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Solid State Fermentation of Soybean Hulls for Cellulolytic Enzymes Production

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1. Introduction

Soybean hulls are the byproduct of soybean processing and accounts for 5-8% of the 96 million metric tons soybean crop of 2006 in USA (Mielenz et al., 2009). It is mechanically removed from the soybeans during the process of dehulling and commercially sold as it is or in form of compressed pellets mostly for the feed industry (Marshall and Wartelle, 2003). Soybean hulls have rich cellulosic composition, notably half of the composition is cellulosic i.e. cellulose and hemicellulose combined (Brijwani et al., 2010). Fungal solid state fermentation (SSF) of soybean hulls for cellulolytic enzyme production is a useful way of value addition of this under-utilized byproduct. A cellulolytic enzyme system is group of enzymes that work synergistically to hydrolyze lignocellulosic biomass. It is composed of endoglucanase (endo-1, 4-β-D--glucanase, EC 3.2.1.4), exoglucanase (1,4-β-D-glucan-cellobiohydrolase, EC 3.2.1.91), and β-glucosidase (β-D-glucoside glucanohydrolase, cellobiase, EC 3.2.1.21) (Holker et al., 2004; Esterbauer et al., 1991). Xylanase (EC 3.2.1.8), though not part of the group, complements the cellulolytic enzyme system as it is needed to elicit complete and efficient hydrolysis of the lignocellulosic biomass, which has an appreciable amount of hemicellulose or xylan (Brijwani et al., 2010; Brijwani, 2011). It has been widely accepted that Solid State Fermentation (SSF) is an attractive means to produce cellulase economically because of its lower capital investment and lower operating cost (Cen and Xia 1999). Further, the ability of SSF to minimize catabolite repression has been already described for several enzymes (Aguilar and Huitron 1986; Archana and Satayanaryana 1997; Siqueira et al. 1997; Solis-Pereyra 1996). SSF is defined as a fermentation process in which microorganisms grow on solid materials without the presence of free liquid and the moisture necessary for microbial growth exists in adsorbed state or complexed with solid matrix (Krishna, 2005). Both bacteria and fungi are known to produce cellulases using complex cellulosic substrates, however, fungal enzymes are generally complete comprising of all the cellulosic activities (Stockton et al., 1991).

The operating conditions like temperature, pH and moisture content are very important for microbial growth and efficient cellulase production in SSF. Optimization of parameters in multifactor experimental designs fall short in locating true optimum especially when there are interactions among independent variables, besides being time consuming (Giovanni 1983; Theodore and Panda 1995). One of the worthwhile techniques to identify the explanatory variable in the system is Response Surface Methodology (RSM) (Maddox and Richer 1977;

Hounsa et al. 1996). RSM is a collection of statistical techniques for designing experiments building models, evaluating the effect of factors and obtaining optimum conditions for desirable responses. RSM can be used to evaluate the significance of several factors especially when interactions exist among factors, and they are complex to determine (Liu and Tzeng 1998). RSM has been widely adopted among investigators in the field of bioprocessing and has been used to quantify complex interplay of parameters affecting biological system.

In SSF for cellulase production, cellulosic substrate acts as both the carbon source and as an inducer for cellulase production (Cen and Xia 1999). The crystallinity of cellulosic substrate influence the rate of hydrolysis, for instance, amorphous cellulose is readily digestible while crystalline cellulose requires synergistic action of both exo- and endo-glucanases (Mes-Hartree et al., 1988). SSF can be viewed as a discrete solid phase in which microorganisms grow on the surface of moist, discrete particles as well as inside and between them. The space between particles is occupied by a continuous gas phase (Botella et al., 2009). Availability of oxygen in the open spaces between particles is a major challenge in SSF (Thibault et al., 2000; Oostra et al., 2001). Physicochemical characteristics, crystallinity, bed porosity and specific volumetric surface, of the substrate (soybean hulls) could be vital for the induction of cellulolytic enzyme system in fungal cultures. In this chapter we have delineated the importance of soybean hulls as a substrate for cellulolytic enzyme production using SSF technique. We have discussed the role of symbiotic association of two fungal species in production of complete and balanced cellulolytic enzyme system from soybean hulls, and the use of optimization technique such as RSM for improved and standardized production of cellulases. Since the substrate features are central to enzymatic synthesis in fungal SSF, the final section of this chapter discusses the role of physicochemical characteristics in cellulolytic enzymes synthesis. There is plethora of information available on compositional features of substrate; however, studies on physical nature of substrate in inducing cellulolytic enzyme system is limited (Brijwani, 2011). We believe elucidating the significance of physicochemical characteristics would provide process designers an efficient tool in directing the production of various enzyme activities of the cellulolytic enzyme system as dictated by their end use application.

2. Soybean hulls as a substrate in solid state fermentation

Soybean hulls have rich cellulosic composition (cellulose – 33.49%, hemicellulose – 17.15%) and are low in lignin (9.88%) (Brijwani et al., 2010). Notably the rich cellulosic composition is appropriate for fungal growth and cellulase production. Carbon to nitrogen ration (C/N) is important determinant of SSF process efficiency (Krishna, 2005). Soybean hulls are lower in protein content (~10%) and often supplementation with good nitrogen source like wheat bran with protein content of approximately 20% is a judicious choice. Brijwani et al. (2010) showed that supplementation of four parts of soybean hulls with one part of wheat resulted in high titers of enzymes with balanced proportion of various activities. The 1:1 ratio of filter paper units and beta-glucosidase is generally recognized as balanced enzyme system that is able to overcome glucose inhibition of cellulases and drives reaction forward (Chahal et al., 1992; Mandels et al., 1981).

3. Mixed fungal solid state fermentation and response surface optimization

Fungal strains that produce cellulases are mainly comprised of *Trichoderma, Aspergillus, Penicillium* and *Fusarium* genera. *Trichoderma reesei* is the most widely employed fungus for

production of cellulolytic enzymes and has been extensively studied (Stockton et al. 1991). Strains of *Trichoderma* can accumulate high activities of endo and exo-glucanase, but are poor in β-glucosidase (Duff et al. 1987) whereas the strains of *Aspergillus* are high in β- glucosidase activity (Grajek 1987). Therefore, strains of both *Trichoderma* and *Aspergillus* can be successfully employed together in solid state fermentation for the production of multicomplex cellulase system. Brijwani et al. (2010) demonstrated that co-culturing of *T. reesei* with *A. oryzae* in 1:1 ratio significantly improved the production of beta-glucosidase levels in SSF of soybean hulls supplemented with wheat bran compared to mono culture of *T. reesei*.

A second order quadratic model (equation 1) comprising of main effects and interaction effects of three vital process variables – temperature (X1, °C), pH (X2), and moisture (X3, %) was fitted to experimental data of mixed culture fermentation of wheat bran supplemented with soybean hulls using central composite rotatable design (CCRD). The process variables were varied at two levels each and five central points were replicated for better estimation of error variance (Brijwani et al., 2010).

$$y = \beta_0 + \sum_{i=1}^{3} \beta_i x_i + \sum_{i=1}^{3} \beta_{ii} x_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} x_i x_j \quad (1)$$

where, y = predicted response, β_0 = constant coefficient, β_i = linear coefficient, β_{ii} = quadratic coefficient, and β_{ij} = interaction coefficient.

The analysis of variance (ANOVA) and three dimensional surface plots were generated using Design Expert 7.1. The optimized values of three independent variables for maximum activities were determined using numerical optimization package of Design Expert 7.1. Numerical optimization searches the design space using fitted model to find the optimized values of independent variables that maximize cellulase and β-glucosidase activities. Optimization was carried out with respect to filter paper units and beta-glucosidase activity. Both filter paper units (indicator of total cellulase activity: exo- and endo-cellulase combined) and beta-glucosidase are the lead activities needed for efficient hydrolysis of lignocellulosic biomass.

3.1 Cellulase optimization and model fitting

Maximum cellulase activity (10.55 FPU/g of dry substrate) was observed at pH 4.5, moisture content of 70%, and temperature of 30°C. The overall second order polynomial equation for cellulase activity as measured in terms of FPU (Filter Paper Units)/g was:

$$Y = 7.95 + 0.76X_1 + 0.34X_2 + 2.97X_3 + 0.21X_1X_2 + 0.82X_1X_3 -$$

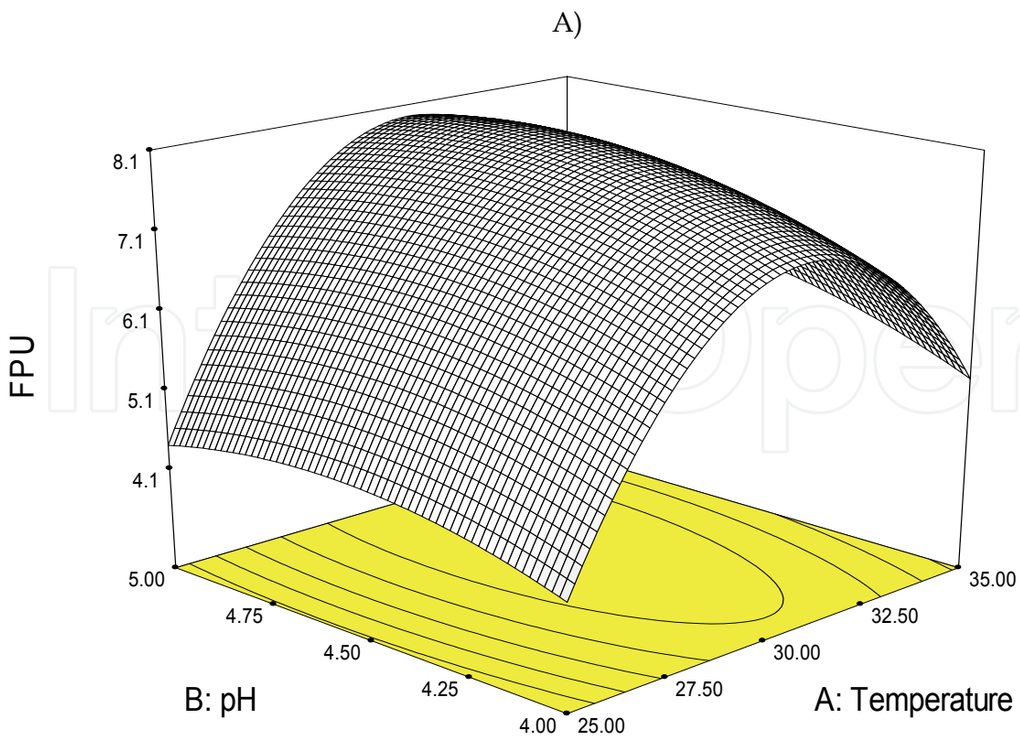
$$- 0.026X_2X_3 - 2.56X_1^2 - 0.40X_2^2 - 1.10X_3^2 \quad (2)$$

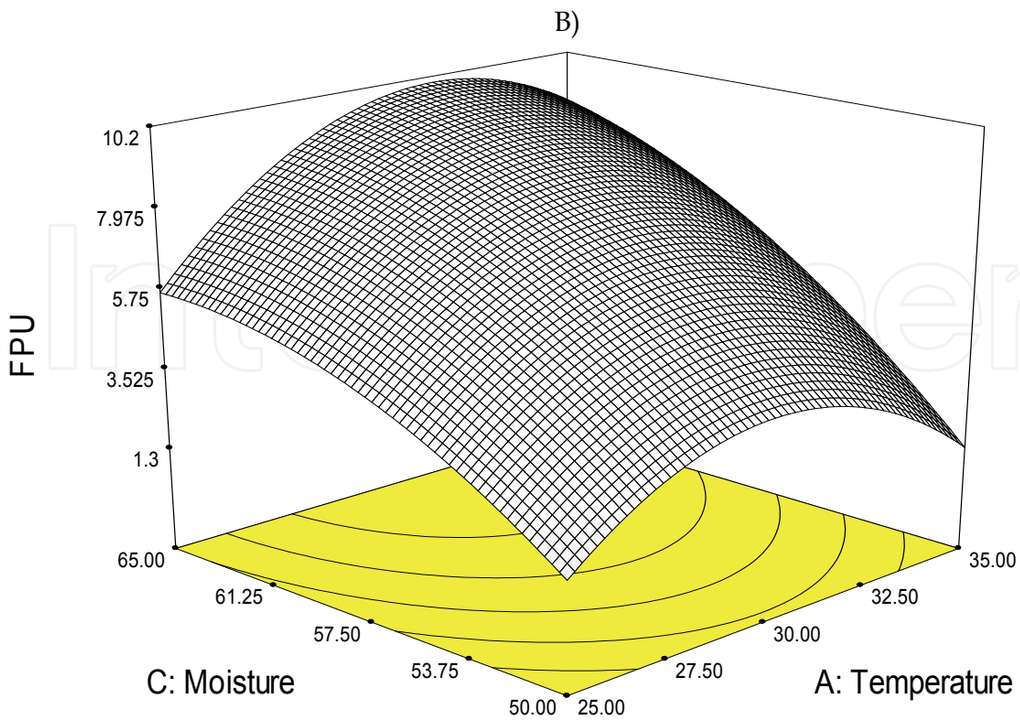
The lower p-value and insignificant lack of fit was obtained with quadratic model that suggested good fit. Higher coefficient of regression (>95%) suggested that there was good agreement between predicted and estimated cellulase activity under different conditions of temperature, pH and moisture content. Coefficients and p-values of the model indicated that temperature and moisture content significantly affected the cellulase production. While, moisture content had linear effect on cellulase activity temperature had quadratic effect. The interaction effect of moisture and temperature was significant as compared to their corresponding interactions with pH.

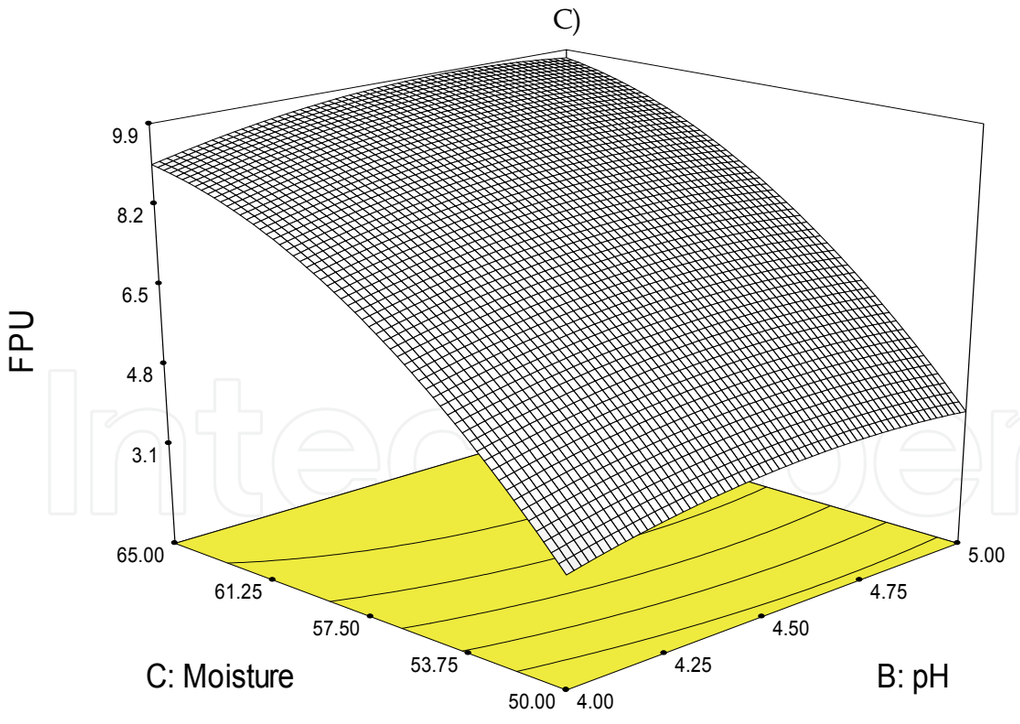
The surface plots were made as function of temperature and pH, moisture content and temperature, and pH and moisture content (Fig. 1). At lowest temperature and low pH (Fig. 1a) the cellulase activity was lowest. Increasing the temperature with moderate increase in pH keeping moisture content fixed at 57.5%, resulted in an increased cellulase activity, which reached its maximum in the vicinity of 30°C. Further increase in temperature resulted in a drop in cellulase activity even at increased pH value. Keeping the pH constant at 4.50 the interaction of moisture and temperature in presented in Fig. 1b. It is evident that with increase in moisture and temperature, the cellulase activity increased. Increasing the temperature beyond 34°C resulted in reduced cellulase activity and maximum activity could be seen in the neighborhood of 70% moisture and 33°C. Similarly, keeping the temperature constant at 30°C and observing for interaction between pH and moisture (Fig. 1c) we found that with increase in moisture and pH, cellulase activity increased and reached maximum around 65% moisture content and pH> 4.25. Our results are in consonance with the results of (Latifian et al. 2007) who have reported that moisture had a significant effect on cellulase production using *T. reesei* QM 9414 and *T.reesei* MCG 77 and maximum enzyme activity was observed at 70% moisture content. They adopted the RSM approach and further reported a temperature and moisture content range of 25-30^{0}C and 55-70%, respectively, for optimal cellulase production.

3.2 Beta-glucosidase optimization and model fitting

The maximum response (8.13 IU/g of dry substrate) occurred at 70% moisture content at pH of 4.5 and incubation temperature of 30°C. β-glucosidase data was fitted to a quadratic model with the following equation:







FPU: Filter Paper Units/g of dry substrate

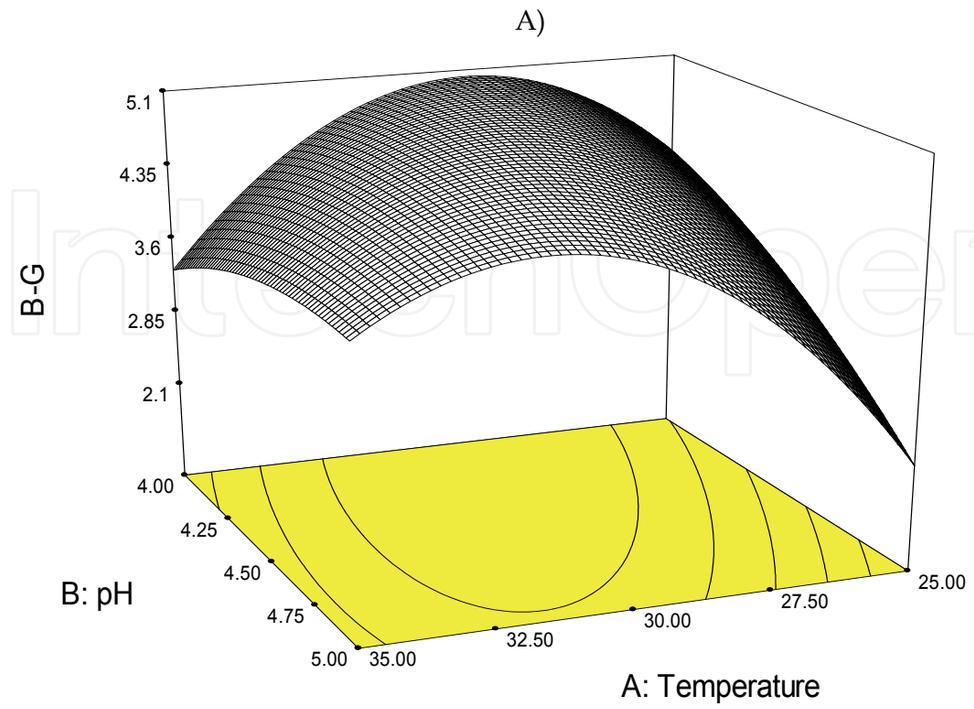
Fig. 1. Surface plots illustrating the effect of A) temperature and pH, B) moisture and temperature, C) moisture and pH on cellulase activity measured as FPU (Filter Paper Units)/g dry substrate

$$Y = 4.90 + 0.25X_1 - 0.33X_2 + 1.73X_3 + 0.69X_1X_2 +$$

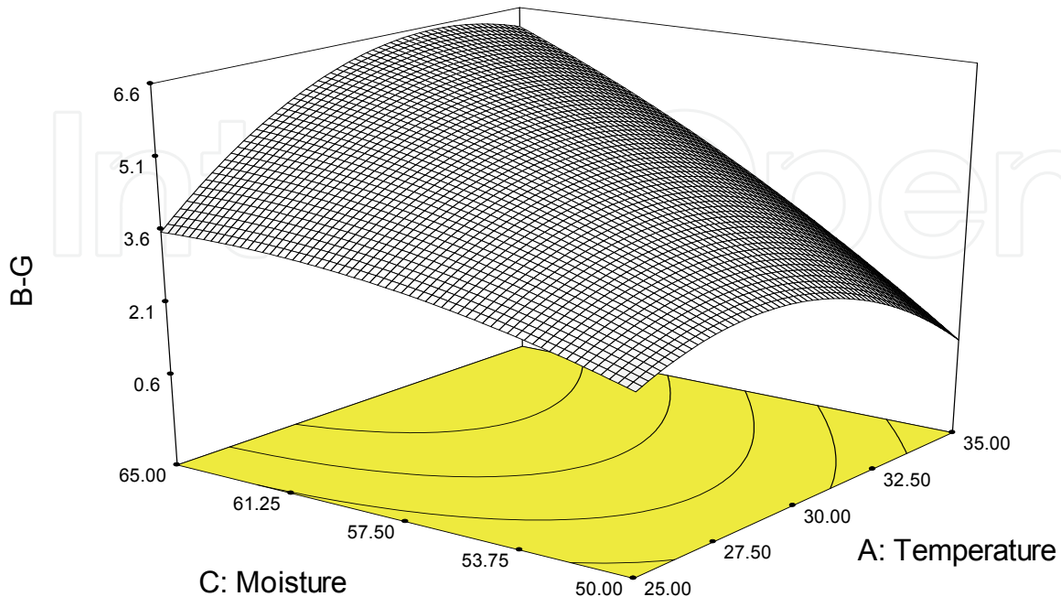
$$+1.03X_1X_3 + 0.42X_2X_3 - 1.32X_1^2 - 0.24X_2^2 - 0.41X_3^2 \quad (3)$$

The lower p-value and insignificant lack of fit was obtained with quadratic model that suggested good fit. Higher coefficient of regression (>85%) suggested that there was good agreement between predicted and estimated cellulase activity under different conditions of temperature, pH and moisture content. Coefficients and corresponding p-values indicated that moisture and temperature had significant effect on β-glucosidase activity. While effect of moisture was linear the effect of temperature was quadratic. The interaction effects of three independent variables were not significant; however, interaction between moisture and pH was considerable.

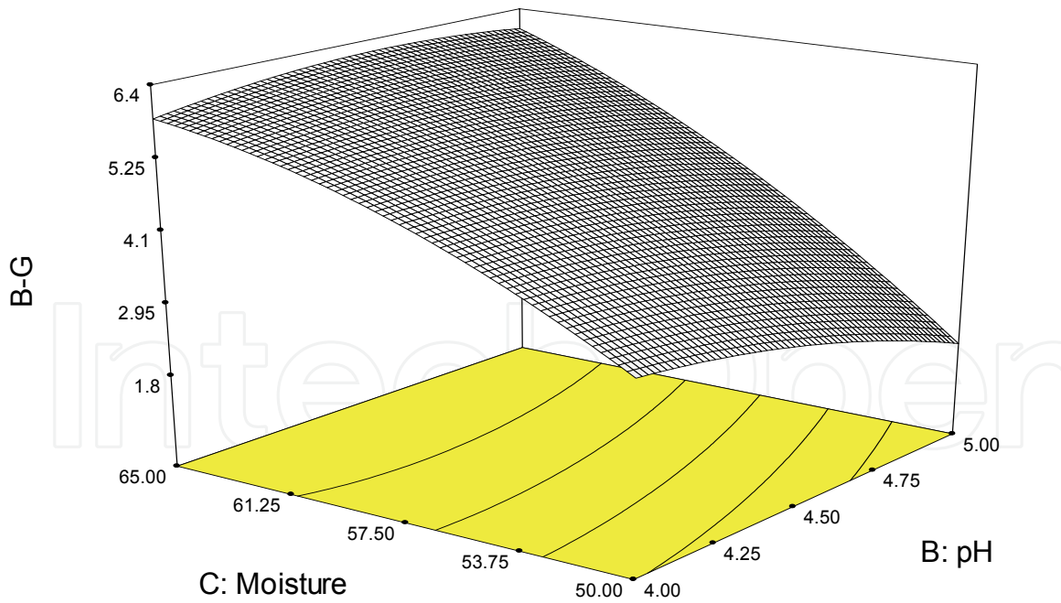
The surface plots were made as function of temperature and pH, moisture content and temperature, and pH and moisture content (Fig. 2). At a fixed moisture content of 57.5% increasing the temperature and pH beyond 27.5 ^{0}C and 4.5, respectively, resulted in increased β-glucosidase activity which was maximum in the neighborhood of 32°C (Fig. 2a). At fixed pH of 4.5, by increasing moisture content and temperature, β-glucosidase activity increased and achieved maxima around 32°C and 65% moisture (Fig. 2b). At constant temperature of 30°C, β-glucosidase activity, increased with increasing moisture levels and showed maximum activity near 62% moisture content (Fig. 2c). The earlier studies conducted on RSM optimization (Singhania et al. 2007) using wheat bran employing *T. reesei* Rut C-30 reported a combination of 37.5% and 30^{0}C as optimal conditions for moisture content and temperature, respectively, for maximum enzyme production; however, enzyme yields reported by them are substantially lower than the values obtained in this study. Further, enzyme yields also depend upon the substrates used and the interaction between substrate and moisture. This interaction depends on the water binding capacity of the substrates and availability of water for the growth of an organism.



B)



C)



B-G: β-glucosidase activity as IU/g of dry substrate

Fig. 2. Surface plots illustrating the effect of A) temperature and pH, B) moisture and temperature, C) moisture and pH on β-glucosidase (B-G) activity measured as IU (International Units)/g dry substrate

3.3 Numerical optimization of independent variables to maximize cellulase and β-glucosidase activities

Using Design Expert 7.1 numerical optimization subroutine design space was explored with fitted quadratic model to arrive at optimum temperature, moisture and pH conditions that maximize cellulase and β-glucosidase activities. In this task, goals were set to achieve maximum activities of cellulase and β-glucosidase by setting independent variables within the range of upper and lower limit. The optimized variables were found using desirability objective function that assigns relative importance to the responses. Solutions with higher desirability gave optimum temperature of 30 °C, pH of 5 and moisture content of 70%, and the corresponding cellulase and β-glucosidase activities were 10.55 FPU/g and 8.13 IU/g, respectively. Evidently, co-culturing of fungal cultures in SSF with proper optimization resulted in balanced production of enzymes with desired 1:1 ratio of filter paper units and beta-glucosidase activity.

4. Physicochemical characteristics of soybean hulls and production of cellulolytic enzyme system in fungal solid state fermentation

In solid state fermentation, substrate acts as both carrier and source of carbon for growth and productivity of fungal cultures. In literature, numerous studies have highlighted the importance of composition of carbon substrate in induction of cellulolytic enzyme system (Aro et al., 2005, Mach and Zeilinger, 2003; Bisaria and Mishra, 1989). Due to the discrete nature of SSF, the particulate nature of substrate becomes directly manifested such that the physicochemical characteristics: crystallinity, bed porosity and volumetric specific surface, can influence the production of cellulolytic enzyme system in fungal cultures (Brijwani, 2011). The relationship of cellulolytic enzyme system with physicochemical characteristics has been investigated in fungal cultures of *T. reesei* and *A. oryzae* present as mono and mixed forms (Brijwani, 2011). By subjecting soybean hulls to mild pretreatments (steam, and ambient temperature acid and alkali pretreatment) it was possible to alter the physicochemical characteristics without changing the total cellulosic composition of soybean hulls (Brijwani, 2011). Before going into details of the relationship of physicochemical characteristics with cellulolytic enzyme production it is vital to shed light on the techniques for measuring the physicochemical characteristics. Measurement of crystallinity is an arduous task especially for heterogeneous substrate like soybean hulls. With interlocking amorphous and crystalline regions there is dispersion in the scattering; consequently, the peak separation becomes challenging and accurate determination of crystallinity is compromised. In the forthcoming section elaborate details on techniques for measuring crystallinity of soybean hulls is presented. For measurement of other two physicochemical properties: bed porosity and volumetric specific surface, readers are directed to the recent work of Brijwani and Vadlani (2010).

4.1 X-ray diffraction studies of soybean hulls for measurement of crystallinity

Wide angle X-ray diffraction has been extensively used to measure the crystallinity of cellulosic substrates. There are several ways to measure crystallinity in the polymeric sample from an X-ray diffractogram; the most common is the Segal method (Segal et al., 1959). The method requires that amorphous material diffracts with the same intensity at 18^{o} (~ 10ī plane) and 22^{o} (002 plane) and that the crystalline cellulose does not contribute to the reflection at 18^{o}. However, all materials give rise to X-ray scattering, and ensuring the

crystalline component does not produce similar reflection at certain scan angles would be difficult. In particular, when large quantities of amorphous hemicellulose and lignin with cellulose are present (as evident in lignocellulosic biomass), the scattering becomes diffusive and spread out. This creates difficulty in separating amorphous reflections from crystalline reflections. Because of the large crystallite size in pure crystalline substances, scattering is quite sharp and diffusive patterns can be avoided or at least minimized. However, in substrates with small crystallite size (20-50 Å), such as lignocellulosic biomass, separating amorphous scattering from crystalline scattering would no longer be trivial and would have serious overlaps (Thygesen et al., 2005; Kasai and Kakudo, 2005). Thus, we briefly present some of the other refined methods described in the literature that can be successfully used to determine crystallinity (a useful parameter in successful utilization of biomass for fuels and chemicals and an important parameter in this study) of lignocellulosic biomass.

Earlier developments in measuring crystallinity of polymeric substances with paracrystalline distortions (owing to amorphous scattering) took place with the work of Ruland (Ruland, 1961) and Vonk (Vonk, 1973). This method involves separation of crystalline peaks from amorphous peaks. The amorphous part of the scattering is obtained from standard amorphous substances, the reflections are then scaled down below the crystalline reflections, and the fraction of integrated intensity of the crystalline phase out of total intensity including the amorphous background is referred to as crystallinity of the sample. That approach was further refined as the Rietveld refinement (Rietveld, 1967; Rietveld, 1969), which uses the full diffraction pattern in a least squares fitting numerical procedure to fit the complete X-ray diffractograms including the background or diffusive scattering due to amorphous regions.

Several shape functions can fit the complete X-ray diffractograms using least squares fitting. However, it is very important to understand the three major sources that contribute to the shape function of the observed X-ray profile (Taupin, 1973): structure of material under consideration, spectral distribution of the X-ray source, and instrumental or non-spectral contributions. The observed profile h(2θ) is a convolution (Θ) of the intrinsic specimen profile f(2θ) with the spectral distribution (W) and the instrumental function (G) superimposed over the background b (Garvey et al., 2005):

$$h(2\theta) = [(W\Theta G)\Theta f](2\theta) + b \tag{4}$$

The Voigt function, which is a convolution of Gaussian and Lorentzian peak functions, would include both Gaussian intrinsic broadening of the specimen along with the Lorentzian instrumental profile that considers the background from amorphous scattering. The Voigt function, therefore, appropriately mimics the three major sources presented above. Using the Voigt function intensity of the reflection is represented by following equation:

$$f(2\theta) = \frac{a_0 \int_{-\infty}^{\infty} \frac{\exp(-(2\theta)^2)}{{a_1}^2 + \left(\frac{x-a_c}{a_g} - 2\theta\right)^2} d(2\theta)}{\int_{-\infty}^{\infty} \frac{\exp(-(-(2\theta)^2)}{{a_1}^2 + (2\theta)} d(2\theta)} \tag{5}$$

Where a_o is the amplitude of the peak, a_c is the center of the peak, a_l is the width of the Lorentzian component, and a_g is the width of the Gaussian component of the peak. The

whole process involves nonlinear least squares fitting of the Voigt function from the Peakfit program to full X-ray diffractograms. The major reflective planes in cellulosic material corresponding to following Miller indices (hkl) were identified: 101, 10ī, 002, 021, and 040 (Liu et al., 2005); 002 was the prominent reflection representing crystalline cellulose (sometimes resolved into 021 plane as well). The program was re-run locking these planes, and, consequently, five Voigt functions were fitted. The goodness of fit was assessed using R^2. Crystallinity was calculated from equation (6) (Wada and Okano, 1997),

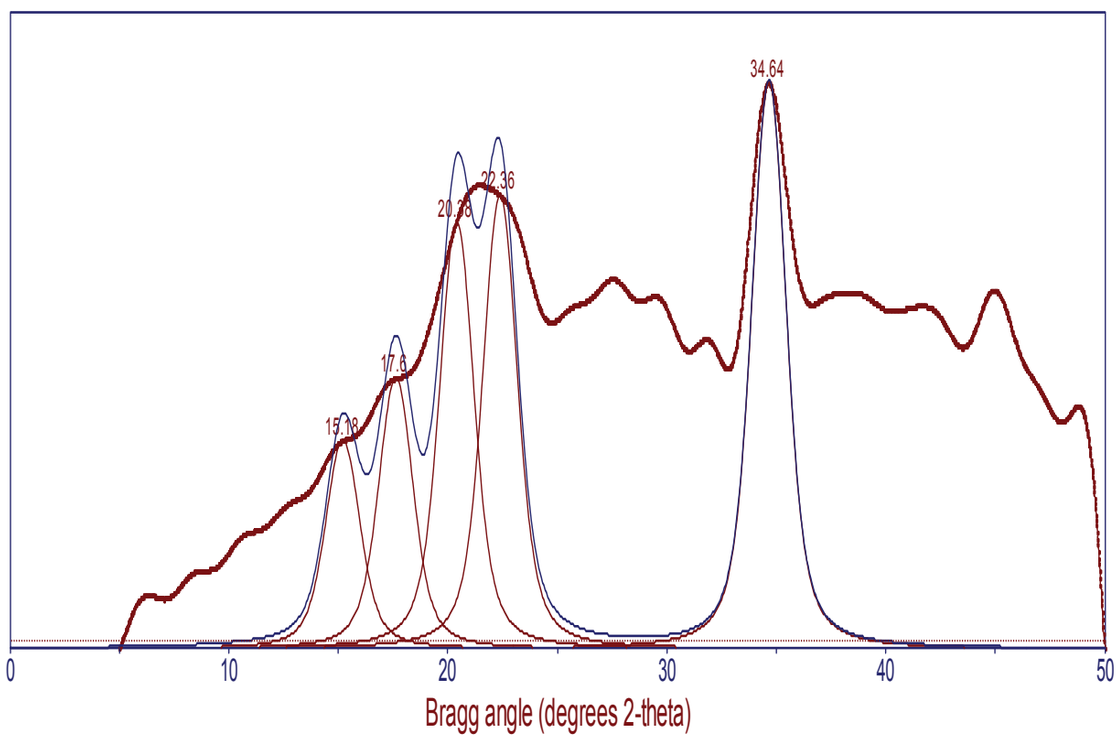
$$X_{cr}(\%) = \frac{I_{002} + I_{021}}{I_{101} + I_{10\bar{1}} + I_{002} + I_{021} + I_{040}} \times 100 \quad (6)$$

Where I followed by a subscript represents the integrated intensity of the particular Bragg plane. Five Bragg planes (subscripts of I in equation 6) have been generally identified in cellulosic samples from plant materials (Liu et al., 2005). Crystallinity, therefore, represents the fraction of α-cellulose represented by planes 002 and 021 present in a particular sample.

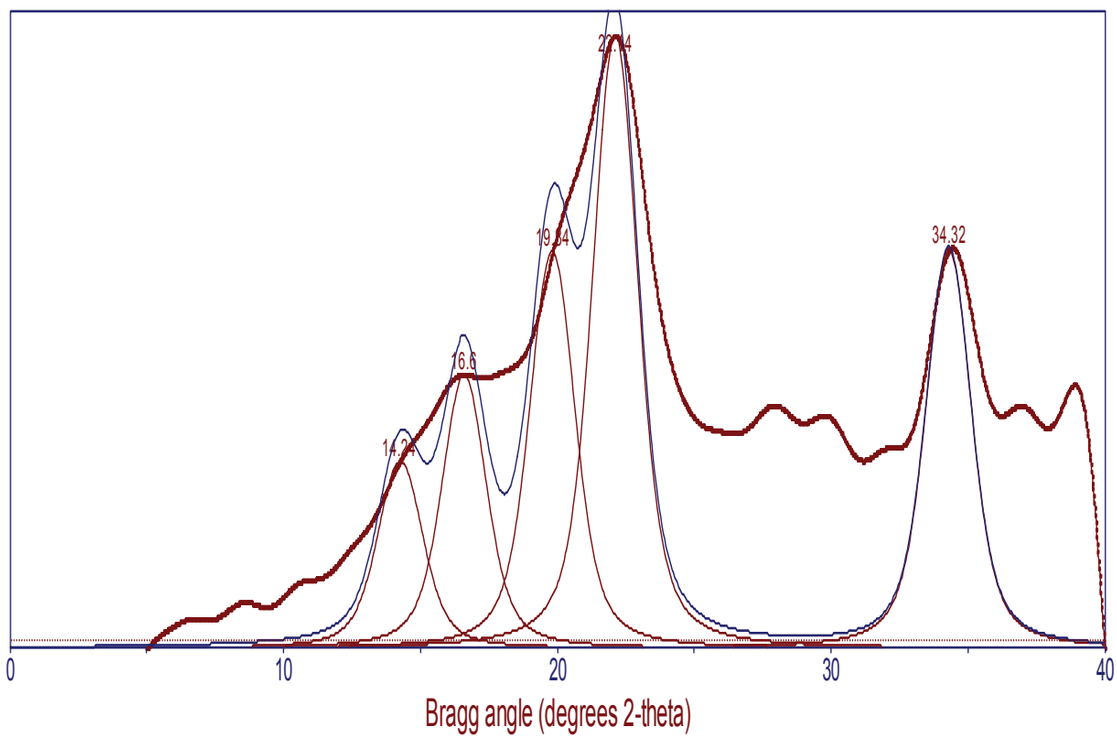
4.2 Effect of various pretreatments on physicochemical characteristics of soybean hulls

The X-ray diffractograms for five substrates: native soybean hulls (untreated), steam pretreated soybean hulls (121 ^oC, 15 psi), HCl pretreated soybean hulls (ambient temperature treatment with 1N HCl using 5% soybean hulls slurry for 24 h), H_2SO_4 pretreated soybean hulls (ambient temperature treatment with 1N H_2SO_4 using 5% soybean hulls slurry for 24 h), and NaOH pretreated soybean hulls (ambient temperature treatment with 1N HCl using 5% soybean hulls slurry for 24 h) is shown in Fig. 3A-E (notice the five peaks corresponding to identified lattice planes). Almost all diffractograms using this scheme had R^2>0.95. For more details on pretreatment, readers are directed to Brijwani and Vadlani (2010). As seen from Fig. 1A-E, the gradual evolution of the 002 plane ($2\theta \sim 22^o$) from native soybean hulls to pretreated hulls is an indication of increased crystallinity in pretreated solids. The pretreated soybean hulls had crystallinity in the range of 57 to 59% compared to untreated native soybean hulls that had 42% crystallinity. The increase is probably due to reduction in the amorphous phase and plausible correction in lattice defects of cellulose during ambient-temperature acid and alkali treatments and steam treatment (Brijwani, 2011). In a similar fashion pretreated substrates were more porous compared to native soybean hulls. The increase in bed porosity is likely due to modification of the internal structure of soybean hulls that led to redistribution and partial solublization of hemicellulose and swelling of the biomass (Kumar et al., 2009). Volumetric specific surface (cm^{-1}), on the other hand, was similar for pretreated and native soybean hulls. It should be noted that volumetric specific surface measurements were the outcome of particle size analysis done on dried substrates without any moisture addition to facilitate efficient sieving. Hence, the volumetric specific surface was more of a function of particle size and, therefore, was exclusively the representation of external surface area per unit volume. It, however, could not be the real representation of internal surface area that is the function of pore size and internal volume. The reason for showing volumetric specific surface in the current study is to identify research directions such that studying pore volume alongside porosity might provide more insight into the dynamics of cellulolytic enzyme production and its relationship with physical properties of the substrate (Brijwani, 2011).

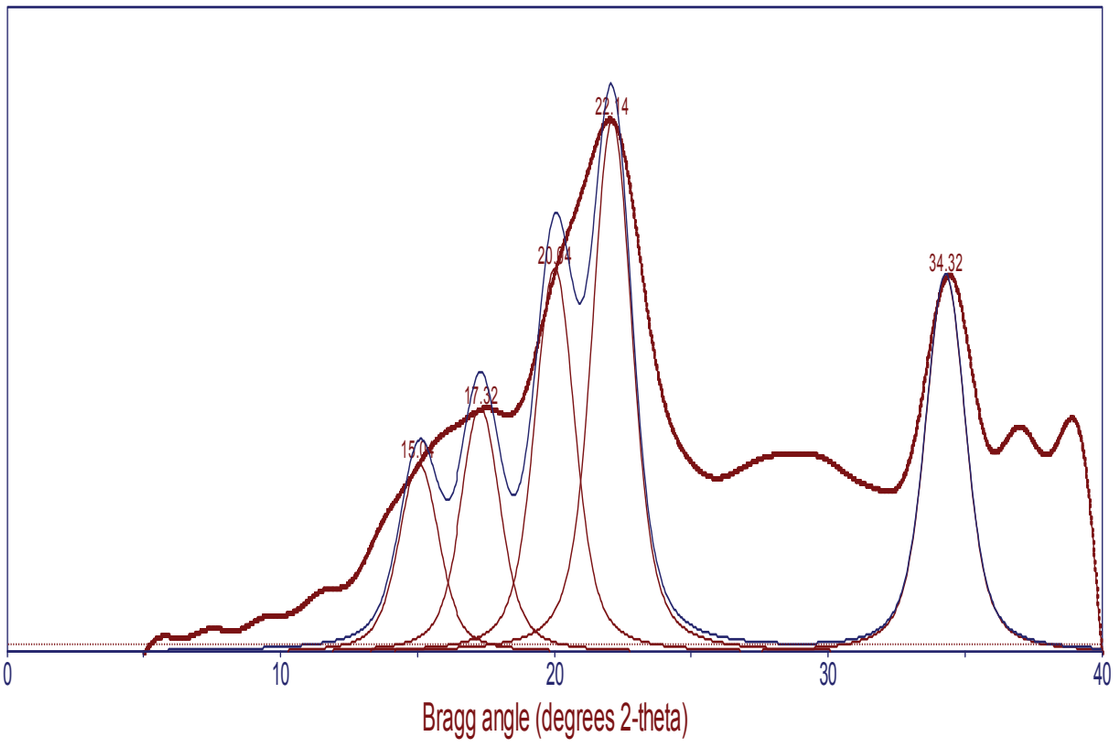
(A)


34.64
22.36
20.36
17.6
15.16
0
10
20
30
40
50
Bragg angle (degrees 2-theta)

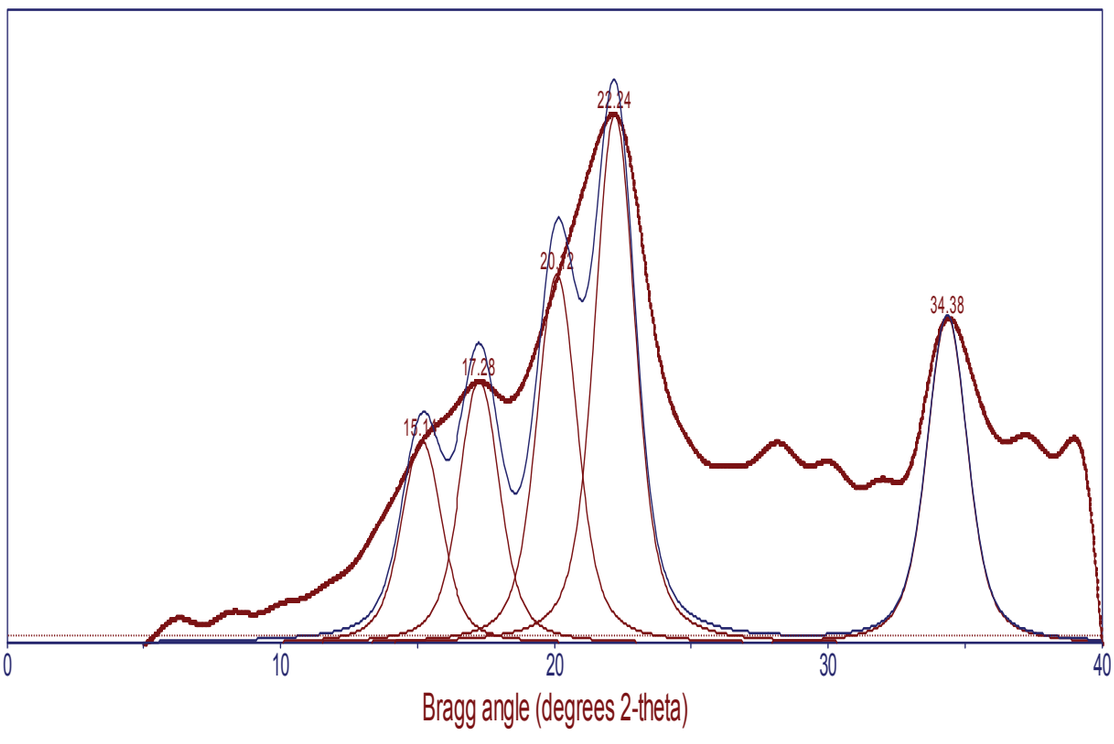
(B)


34.32
19.84
16.6
14.24
0
10
20
30
40
Bragg angle (degrees 2-theta)

(C)


22.14
20.04
34.32
17.32
15.04
0
10
20
30
40
Bragg angle (degrees 2-theta)

(D)


22.24
20.22
34.38
17.28
15.14
0
10
20
30
40
Bragg angle (degrees 2-theta)

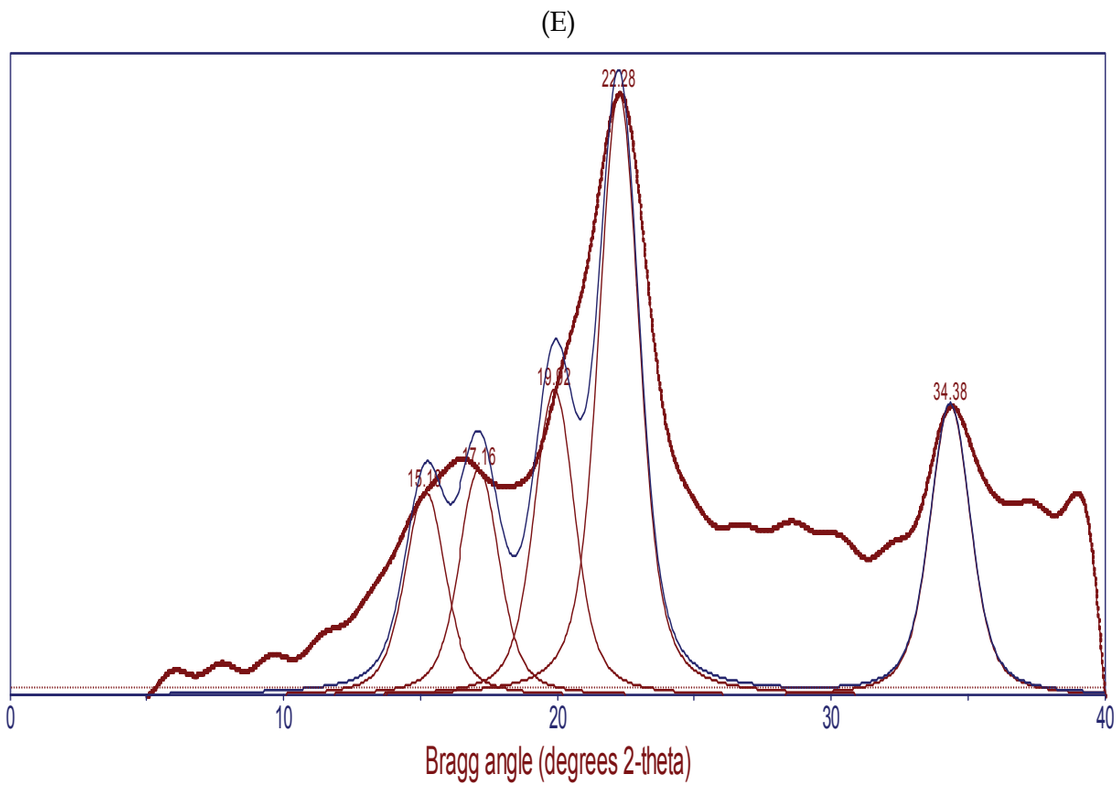


Fig. 3. X-ray Signatures. Gaussian smoothing followed by Voigt function was used to fit the diffractogram output of the instrument. (A) Native soybean hulls. The characteristics peaks identified were: 2θ = 15.18° (101 plane), 17.6° (10ī), 20.38° (021 plane), 22.36° (002 plane), and 34.64° (040 plane). (B) Steam treated soybean hulls. The characteristics peaks identified were: 2θ = 14.24° (101 plane), 16.6° (10ī), 19.84° (021 plane), 22.14° (002 plane), and 34.32° (040 plane). (C) HCl treated soybean hulls. The characteristics peaks identified were: 2θ = 15.04° (101 plane), 17.32° (10ī), 20.12° (021 plane), 22.24° (002 plane), and 34.38° (040 plane). (D) H_2SO_4 treated soybean hulls. The characteristics peaks identified were: 2θ = 15.11° (101 plane), 17.28° (10ī), 20.04° (021 plane), 22.14° (002 plane), and 34.32° (040 plane). (E) NaOH treated soybean hulls. The characteristics peaks identified were: 2θ = 15.18° (101 plane), 17.16° (10ī), 19.92° (021 plane), 22.28° (002 plane), and 34.38° (040 plane).

4.3 Effect of physicochemical characteristics on cellulolytic enzyme production in mixed and mono cultures of *T. reesei* and *A. oryzae*

In order to explicitly demonstrate the effect of crystallinity and bed porosity on cellulolytic enzyme production, we modeled both crystallinity and porosity using the general linear model of SAS with the following expression:

$$y_{ijk} = \mu + ab_{ij} + \epsilon_{ijk} \tag{7}$$

Where y_{ijk} is one of the enzyme activities as the dependent variable, μ is the grand mean, ab_{ij} is the interaction effect of crystallinity and porosity, and ϵ_{ijk} is random error with mean 0 and experimental error variance as its variance. The composition was not included as it

was fairly constant across treatments i.e., the total cellulosic composition (cellulose + hemicellulose) was not significantly different among the pretreatments (Brijwani, 2011). This was attributed to mild nature of the pretreatments. Similarly, volumetric specific surface was not included in the model as it did not change significantly upon pretreatment. Considering crystallinity and porosity together became necessary because of availability of a reduced number of degrees of freedom that refrained their independent analysis. It was very difficult to generate substrates for which one effect was varied while the other effect remained constant. Future studies in this direction will be beneficial, provided such substrates are available or easy to generate. Broadly speaking, the model represented by equation (7) is more reflective of one-way variance analysis than factorial variance analysis.
On the basis of results in Table 1, both crystallinity and porosity had positive and significant improvements in all enzyme activities except xylanase production for *T. reesei*. Notice that only native and steam-treated soybean hulls were included in determining out the

Interaction	Culture	Cellulolytic enzyme system				Treatments considered
		Filter paper units (FPU/g-ds)	Beta-glucosidase (IU/g-ds)	Endoglucanase (IU/g-ds)	Xylanase (IU/g-ds)	
Crystallinity × Porosity	*Trichoderma reesei*	<0.0001*	0.0388*	<0.0001*	0.0472	Native, Steam
Crystallinity × Porosity	*Aspergillus oryzae*	<0.0001*	0.2736	<0.0001*	0.0065*	Native, Steam, HCl, H_2SO_4
Crystallinity × Porosity	*Aspergillus oryzae*	0.4629	0.9218	0.0005*	0.9912	Native, Steam
Crystallinity × porosity	Mixed	<0.0001*	0.0140*	<0.0001*	<0.0001*	Native, Steam, HCl, H_2SO_4
Crystallinity ×porosity	Mixed	0.0044*	0.0449	0.0257*	0.9061	Native, Steam

"*" indicates probability for a particular interaction is significant at 95% confidence Abbreviations
Native: - Untreated soybean hulls
Steam: - Steam-pretreated soybean hulls
HCl: - Soybean hulls pretreated with 1N HCl
H2SO4: - Soybean hulls pretreated with 1N H2SO4
NaOH: - Soybean hulls pretreated with 1N NaOH

Table 1. Effect of interaction between physical characteristics on production of cellulolytic enzyme system for three cultures

differences. This was a result of the higher sensitivity of *T. reesei* toward inhibitors present in acid- and alkali-treated substrates. In *A. oryzae*, when all four substrates were considered, we noticed that probabilities were significant in all cases. In general, acid-pretreated substrates, except in β-glucosidase production, performed lower than native and steam-pretreated substrates, which is probably the reason for the lower probabilities. Therefore, when acid substrates were removed from the model, the real effect of crystallinity and porosity on production of endocellulase became evident, and the effect increased significantly with increases in crystallinity and porosity. In mixed culture (Table 1), the effect was more or less level, and performance was nearly identical except that filter paper and beta-glucosidase activities were significantly higher in less crystalline and porous native soybean hulls than in highly crystalline and porous steam-treated soybean hulls.

Porosity is a physical parameter that ensures oxygen availability between the moist substrate particles. It is plausibly implicated in the growth of fungal cultures and, therefore, affects enzyme production from a growth point of view. Rahardjo et al. (2005a; 2005b) explained this phenomenon by using different kinds of model substrates that differed in the amount of open spaces for production of α-amylase in solid-state cultures of *A. oryzae*. Crystallinity, on the other hand, might be the decisive factor in influencing expression levels of enzymes within a cellulolytic enzyme system, suggesting it might enrich one activity over another. This phenomenon was noticed in *T. reesei*, in which production of cellulase enzymes increased substantially in pretreated soybean hulls compared with native hulls but xylanase remained constant. We made similar observations in endocellulase production in *A. oryzae*. A positive effect of crystallinity was also reported for liquid cultures of *Trichoderma* spp. (Acebal et al., 1986; Evans et al., 1992; Aiello et al., 1996). Although no specific variation in different activities with varying crystallinity were mentioned in these studies, an overall increase in cellulase activity was reported during growth on pretreated crystalline biomass or standard crystalline cellulose.

5. Conclusions

The present study demonstrated for the first time the suitability of mixed-culture, solid-state fermentation in the production of an efficient cellulase enzymes complex from soybean hulls. Further, the role of physicochemical characteristics in production of cellulolytic enzymes in fungal SSF was comprehensively investigated. With recent advances in computation capability, whole X-ray diffractograms can be fitted to obtain reliable measures of crystallinity of heterogeneous material such as lignocellulosic biomass. Production of cellulase enzyme using co-cultures of *T. reesei* and *A. oryzae* grown on solid media containing 4:1 of soybean hulls and wheat bran were fitted to quadratic model using RSM. Both cellulase and β-glucosidase production showed significant dependence on temperature and moisture content as compared to pH. Temperature showed quadratic effect on the production of both cellulase and β-glucosidase while moisture had linear effect on cellulase and β-glucosidase production. The interaction effects of three independent variables were not significant though interaction of temperature and moisture was considerable for cellulase production, while interaction of moisture and pH was considerable for β-glucosidase production. The optimized values of three independent variables viz. temperature, pH and moisture content were predicted using numerical optimization that maximized cellulase and β-glucosidase activities. Results showed that co-culturing of fungal

species with proper optimization resulted in complete and balanced production of enzymes. Both bed porosity and crystallinity were significantly implicated in production of cellulolytic enzymes. We explicitly showed that exposure of crystallinity made *T. reesei* a robust producer of cellulolytic enzymes, whereas *A. oryzae* responded to crystallinity by being a good producer of endocellulase activity. The changes brought about by physical characteristics are an important design tool for process development of the SSF. By changing the physical attributes expression levels of enzymes can be varied such that physicochemical characteristics become the leading force in directing synthesis of the cellulolytic enzyme system in SSF.

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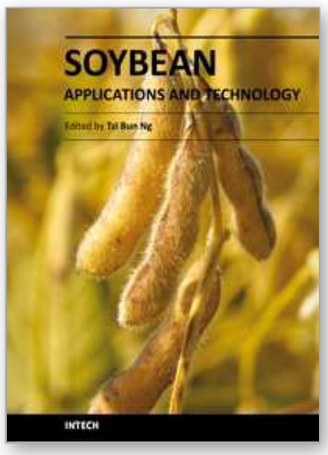

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Soybean is an agricultural crop of tremendous economic importance. Soybean and food items derived from it form dietary components of numerous people, especially those living in the Orient. The health benefits of soybean have attracted the attention of nutritionists as well as common people.

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