determined by the value of R^2.

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \beta_{ij} X_i X_j + \sum_{i=1}^{n} \beta_{ii} X_i^2 \quad (2)$$

$$R^2 = 1 - \frac{\sum_{i=1}^{n} (y_i - \hat{y}_1)^2}{\sum_{i=1}^{n} (y_i - \bar{y}_1)^2} \quad (3)$$

$$R^2_{adj} = \frac{(1 - R^2)(1 - N)}{(N - p - 1)} \quad (4)$$

Results and Discussion

Reducing sugar, protein content and cellulase activity were estimated using analytical methods described in Materials and Methods (Estimation of reducing sugar, Estimation of protein and Determination of enzyme activity respectively). The responses obtained for different sets of process conditions have been shown in Table 3. The significance of the effect of individual parameters on the responses has been shown in Table 4. Cellulase produced amounted to 0.172 mg from 10 mL of partial purified sample of fermented peahull slurry (100 g). The purified enzyme had specific cellulase activity 13.8 U/mL. These results were higher or in close agreement with reported findings [16, 24–26].

Cellulase Activity

Cellulase production was compared under various conditions with *T. reesei*. Maximum cellulase activity of 0.315 ± 0.019 U/mL was obtained at 110 rpm, 7% H_2O_2 concentration and 72 h harvesting time (Table 3) which was comparable to the findings of Bhavna [21] and Nema [27] but lower than Mrudula [28] and Tallapragada [29]. The reported difference in cellulase activity under submerged fermentation may vary due to variation in substrate considered. The results show that the production of enzyme is slow at the beginning of fermentation and then increased with fermentation. As the alkaline concentration increased upto a particular range, the growth of *T. reesei* also increased. This could be attributed to the increase in crystallinity of cellulose and removal of lignin and other inhibitors. Thereby, cells utilized cellulose as a carbon source and increased the production of cellulase. At lower harvesting time, the cellulase activity was reduced. This indicated that short reaction times may not be sufficient for the degradation of cellulose or crystalline fraction of cellulose. Thus, the hydrolysis time should be sufficient for the degradation of cellulose to increase the enzyme production [30–32]. The effect of harvesting time on cellulase activity was observed to be statistically significant ($p < 0.01$). The interaction effect of variables were found to be non-significant ($p > 0.05$) which shows no synergy between variables which could contribute to increase in cellulase activity. However, minor interaction between H_2O_2 concentration and harvesting time ($p < 0.1$) was observed

Table 3 Responses obtained for dependent variables for 20 experimental runs

Run no.	X_1: agitation speed (rpm)	X_2: H_2O_2 conc. (%)	X_3: harvesting time (h)	Protein (mg/mL)	Reducing sugar (mg/mL)	Cellulase activity (U/mL)
1	100	5	48	0.95	3.28	0.304
2	120	5	48	0.98	3.13	0.134^{L}
3	100	9	48	1.05	3.27	0.303
4	120	9	48	1.06	3.19	0.138
5	100	5	96	1.56	1.65	0.305
6	120	5	96	1.62	1.70	0.137
7	100	9	96	1.45	1.56	0.304
8	120	9	96	1.70	1.6	0.181
9	93	7	72	1.02	2.33	0.163
10	127	7	72	1.34	2.15	0.253
11	110	3.64	72	0.96	2.25	0.314
12	110	10.36	72	1.25	2.34	0.309
13	110	7	31	0.89^{L}	3.55^{H}	0.158
14	110	7	112	1.78^{H}	1.03^{L}	0.296
15	110	7	72	1.48	2.19	0.315^{H}
16	110	7	72	1.47	1.99	0.290
17	110	7	72	1.48	2.23	0.305
18	110	7	72	1.30	2.11	0.264
19	110	7	72	1.46	2.42	0.287
20	110	7	72	1.39	2.32	0.270

L minimum, *H* maximum

Table 4 Regression coefficients of polynomial model for responses of pea hull fermentation

Source	Protein (mg/mL)		Reducing sugar (mg/mL)		Cellulase activity (U/mL)	
	Coeff	p value	Coeff	p value%	Coeff	p value
X_1: agitation speed	0.065	0.0321**	− 0.032	0.4125	− 2.28E-03	0.460
X_2: H_2O_2 conc	0.047	0.104*	7.4E-004	0.98	− 3.34E-03	0.288
X_3: harvesting time	0.28	< 0.0001***	− 0.78	< 0.0001***	0.033	< 0.0001***
$X_1 X_2$	0.021	0.547	7.5E-003	0.88	− 1.0E-03	0.802
$X_1 X_3$	0.034	0.345	0.040	0.437	6.00E-03	0.153
$X_2 X_3$	− 0.026	0.459	− 0.030	0.55	7.00E-03	0.101*
X_1^2	− 0.067	0.027**	0.042	0.29	1.68E-03	0.578
X_2^2	− 0.094	0.004***	0.061	0.130	− 6.60E-03	0.827
X_3^2	− 0.011	0.68	0.060	0.1407	− 8.43E-04	0.780
R^2	93.45%		97.72%		92.87%	
R^2_{adj}	88%		96%		86%	
F value	15.86		47.61		14.47	

***,**,*1, 5 and 10% level of significance

(Table 4) which indicate that substrate pre-treatment may alter enzyme production and activity. A linear model (Fig. 1) showed better fit (92.87%) than a non-linear model (86%).

Reducing Sugar

Results of reducing sugar of different experimental runs are shown in Table 3. During fermentation, *T. reesei* utilized reducing sugar for their growth. Utilization of sugar is an important consideration during fermentation, as it is linked to cellulase yield. Reports show that the accumulation of reducing sugar represses the production of cellulase [23]. The reducing sugar ranged from 1.03 to 3.55 ± 0.15 mg/mL which was in accordance with the findings of John [33] and Wang [34]. The maximum reducing sugar was observed for the samples having 31 h harvesting time, H_2O_2 conc. of 7%

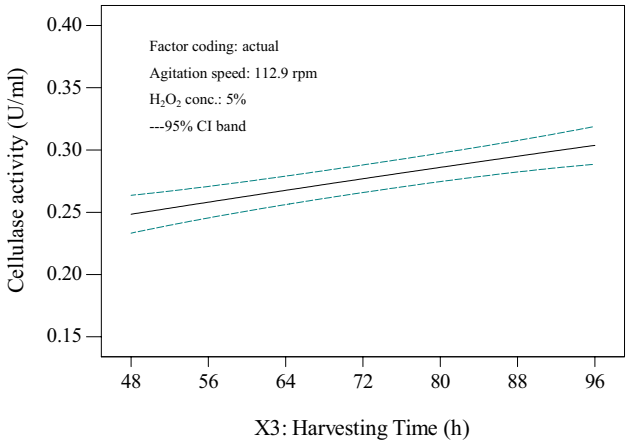

Fig. 1 Effect of harvesting time (h) on cellulase activity (U/mL) at constant agitation speed and H_2O_2 concentration

and 110 rpm agitation speed. The variation in reducing sugar depended on the cell growth as suggested by Raimbault [35]. The utilization of reducing sugar increased with fermentation and harvesting time to increase the desired production. The growth of *T. reesei* lowered the pH which complimented the utilization of reducing sugar rapidly thereby increasing the cellulase production [36]. Significant decrease ($p < 0.01$) in reducing sugar was observed with increase in harvesting time. As no interaction terms were found significant (Table 4), a linear model was found best suited (Fig. 2) to fit the data (97.72%). Comparable values of R^2_{adj} and R^2 further pointed towards non-linearity in data.

Protein Content

Results of different experimental conditions and observed values of protein are shown in Table 3. The protein content

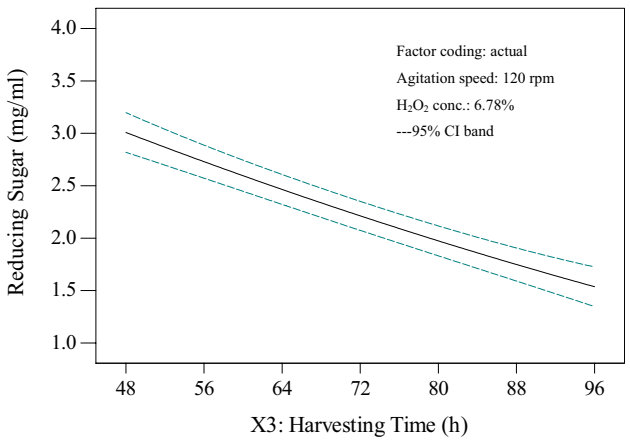

Fig. 2 Effect of harvesting time (h) on reducing sugar (mg/mL) at constant agitation speed and H_2O_2 concentration

ranged from 0.89 to 1.78 ± 0.07 mg/mL which was higher than that reported by Ghosh [37]. Maximum protein for the fermented peahull slurry was observed for an agitation speed of 110 rpm, H_2O_2 conc 7% and harvesting time 112 h, while minimum protein was observed for experimental condition with agitation speed 110 rpm, H_2O_2 conc 7% and harvesting time 31 h. As per observations, the protein content of the biomass increased with harvesting time. These finding are in accordance with [36] who reported that the maximum protein production was observed on the fifth day of fermentation by *Aspergillus niger* (KA-06) and thereafter the protein yield declined. The protein content increased with increase in agitation speed upto a threshold level beyond which it decreased gradually (Fig. 3a). This could be explained due to increase in shear stress on higher speeds which could damage the fungal hyphae thereby affecting the overall protein content [38]. All process variables affected the protein content significantly (Table 5) with higher influence of harvesting time ($p < 0.01$) and agitation speed ($p < 0.05$) than

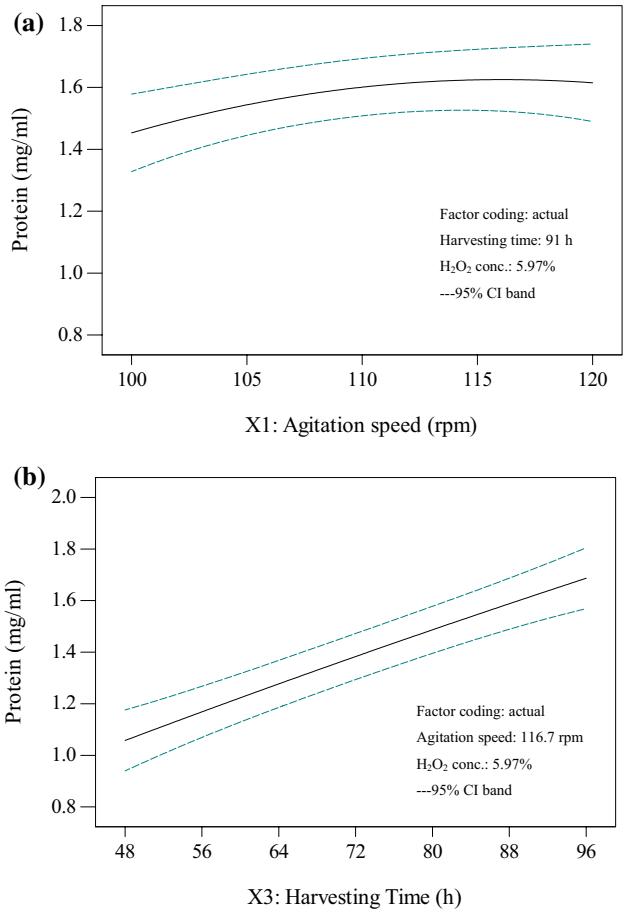

Fig. 3 **a** Effect of agitation speed (rpm) on protein content (mg/mL) at constant harvesting time and H_2O_2 concentration. **b** Effect of harvesting time (h) on protein content (mg/mL) at constant agitation speed and H_2O_2 concentration

H_2O_2 concentration. Figures 3a and b shows the variation in protein content with X_1 and X_3 respectively. The interaction effects were non-significant ($p > 0.1$). H_2O_2 concentration contributed to declining non-linear variations in data significantly ($p < 0.01$) followed by agitation speed ($p < 0.05$). This showed that lower pre-treatment concentrations and agitation speed are beneficial for higher enzyme production. A non-linear polynomial is therefore suggested in prediction of protein content (Fig. 3a).

Optimization of Fermenting Parameters

The objective of the study was to optimize the process parameters to get the best possible combination of independent variables that would result in a cellulase production with better efficiency. Design Expert software 9.0.6.2 was used to analyze data to obtain the optimized solution for cellulase production. The optimized conditions could be a single point or a range of points in which all the possible combinations would yield good results. While using any optimization technique, some constraints set the guideline to get the desired results. The response values and analysis of models give the valuable information in deciding the constraints for independent variables and responses. In the present study, experiments were carried out using CCRD in realistic vicinity to locate the true optimal values of multiple independent variables. Table 5 represents the goals which were fixed for all independent variables and dependent variable as per the objective of the study.

Optimization of the process variables yielded 58 solution sets out of which a single set most suited to the criteria was selected. The optimum level of independent variables in coded and actual form is given in Table 6.

Table 5 Criteria selected for optimization of fermenting parameters

Variables	Goal	Lower limit	Upper limit	Importance
Agitation speed	In range	− 1	+ 1	+++
H_2O_2 conc	In range	− 1	+ 1	+++
Harvesting time	In range	− 1	+ 1	+++
Reducing sugar	Minimize	1.03	3.55	+++
Protein	Maximum	0.89	1.75	+++
Cellulase activity	Maximum	0.219	0.32	+++

The optimized set of independent variables (agitation speed, H_2O_2 conc and harvesting time) obtained from Design-Expert software, was used to verify the results. The values of the responses (reducing sugar, protein and cellulase activity) were compared with the values given by the software after optimization. The results revealed that the actual values of all attributes were close to the predicted value (Table 7) and ANOVA showed that variation between predicted and actual score was highly non-significant ($F_{tab} > F_{cal}$). Thus, the production of cellulase enzyme from pre-treated peahulls at 120 rpm agitation speed, 5% H_2O_2 concentration and 91 h harvesting time may be considered optimum for cellulase production. The final cellulase obtained after dialysis and lyophilization is shown in Fig. 4.

Conclusions

A novel, environment friendly and low-cost approach to pea hull waste utilization for enzyme production has been discussed. Optimized fermentation parameters were identified for enhanced production of cellulase from pre-treated pea hulls. The described work may aid to provide relevant information to food process industries for solid and liquid waste management while enhancing their profit margin through value-added products. Moreover, the cellulase produced could have numerous applications in textile, detergent, paper and food industries. Application of recombinant cultures or phenol degrading microbes along with cellulase producing cultures could be investigated as another effective technique for utilization of lignin enriched waste.

Table 7 Comparison between actual and predicted values of responses

Responses	Predicted score*	Actual score**	F value	t value***
Protein	1.53 mg/mL	1.66 mg/mL	5.06	3.97
Reducing sugar	1.73 mg/mL	1.6 mg/mL	4.18	1.23
Cellulase activity	0.302 U/mL	0.372 U/mL	7.10	1.02

*Obtained from Design Expert 9.0.6.2

**Actual values of optimized conditions

***t value found non significant (at 5% level of significance)

Table 6 Optimized responses for fermentation of peahull slurry

Independent variables	Optimum value		Responses	Optimum value
	Uncoded	Coded		
Agitation speed (rpm)	120	1	Protein	1.53 mg/mL
H_2O_2 concentration (%)	5	− 1	Reducing sugar	1.74 mg/mL
Harvesting time (h)	91	0.805	Cellulase activity	0.302 U/mL

Fig. 4 Cellulase (stored at 4 °C) in powdered form after dialysis and lyophilization

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