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Statistical Optimization of *Prosopis juliflora* Containing Medium for the Enhanced Production of Cellulase by *Cellulomonas uda* NCIM 2353

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Cellulases are the multi enzyme complexes that are capable of degrading lignocellulosic substances. They have wide range of applications in textile, leather, and food industries and in particular in biofuel production. *Prosopsis juliflora*, the drought resistant evergreen spiny tree is the richest source of carbon (40% of total sugar). Hence this lignocellulosic waste can be exploited for the growth of bacteria and for its conversion into value added products. In the present study *Prosopis juliflora* pods is being exploited for cellulase production from *Cellulomonas uda* NCIM 2353. Growth and production profiling of cellulase of *Cellulomonas uda* on pods containing media was studied. Further the medium was optimized for the enhanced production of cellulase using a response surface methodology. For this the wide significant parameters were found out to be carbon source (pods concentration), xylose, yeast extract and initial media pH. The interactive effect of these four significant variables at five levels on the cellulase production was studied in 26 trails as given by central composite design. The linear, quadratic and interactive effects of all these parameters on the cellulase activity were analysed. The observed coefficient of determination is 0.96224 and the critical values obtained are (g/100 mL): pods concentration, 5.0667%w/v; xylose, 0.5514 %w/v; yeast extract,0.6972 %w/v, pH, 7.401.

Key words: Cellulase, *Prosopis juliflora*, Central Composite Design, Optimization.

Lignocellulosic materials comprises agricultural residues, wood, paper and yard waste in municipal solid waste and other dedicated energy crops[1]. Cellulose, hemicellulose and lignin are the predominant polymers present in lignocellulosic waste. Cellulose accounts as 50% of the dry weight of plant biomass[2]. Cellulose is the homologues biopolymer linked together by β 1-4 glycosidic bonds. The conversion of lignocellulosic waste into value added products is done through cellulase which cleaves cellulose into simple sugars. Cellulase is the multi enzyme complex that constitutes endocellulase (EC 3.2.1.4) that randomly cleave internal bonds to create new chain ends, exocellulase (EC 3.2.1.91) which cleaves two to four units from the ends of the exposed chains produced by endocellulase, resulting in oligosaccharides and cellobiases (EC 3.2.1.21) that hydrolyse the exocellulase product into individual monosaccharides[3].

Cellulase contributes extensively to worldwide industrial enzyme demands. During the period 2004-2014 an increase of approximately 100% in use of these enzymes has been projected[4]. It finds its extensive applications in textile

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industries as laundry detergents for deinking and for improving fiber brightness, strength properties[5]. In biofuel production for enzymatic hydrolysis of agricultural wastes thereby reducing the sugar loss through side – reactions and making the reaction milder and more specific[6]. In food industries for clarification of fruit juices; improving the yields in starch and protein extraction; improving maceration, pressing, and for color extraction of fruits and vegetables, in poultry cereal-based diets for improving the nutrient utilization to increase their feed conversion efficiency[5]. In paper industries for deinking the paper[7,8] and in sewage treatments for decomposition of wastes and residues.

Prosopis juliflora (vernacular names: vilayti, pardesi, babul or mesquite) is a drought resistant evergreen spiny tree is native to central and south America and spreaded to north America[9]. The pods are flattened, multi-seeded and curved with hardened pericarp. The productivity of the pods is 2-4 million metric tons worldwide[10]. It is one of the largest contributors of municipal waste. Further invasion of this waste makes the land unavailable for grazing of cattles thus decreasing livestock productivity.The leaves of *Prosopis* are unpalatable. It has restricted usage as fodder since it causes deleterious effects on cattle[11]. This second generation feed stock is rich in carbon content (cellulose 0.39 g/g, total organic carbon 0.06 g/g, total reducing sugar 0.009 g/g) and hence provides the better platform for the bacterial growth and for its conversion into value added products[12].

For utilization of cellulosic substrates in lignocellulosic wastes, micro organisms produce cellulase. *Bacillus*, *Cytophaga, Cellulomonas, Streptomyces, Microbacterium species are* known for cellulase production. Among fungi *Acremonium, Aspergillus, Fusarium solani, Penicillium, Sclerotium rolfsii, Trichoderma* are well known for its cellulolytic property. Among the bacterial species reported, *Cellulomonas* is being widely exploited for cellulase production as it exhibits better titers[13-17]. *Cellulomonas uda* NCIM 2353 is gram positive rod shaped facultative anaerobic bacteria that can utilise the both the constituents of carbohydrates, cellulose and hemicellulose under aerobic conditions during fermentation[15, 18]. *Cellulomonas* species contains large multigenes for hydrolyzing both cellulose (cenA, cen B) and hemi cellulose (cex) genes. These multiple domains are joined by characteristic linker sequences made of proline and threonine residues[17]. These effective cellulolytic secretors are considered superior over fungi because of high doubling rate, high productivity, thermo stable and alkali stable properties[19].

Submerged fermentation is the traditional method followed for cellulase production. It is more suitable for the bacteria that require high moisture content. The high rate of homogenization with easier downstream processing steps makes this the suitable technique for cellulase production[20].

Response Surface Methodology (RSM) is the collection of statistical and mathematical tools to design the experiments for evaluating the factor effects to get the desired production yield[21]. The conventional experiments involving optimization of one variable at a time is time consuming since it involves more number of experiments. Further the interactive effects of all independent variables over the dependent variable cannot be found by conventional optimization. RSM involves full factorial search where design variables are varied simultaneously. It minimizes the error in determining the factor effects since it shows the simultaneous, systematic, and efficient variation of all parameters at a time[22]. This statistical tool can be used even if the prior knowledge of relationship between response elements and the variables is unknown[23]. The response of the dependent variable is visualized by contour plots. They are the three dimensional graphs obtained by varying any two independent variables simultaneously while having other variables at a fixed point. Central Composite Design (CCD) is done to fit a second-order polynomial by least squares technique. The second order polynomial equation is used to describe the test variables and describe the combined effect of all the test variables in the response[12].

The prominent aim of the present study was to study the cellulase production by *Cellulomonas uda* in a *Prosopis juliflora* containing medium and its further optimization using central composite design.

MATERIALS AND METHODS

Microorganism and maintenance

Cellulomonas uda NCIM 2353 was obtained from National Chemical Laboratory (NCL) Pune. *Cellulomonas uda* NCIM 2353 stock culture was maintained in Dubos media (g/L): sodium nitrate, 0.5; dipotassium hydrogen phosphate, 1; magnesium sulphate, 0.5; potassium chloride, 0.5; yeast extract, 0.5; cellulose, 5.0 and ferrous sulphate, 0.01. The pH was adjusted to 6.5. Sub culturing was done once in three weeks and the culture was stored at 4°C. All the media components, chemicals and reagents used in the study were obtained from Hi media (India), Sigma Aldrich (USA) and Merck (Germany).

Collection and preparation of *Prosopis juliflora* pods

The pods of *Prosopis juliflora* were collected from Tanjore, India. The pods were collected from the same region in order to prevent the changes occurring due to different nutritional content. The pods were dried and finely grounded to mesh size #40.

Production of cellulase using Pods containing medium

The cellulase production by *Cellulomonas uda* NCIM 2353 was carried out in 250 ml Erlenmeyer flask with production media of 100 ml. The components in production media: *Prosopsis juliflora* pods, 5 g/L; yeast extract, 2.5 g/L; ammonium Sulphate, 7 g/L; potassium dihydrogen phosphate, 10g/L; magnesium sulphate, 0.3 g/L; calcium chloride, 1.5g/L; urea, 1.5g/L; peptone, 0.1g/L; ferrous sulphate, 5 mg/L; manganese sulphate, 1.56 mg/L; cobalt chloride, 3.66 mg/L; tween 80, 0.1ml/L. The initial pH was adjusted to 7.4 and then the media was sterilized at 121°C for 15 minutes. It was inoculated with 5% seed culture and incubated in orbital shaker for 150 rpm at 30°C. Growth of the *C.uda* was monitored by withdrawing samples in every 4 hours.

The carbon source concentration is optimized for the cellulase enzyme activity by varying the substrate concentration from 1% - 6% (W/V). Quantitative estimation of cellulase was determined every 6 hours. Fermented broth was centrifuged at 10000 rpm for 10 minutes at 4 °C. Supernatant was used as crude enzyme extract for determining cellulase activity.

Cellulase assay

Cellulase activity was determined by measuring the reducing sugar released by the enzyme from the cellulose substrate by Dinitro salicylic acid (DNS) method. 1ml of the crude enzyme extract was added to the reaction mixture containing1 ml of substrate (1% Carboxy methyl cellulose) and 1ml of citrate phosphate buffer of pH 7.2. After the reaction time of 30 minutes at 30°C, 3 ml DNS was added and the mixture was incubated at 90°C for 10 minutes. Absorbance was measured at 540 nm[24]. One International unit of cellulase activity is defined as one micromole of reducing sugars released equivalent to glucose per minute under the assay conditions described. Cellulase activity is calculated as follows:

Cellulase activity (U/ml) = $[(OD_{540}/\text{Std.slope})/(180X30X1)]$

Here, 180, molecular weight of glucose is in µg/µmole; 30, reaction time in minutes; 1, reaction volume in mL of crude enzyme solution taken for assay activity.

Statistical optimization

The levels of the significant parameters and their interaction effects that influence cellulase production was determined and optimized by Response surface central composite design using the software package Statistica 8.0 (evaluation version). According to the design, the total number of treatment combinations is $2^k+2^k+n_0$, where k is the number of independent factors and n_0 is the number of repetition of experiments at the centre point. Each factor in the design was studied at five different levels (-2,-1, 0, 1, 2). The dependent variable is cellulase activity and the independent variables chosen in this study includes pod concentration, xylose, yeast extract and the fermentation pH. The independent variables are varied in the range of (g/100 mL): pods, 3 to 7; xylose, 0 to 0.8; yeast extract, 0 to 2 and pH 6.6 to 8.2.The fixed centre points are (g/100 mL): pods 5%, xylose 0.4 g, yeast extract 1 g, and pH 7.4. These four independent variables were studied at five different levels and hence 26 runs were done. The enzyme production was analyzed and the data was fitted into a polynomial equation as below

$$Y=\beta_0+\beta_1 F_1+\beta_2 F_2+\beta_3 F_3+\beta_4 F_4+\beta_{11} F_1^2+\beta_{22} F_2^2+\beta_{33} F_3^2+\beta_{44} F_4^2+\beta_{12} F_1 F_2+\beta_{13} F_1 F_3+\beta_{14} F_1 F_4+\beta_{23} F_2 F_3+\beta_{24} F_2 F_4+\beta_3^4 F_3 F_4 \quad ...(1)$$

Where Y is the response, β_i, β_{ij} are the regression

coefficients for linear and quadratic effects respectively.

RESULTS AND DISCUSSION

Growth pattern of *Cellulomonas uda* NCIM 2353

The growth pattern of *Cellulomonas uda* NCIM 2353 on the production media was studied. *Cellulomonas* displayed typical growth curve with the long exponential phase from 10h to 56h and stationary phase from 56h (Fig 1). *Cellulomonas* was found to utilize the *Prosopis juliflora* pods as the sole carbon source for its growth and reproduction. The inoculated media was analyzed for their time dependent cellulase production. The enzyme activity was found to be optimal in the late exponential phase at 54th hour for all different trials carried out (Fig 2). This is the clear indicative of the fact that cellulase, the primary metabolite is produced at late exponential phase.

Optimization of pod concentration

As the pod concentration increased the cellulase activity also increased (Fig 3). Hence it can be concluded that *Prosopsis juliflora* pods are the inducers for the cellulase production. However the decrease in enzyme activity after 5% pods concentration was observed. This decrease in activity may be due to the inhibitors. This is supported by the findings of Oguntimein and Moo-young who reported the inhibitory effect of accumulated cellobiose and cellodextrin of low degree of polymerization[25].

Statistical optimization

Among the 26 experiments, two experiments were repetition of the central point (trial number 25 and 26). The closeness of the yield for these two experiments can be a sign for the accuracy of the model and it helps to estimate the experimental error (Table 1). ANOVA and least square techniques were used for evaluating the

Table 1. Central Composite Design of the variables in real units for the response of Cellulase activity along with its predicted & observed values

Trial no	Pods (g/100 mL)	Xylose (g/100 mL)	Yeast extract (g/100 mL)	pH	observed values (U/mL)	predicted values (U/mL)
1	4	0.20	0.5	7.0	0.252	0.221
2	4	0.20	0.5	7.8	0.193	0.203
3	4	0.20	1.5	7.0	0.157	0.171
4	4	0.20	1.5	7.8	0.119	0.125
5	4	0.60	0.5	7.0	0.357	0.364
6	4	0.60	0.5	7.8	0.382	0.359
7	4	0.60	1.5	7.0	0.334	0.300
8	4	0.60	1.5	7.8	0.272	0.267
9	6	0.20	0.5	7.0	0.206	0.211
10	6	0.20	0.5	7.8	0.203	0.216
11	6	0.20	1.5	7.0	0.158	0.161
12	6	0.20	1.5	7.8	0.145	0.139
13	6	0.60	0.5	7.0	0.388	0.361
14	6	0.60	0.5	7.8	0.394	0.380
15	6	0.60	1.5	7.0	0.307	0.298
16	6	0.60	1.5	7.8	0.278	0.288
17	3	0.40	1.0	7.4	0.253	0.271
18	7	0.40	1.0	7.4	0.280	0.283
19	5	0.0	1.0	7.4	0.078	0.061
20	5	0.8	1.0	7.4	0.316	0.353
21	5	0.4	0.0	7.4	0.331	0.351
22	5	0.4	2.0	7.4	0.210	0.210
23	5	0.4	1.0	6.6	0.239	0.265
24	5	0.4	1.0	8.2	0.243	0.237
25	5	0.4	1.0	7.4	0.425	0.406
26	5	0.4	1.0	7.4	0.388	0.406

statistical significance of the constructed model. Using Fisher-statistical test (F-test), ANOVA determines the factor(s) that significantly affects the response. Effects having a confidence level less than 95% (p-value higher than 0.05) were rejected and pooled into the error term and new ANOVA was performed with the reduced model[26].

In the present study, the linear effects of xylose, yeast extract, quadratic effects of pods, xylose, yeast extract and pH was found to be significant (Table 2). The R square value gives the accuracy of fit[27, 28]. The R square obtained in this study is 0.96224 which proves to be the good fit. Since coefficient of determination (R^2) decreases when a regression variable is eliminated from the model, R^2_{adj} is also preferred as it consider the number of regression variables[26]. R^2_{adj} obtained in the present study is 0.91419 that confirms the fitness of the model.

The significance and the magnitude of the estimated coefficients and their possible interactions were also determined and their values show the improvement in the response as those variables changes from its low to high values. The high co efficient value indicate that the particular variable has the greatest impact on response element while the coefficient factor close to zero signifies little or no significance[29]. The sign of the co efficient indicates the level which is required for further optimization. In the present study yeast extract and pH showed negative effects; hence decreasing its concentration further could enhance the enzyme activity whereas pods and xylose has positive co efficient hence increasing its concentration might have positive response over the enzyme activity.

In order to construct a model to optimize the media composition, by back elimination method the terms which are not significant enough are discarded from the equation (1). Each term with a $p > 0.05$ was removed from the model. The reduced model with all the significant linear, quadratic and interaction parameters is expressed in the form of polynomial equation asfollows:

Table 2. Effect estimates of central composite design

Factor	Coeff.	Std.Err.	t	p
Mean/Interc.	0.406248	0.019390	20.95172	0.000000
(1)pods (L)	0.002853	0.011195	0.50966	0.620359
pods (Q)	-0.032304	0.013127	-4.92179	0.000456
(2)xylose (L)	0.073081	0.011195	13.05641	0.000000
xylose (Q)	-0.049757	0.013127	-7.58087	0.000011
(3)yeast extract(L)	-0.035277	0.011195	-6.30238	0.000058
yeast extract(Q)	-0.031365	0.013127	-4.77868	0.000573
(4)pH (L)	-0.006919	0.011195	-1.23617	0.242142
pH (Q)	-0.038728	0.013127	-5.90061	0.000103
1L by 2L	0.001913	0.013711	0.27912	0.785334
1L by 3L	0.000012	0.013711	0.00169	0.998681
1L by 4L	0.005961	0.013711	0.86949	0.403155
2L by 3L	-0.003549	0.013711	-0.51763	0.614965
2L by 4L	0.003282	0.013711	0.47873	0.641508
3L by 4L	-0.006993	0.013711	-1.02004	0.329620

*Highlighted variables are significant

Table 3. Critical values at predicted value 0.445IU/ml

Factor	Observed - Minimum	Critical - Values	Observed - Maximum
Pods concentration	3.000000	5.066711	7.000000
Xylose	0.000000	0.551468	0.800000
Yeast extract	0.000000	0.697262	2.000000
pH	6.600000	7.401021	8.200000

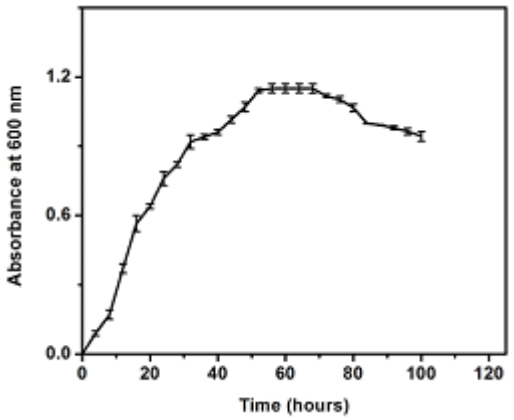

Fig. 1. Growth pattern of *C. uda* in pods containing medium

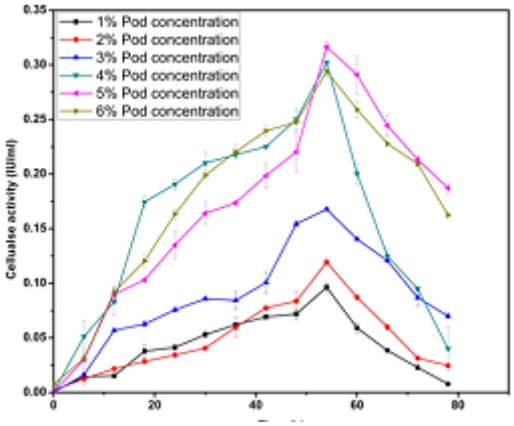

Fig. 2. Production profiling of *C.uda* in media containing various concentrations of pods

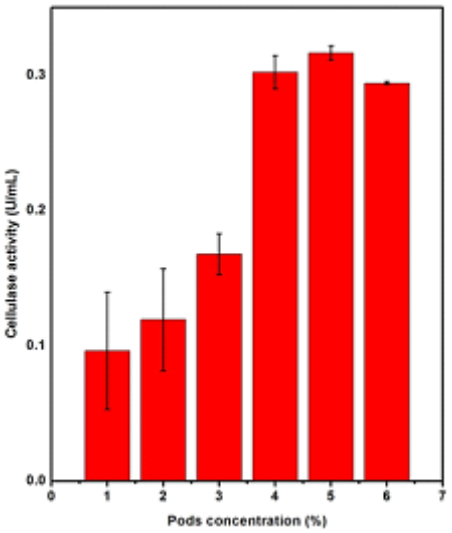

Fig. 3. Effect of Pods concentration on the production of cellulase activity

$Y=0.406248+0.073081F_2-0.035277F_3-0.032304F_1^2-0.049757F_2^2-0.031365F_3^2-0.038728F_4^2$

Contour plots generated (figures not shown) shows the combined effects of process variables over the response element. The linear and interactive effects studied through these plots gives the optimum conditions for the maximum enzyme production. The graph is plotted between any two independent variables and the response (cellulase activity) on the third axis while keeping other independent variables at fixed central point.

Validation of experiment model

Response analysis revealed that maximum enzyme activity by *Cellulomonas uda* NCIM 2353 could be produced in the following optimal conditions (g/100 mL): pods, 5.0667; xylose, 0.5514; yeast extract, 0.6972; pH, 7.401 (Table 3). Validation of experimental model was done by carrying out the batch fermentation at the above mentioned optimized conditions. Under these conditions the optimized enzyme activity was found to be 0.398 U/mL which was close to the enzyme activity predicted by regression models (0.406 U/mL).

The results obtained in the present study are comparable with those obtained by Ladeira and Cruz who used Sugarcane baggase as the carbon source and corn steep liquor as the nitrogen source to produce cellulase from *Bacillus sp.* SMIA-2 [30]. The maximum cellulase activity achieved was 0.29 U/m L at 168th hour of culture time. The enzyme produced showed wide range of pH and temperature stability and hence had its potential application in detergent industry. Fungsin inoculated *T. reesei* in cultivation medium containing of 15% (w/v) cassava waste. After cultivation at 35 °C with shaking speed of 150 rpm for 72 hours, the maximum yield of cellulase was 0.3 U/mL. The optimum temperature, pH and conversion period for bioconversion were 60 °C, 6.0, and 6 hrs, respectively[31]. Patil *et al.*, isoalted *Colletotrichum sp* from medicinal plants to produce cellulase. In the yeast extract peptone agar synthetic medium containing 0.5% CMC, the yield of cellulase is 0.013U/ml[32]. Yanna Liang isolated *Brevibacillus* sp. strain JXL from swine waste. When this strain was grown in cellulolytic medium containing cellulose and glucose the cellulase activity attained was 0.015 FPU/mL[33].

CONCLUSION

This is the first report regarding exploiting the *Prosopsis juliflora* for cellulase production by *Cellulomonas uda* NCIM 2353. From this study it can be concluded that *Prosopsis juliflora* pods plays the critical role in enzyme production. The enzyme produced can be immobilized into the matrixes to extend its applications in biofuel production. The Response surface methodology was found to be a great tool to optimize the substrate concentration along with other media components. After central composite design the enzyme activity increased from 0.31637 U/mL to 0.406 U/mL. A polynomial equation was constructed and the adequacy, fitness and validity of the model were checked.

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