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Research Article

Experimental Validation of Optimization by Statistical and CFD Simulation methods for Cellulase Production from Waste Lignocellulosic Mixture

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Abstract

Cellulase production poses a challenge to the biofuel industries. In the present work, a mixture of surgical waste cotton and packaging card board was used for cellulase production, employing *Trichoderma harzanium* ATCC 20846. For a Submerged Fermentation (SMF), a statistical optimization was performed using Response Surface Methodology (RSM) for the following parameters: agitation, Dissolved Oxygen% (DO), aeration, viscosity, and temperature. Additionally, a Computational Fluid Dynamic (CFD) simulation was performed to study the optimum broth viscosity. A cellulase production SMF (model validation) performed using the parameter values given by the design and simulation yielded enzyme activities of: 1.85±0.1 FPU/mL; 12.4±0.2 CMCase/mL; 743±0.1 Xylanase/mL; and 3165.8±0.25 Beta-glucosidase/mL. 12% variation was seen from the predicted results. Furthermore, the biomass yield coefficients ($Y_{x/s}$, $Y_{x/O2}$); Oxygen Uptake Rate (OUR); maintenance coefficients (mO2x);mass transfer coefficient (K_La); Oxygen Transfer Rate (OTR); and the effects of viscosity and sugar accumulation on cellulase production were studied for the SMF.

Keywords: Statistical optimization; Biomass yield coefficient; Mass transfer coefficient; Oxygen uptake rate; Simulation.

Introduction

Cellulases sequentially hydrolyze cellulose to individual beta-D-glucose units [1]. Cellulase production incurs 40% of the total bio-ethanol production cost. Researchers have been working on process improvements [2,3]. *Trichoderma harzanium*, a noted producer of cellulase, can grow at various temperatures. It falls under Phylum Ascomycota and the family Hypocreaceae and multiplies using asexual spores [4].

A novel surgical cotton-waste cardboard mixture was chosen as the substrate for the following reasons: (a) Waste re-usability (b) the Higher cellulose content of cotton (c) Cardboard with varying lignin content that helps in providing mechanical support to aid complete pulverization of cotton and (d) Prevention of the competition to animal fodder by avoiding the use of rice/wheat straws. Moreover, improper disposal (usually in landfills) of municipal [5] and packaging wastes [6] has been posing a huge problem, globally. Since these wastes are rich sources of biomass [5], they could be used for energy production processes [7]. Cellulase production from waste has been attempted earlier from sources such as municipal waste by Solid State Fermentation (SSF) [8,9] coir using SSF and Submerged fermentation (SMF) [10] soybean hull and waste paper using SSF [11].

The SMF in a bioreactor is an extension of the microbial cultivation in an Erlen Meyer flask, the only difference being the controllable parameters such as temperature, aeration, pH, foaming, and agitation [12]. Apart from the reactor parameters, the media must possess the optimum C/N ratio [13] to enhance the microbial growth [14]. Scale up of the SMF from a lab scale bio reactor to a pilot scale/ production scale bioreactor can be performed using the calculation of various data such as the Biomass yield coefficient ($Y_{x/s}$, $Y_{x/O2}$); Oxygen Uptake Rate (OUR); maintenance coefficients (mO_2x); mass transfer coefficient (K_La); Oxygen Transfer Rate (OTR) [15,16].

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Process optimizations may aid in the enhancement of process economy [17]. Design of experiment (DoE) is widely applied in bioprocess engineering [18]. Response Surface Methodology (RSM) uses a factorial design to analyze the statistical results [19]. In this work, the Response Surface Methodology using a Central composite design (CCD) has been employed for the process optimization. Though many contemporary researchers have optimized cellulase production media [20], the current work presents an innovative approach of optimizing the bioreactor's operational parameters (they impact each other) [21-24]. The impact of the different factors (aeration, temperature, viscosity, DO%, and agitation) (obtained from RSM-CCD) on the response (enzyme activities) was studied. The regression fit model provides a simple equation that elucidates the fit of the predicted model [25].

CFD helps in providing a numerical modeling of the field of flow impacted by an impeller and an aeration setup [26. CFD analyses and mixing designs provide considerable possibility and ideas for bioreactor scale-up [27, 28]. In the present work, three values of viscosities were chosen to simulate the DO% in the SMF for cellulase production (within the bioreactor). A higher DO% implies better fungal growth and higher cellulase production [29]. The varying viscosities of the culture broth were the chosen values for the CFD simulation of the DO%.

In the present work, SMF for the production of cellulases was carried out in a bioreactor using a novel cotton-cardboard mixture. Prior to the SMF, the reactor's optimum operational parameter values were obtained using statistical optimization techniques. It was hypothesized that after statistical optimization, the various parameters that influence an SMF for cellulase production would yield enhanced enzyme activities in an experimental validation (using the model predicted values).Furthermore, a CFD simulation was performed with anticipation of achieving the optimum viscosity value that needs to be maintained in the SMF for cellulase production. The results predicted by the experimental design, and the results of the actual experiment were compared.

Materials and methods

Statistical optimization of cellulase production from waste lignocellulosic mixture

The model was developed using the following parameters: temperature (X_1), viscosity (X_2), aeration (X_3), dissolved oxygen (X_4) and agitation (X_5). The response factor considered in the model was the enzyme activity (Y). The low and high factors were coded as (-1), and (+1), respectively; the mid-point setting was 0. The optimal factor values were obtained from the central composite face centered design. The Central Composite Design's (CCD) experimental runs are represented as $2^{k,}$ (k - the number of factors chosen for the process optimization) mathematically that resulted in 32 runs as shown in table 1.

Regression analysis

The regression and graphical analyses of the data obtained were performed using MINITAB software (version 18). Response surface regression analysis is performed with a set of data to estimate the relationship between the parameters that are involved in the process. The statistical analysis of the model is presented in the form of an Analysis of Variance (ANOVA).

Computation Fluid Dynamics (CFD) simulation

Three varying culture broth viscosities of: 0.8 cP – media viscosity at the start of the fermentation; 2.075 cP- suggested by the statistical optimization design; and 3.25 cP- obtained at the end of a batch cellulase production; were chosen for the simulation of DO% using CFD version 2.2. For the simulation, the conditions assigned were unsteady state fluid flow; and a liquid with varying viscosity.

Preparation of the novel cellulosic substrate mixture

Surgical waste cotton used in this work includes ethanol cotton wipes for the topical procedure, bandages, shredded cellulosic lab gowns, cotton gauzes, which are devoid of heavy biological fluids. The substrate preparation techniques are as mentioned in our previous works with the mentioned substrate [30,31]. The cardboard and cotton mixture were combined in a 1:1 ratio (weight basis), as the other ratios would not facilitate complete pulverization of cotton. In lesser ratios, the cotton could not be pulverized

(20-minute operation) leading to it forming thin strands and winding around the pulverizer's (M/s Classic, India, SKU SSM GM 001) blades, making it difficult for harvest. The lignocellulosic mixture, after pulverization was used as the substrate for cellulase production.

Table 1. A central composite design for cellulase enzyme production

Run order	X1	X2	X3	X4	X5	Experimental cellulose activity, FPU/mL
1	33	0.9	0.5	40	80	1.62
2	27.5	2.075	2	70	130	1.8
3	33	0.9	3.5	40	180	1.74
4	33	0.9	0.5	100	180	2.2
5	22	3.25	3.5	40	180	1.6
6	27.5	2.075	2	70	130	1.9
7	27.5	2.075	2	70	130	1.9
8	22	3.25	3.5	100	80	2.2
9	27.5	2.075	2	100	130	2.2
10	27.5	2.075	2	40	130	1.8
11	27.5	2.075	2	70	180	1.9
12	27.5	2.075	2	70	80	1.7
13	22	0.9	0.5	100	80	2.2
14	27.5	2.075	3.5	70	130	1.9
15	27.5	2.075	2	70	130	1.9
16	27.5	3.25	2	70	130	2
17	33	3.25	3.5	100	180	1.9
18	33	0.9	3.5	100	80	2.2
19	33	3.25	0.5	40	180	1.6
20	22	3.25	0.5	40	80	1.6
21	27.5	2.075	2	70	130	1.87
22	33	2.075	2	70	130	1.87
23	22	0.9	3.5	40	80	1.7
24	27.5	2.075	0.5	70	130	1.8
25	22	2.075	2	70	130	1.9
26	27.5	2.075	2	70	130	1.9
27	22	3.25	0.5	100	180	2
28	27.5	0.9	2	70	130	2.2
29	22	0.9	0.5	40	180	1.8
30	33	3.25	3.5	40	80	1.6
31	22	0.9	3.5	100	180	2.2
32	33	3.25	0.5	100	80	1.8

Fermentation media and inoculum

T. harzanium ATCC 20846 was purchased from The ATCC. The primary inoculum for the SMF was prepared in 200 mL Vogel's media, containing 1%(w/v) Microcrystalline cellulose (99% purity analytical, 50 um, M/s Sigma Aldrich), as the substrate (for the primary inoculum). A spore suspension containing ($3x10^9$/mL) was used for inoculation of the primary culture. It was incubated for 5 days, at 28°C.

The composition of Vogel's media (g/L) is as follows: Tryptone (1g/L) (Casitose Type I); Tri-sodium citrate (2.5 g/L) (Cell culture grade); Di-Potassium hydrogen phosphate (5 g/L) (AR

grade); Ammonium nitrate (2 g/L) (AR grade); Magnesium sulphate heptahydrate (1.4 g/L) (AR grade); finely powdered surgical waste cotton-card board mixture in 1:1 ratio (1% w/v); Calcium chloride dehydrate (0.1g/L) (AR grade); and Tween 80 – 0.2 % (v/v) (AR grade). A Trace element solution containing Citric acid monohydrate (5g/L) (AR grade); Zinc sulphate heptahydrate (5 g/L) (AR grade); ferrous ammonium sulphate (1 g/L) (AR grade); Copper sulphate (250 mg/L) (AR grade); Manganese sulphate (50 mg/L) (AR grade); Boric acid (50mg/L) (AR grade); Sodium molybdate (50 mg/L) (AR grade); was prepared. 1 mL/L of the Trace element solution was added to the media components [32]. The pH was set at 5.5, before autoclaving.

Submerged Fermentation on the basis of statistical design predictions

The submerged batch fermentation of 1 L culture volume was carried out in a 3.2 L Bioengineering KLF Advanced Bioreactor (M/s Bioengineering, Switzerland) with automated controllers.

Cultivation in the bioreactor

A working volume of 1 litre (Vogel's media + inoculum) was to be used in the bioreactor. 1% (w/v) surgical waste cotton-card board mixture (1:1 ratio) was used as the substrate in the fermentor. 20% (v/v) inoculum was used. The fermentor was set for the pH of 5.5. The other parameter values such as, agitation, aeration, DO%, temperature, and viscosity, were used from the statistical model. 100 mL of fresh autoclaved Vogel's minimal media was added to the reactor, every 24 h, after 36-72 h of growth, to maintain the viscosity at 2 cP (as suggested by the experimental design).

Estimation of the characteristics of the SMF

The biomass yield coefficients based on substrate and oxygen consumptions ($Y_{x/s}$ and $Y_{x/O2}$) of the fungal biomass were determined by estimating the amount of substrate and oxygen consumed, respectively. Dynamic gassing out method was used to estimate the Oxygen Uptake Rate (OUR), and the Oxygen Transfer Rate (OTR) [33] within the bioreactor. The KLa and the OTR varied as a result of the increase in the broth's viscosity after 36-72 h of the submerged fermentation [34, 35]. The viscosity changes during the fermentation were monitored using a Viscometer (M/s Cole Parmer WW98965, Illinois). The amount of glucose, cellobiose, and xylose, accumulated in the broth was estimated using a HPLC (Agilent 1290, with Hi-PLex H column) [36] during the course of the fermentation. The KLa, OTR, and OUR, were calculated using the following relation (Eq. 1) [37].

$$OTR = \{KLa\ (C^* - -C_L\} - OUR \quad (1)$$

OTR – Oxygen Transfer Rate (ppm O_2/hr)
KLa – volumetric mass transfer coefficient (hr^{-1})
C* - Saturated oxygen concentration (ppm O_2)
C_L- Dissolved oxygen concentration within the reactor (ppm O_2)
OUR - Oxygen Uptake Rate (ppm O_2/hr)

Enzyme harvest and enzyme activity estimation

Sampling was done at an interval of 24 h to estimate the enzyme activity. The broth was centrifuged (Centrifuge: M/s. Beckman Coulter) at 6000 rpm, at 4°C, for 20 min. The enzyme activity (enzyme from the supernatant) was measured using the standard IUPAC DNSA method [38].

Dry mycelial weight measurement

The dry mycelial weight was measured using a slightly modified method of Aftab and Patrick, 2008 used in a previous work of ours [37]. The residual substrate concentration was also measured in this method.

Measurement of enzyme activity

The activity of the secreted cellulases was measured using the standard DNSA method of IUPAC [38]. Exoglucanases/FPases; Endoglucanases/CMCases; Beta-glucosidases/Cellobiases; have a threshold value of sugar release. Xylanase, a class of hemicellulase, increased in yield. The activities were estimated by quantifying the amount of reducing sugar released from xylan using dinitrosalicylic acid (DNS) method.

Results and discussion

Statistical analysis

The response surface contains a curvature; so a polynomial model of higher degree is used (MINITAB VERSION, 2018). The second-order polynomial equation obtained for the process optimization is as given in Eq. 2:

$$Y = -0.246 + 0.0877\ X1 - 0.398\ X2 + 0.1613\ X3 + 0.00316\ X4$$

*+ 0.01499 X5 - 0.00162 X1*X1*
*+ 0.1203 X2*X2*
*- 0.0373 X3*X3 + 0.000073 X4*X4 -*
*0.000054 X5*X5*
*- 0.00348 X1*X2 + 0.00091 X1*X3*
*- 0.000136 X1*X4 + 0.000073 X1*X5*
*+ 0.00993 X2*X3 - 0.000780 X2*X4 -*
*0.000340 X2*X5*
*+ 0.000389 X3*X4 - 0.000533 X3*X5 -*
*0.000013 X4*X5* (2)

Where, X1 = Temperature, X2 = Viscosity, X3 = Aeration, X4 = Dissolved Oxygen and X5 = Agitation& Y – Response (Enzyme activity).

The regression analysis of the process is shown in table 2. The term coeff represents the change in the mean response. In terms of linear model, the factors temperature and viscosity have a negative value, which indicates that when the temperature and viscosity increase by 1, the response (enzyme activity) decreases approximately by 0.0372 and 0.0867, respectively accounting for the change. The p – value less than 0.05 (Level of significance) demonstrates that the relationship between the response and parameter is more statistically significant (MINITAB VERSION, 2018). Therefore, temperature, viscosity, and DO%, are considered to be more significant in terms of linear model.

Table 2. Estimated regression coefficient for second-order polynomial model

Term	Coeff	SE Coeff	t-Value	p-Value
Constant	1.9101	0.0169	112.71	0.000
Temperature	-0.0372	0.0140	-2.66	0.022
Viscocity	-0.0867	0.0140	-6.20	0.000
Aeration	0.0233	0.0140	1.67	0.123
DO	0.2133	0.0140	15.26	0.000
Agitation	0.0178	0.0140	1.27	0.230
Temperature*Temperature	-0.0489	0.0378	-1.29	0.222
Viscocity*Viscocity	0.1661	0.0378	4.39	0.001
Aeration*Aeration	-0.0839	0.0378	-2.22	0.048
DO*DO	0.0661	0.0378	1.75	0.108
Agitation*Agitation	-0.1339	0.0378	-3.54	0.005
Temperature*Viscocity	-0.0225	0.0148	-1.52	0.157
Temperature*Aeration	0.0075	0.0148	0.51	0.623
Temperature*DO	-0.0225	0.0148	-1.52	0.157
Temperature*Agitation	0.0200	0.0148	1.35	0.204
Viscocity*Aeration	0.0175	0.0148	1.18	0.263
Viscocity*DO	-0.0275	0.0148	-1.86	0.091
Viscocity*Agitation	-0.0200	0.0148	-1.35	0.204
Aeration*DO	0.0175	0.0148	1.18	0.263
Aeration*Agitation	-0.0400	0.0148	-2.70	0.021
DO*Agitation	-0.0200	0.0148	-1.35	0.204

The regression model was highly significant with an R^2 value of 0.9689, which implies that 96.89% variability of the response could be explained by the model [39]. The term "variation" stated in the above sentence refers to the variation of the response (Enzyme activities) obtained using the optimized reactor parameters. The predicted response (Enzyme activity) is in

96.89% agreement with the predicted optimization model. Dhaliwal Maninder and More, 2016 [40] reported 95.15% variability in the response influenced by the factors for optimization of cellulase production using soil bacteria. Mani et al. 2017 [41] reported 90.12% variability in the response when considering components for media optimization for cellulase production using *Bacillus cereus*.

The adequacy and significance of the model was examined using an ANOVA. The results of the ANOVA are as summarized in table 3.

Table 3. ANOVA for process optimization

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	20	1.20431	0.060215	17.13	0.000
Linear	5	0.99483	0.198966	56.59	0.000
Temperature	1	0.02494	0.024939	7.09	0.022
Viscosity	1	0.13520	0.135200	38.45	0.000
Aeration	1	0.00980	0.009800	2.79	0.123
DO	1	0.81920	0.819200	232.98	0.000
Agitation	1	0.00569	0.005689	1.62	0.230
Square	5	0.12568	0.025136	7.15	0.003
	1	0.00589	0.005890	1.68	0.222
Temperature*Temperature					
Viscosity*Viscosity	1	0.06787	0.067871	19.30	0.001
Aeration*Aeration	1	0.01733	0.017332	4.93	0.048
DO*DO	1	0.01074	0.010744	3.06	0.108
Agitation*Agitation	1	0.04414	0.044136	12.55	0.005
2-Way Interaction	10	0.08380	0.008380	2.38	0.085
Temperature*Viscosity	1	0.00810	0.008100	2.30	0.157
Temperature*Aeration	1	0.00090	0.000900	0.26	0.623
Temperature*DO	1	0.00810	0.008100	2.30	0.157
Temperature*Agitation	1	0.00640	0.006400	1.82	0.204
Viscosity*Aeration	1	0.00490	0.004900	1.39	0.263
Viscosity*DO	1	0.01210	0.012100	3.44	0.091
Viscosity*Agitation	1	0.00640	0.006400	1.82	0.204
Aeration*DO	1	0.00490	0.004900	1.39	0.263
Aeration*Agitation	1	0.02560	0.025600	7.28	0.021
DO*Agitation	1	0.00640	0.006400	1.82	0.204
Error	11	0.03868	0.003516		
Lack-of-Fit	6	0.03059	0.005099	3.15	0.114
Pure Error	5	0.00808	0.001617		
Total	31	1.24299			

The p-value less than the level of significance of 0.05 imply that the factor is more significant [42]. Larger f-value denotes the better fit of the RSM model to the experimental data [43]. The f value with low p-value indicates that the model is statically significant (MINITAB VERSION, 2018). The largest F-value with lowest p-value was observed for DO% and Viscosity in linear terms. DO% is a major parameter influencing the fungal growth and cellulase production. As cellulase production increases, the viscosity of the culture broth increases, in turn negatively influencing the oxygen hold up of the broth, thereby decreasing oxygen availability to the growing cellulolytic fungus and reducing the enzyme activity (response) [44]. The largest f-value with lowest p-value was observed for aeration*agitation in interaction model. This confirms with the scientific fundamental that aeration and agitation are interrelated and contribute to the major parameter DO%, within the bioreactor [44, 45].

Aeration is maintained by supplying air through the sparger line while the agitator breaks the air bubbles and helps in mixing and diffusion of the gas and uptake by the actively dividing culture [44]. The ANOVA study suggested that linear and square models were significant with low p-values. The lack of fit test was also performed to describe the variation in the model. The f-value and p-value of lack of fit was found to be 3.15 and 0.114 respectively which implies insignificant lack of fit. Insignificant lack of fit confirms that the model accounts the regress-response relationship [46].

The absolute values of the standardized effects and the magnitude of the effects are shown in fig. 1. The reference line 2.20 indicates the standardized response at level of significance 0.05. The bars that represents D, B, BB, EE, CE, A, and CC, cross the reference line 2.20 (enzyme activity response) where these factors are statistically significant at the 0.05 level with the current mode terms (MINITAB, 2018).

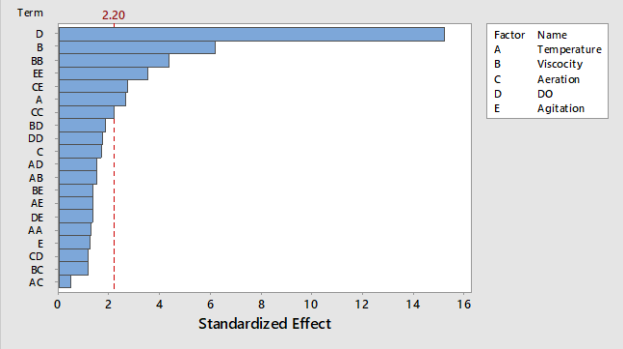

Fig. 1. Pareto chart with response (enzyme activity) with level of significance of 0.05

The parameter hold values (optimum suggested values obtained from the RSM curves) postulated were temperature = 27.5^{o}C; Viscosity = 2.075 cP; agitation = 130 rpm; aeration = 2 SLPM (Standard Litre Per Minute); and Dissolved Oxygen = 70%.The values of the combination are represented in the fig. 2. At temperatures lesser than 27.5-28 ^{o}C, constant FPU values close to 1.78 FPU/mL were obtained. As the temperature was raised from 27.5-33 ^{o}C, the FPU values decreased to 1.7 FPU/mLas seen from various RSM curves in fig.2. The optimum temperature was 27.5 ^{o}C, which can be supported by the fact that the cultivable temperatures of *Trichoderma* specie are within the range of 25-28 ^{o}C. It implies that the organism could grow well at the optimum suggested temperature [42]. A variation in the temperature was checked to perceive if a slight increase in the bioreactor's cultivable temperature could reduce the viscosity of the culture broth [47], which usually causes a reduction in the dissolved oxygen concentration, and oxygen transfer rates [44]. Another reason for the fall in the enzyme activity with the raise in temperature could be because, at elevated temperatures, saccharification of cellulose may occur, causing catabolite repression due to accumulated glucose (as shown in the fig. 4, and cellobiose. These reducing sugars may be consumed by the organism in the bioreactor and as a result further production of cellulases may be inhibited [48]. Therefore, the optimum temperature that the design suggested was 27.5 ^{o}C, which could give an enzyme activity of 1.9 FPU/mL as seen in the fig. 2.

The suggested optimum viscosity to be maintained was 2.075 cP, which could yield an enzyme activity of 1.9 FPU/mL as shown in the fig. 2. At lower values of viscosities such as 0.8 cP, at the start of the fermentation, higher cellulase yields could be obtained close to 2.2 FPU/mL as shown in fig. 2. However, it is impractical to maintain the viscosity at 0.8 cP as the production of cellulases and the accumulation of exopolysaccharides [48] due to the produced cellulases between 36-72 h of fermentation results in viscosity increases close to 2 cP as shown in (fig. 5). At values of viscosities higher than the optimum of 2.07 cP, such as 3.5 cP (at the end of a cellulase production batch), 2 FPU/mL could be obtained as seen in RSM fig. 2, which could be attributed to the increase in agitation to match up the set 80% Dissolved Oxygen values within the fermentor. An increase in agitation would break the mycelia and release the cellulases from the hyphal tips [49] contributing to more cellulases, and thereby an increased enzyme activity of 2 FPU/mL.

The aeration optimum suggested was 2 SLPM (Standard Litre Per Minute), which could yield 1.9 FPU/mL, as seen in fig. 2. At lower values of aeration such as 1 SLPM, 1.7 FPU/mL shown in fig. 2 was obtained, which could be attributed to the fact that, as the biomass within the reactor increase, the Oxygen Uptake Rate (OUR) [50] of the organism increases and depletes the dissolved oxygen. Furthermore, the increase in viscosity of the culture broth due to

cellulase accumulation after 36-72 h of cultivation as shown in fig. 5, would result in the decreased oxygen solubility in the broth and decreased Oxygen Transfer Rates (OTR) [50] thereby resulting in retarded growth and decreased cellulase production. At higher values of aeration of 3 SLPM and more, excessive air passes through the reactor resulting in inevitable foaming. Though the foam sensor disburses a mild concentration of antifoam (Polypropylene glycol less than 200 ppm), repeated foaming causes frequent addition of antifoam and negatively influences the K_La (mass transfer coefficient) of the bioreactor. As a result, growth is hampered and 1.8 FPU/mL may be obtained as seen in fig. 2.

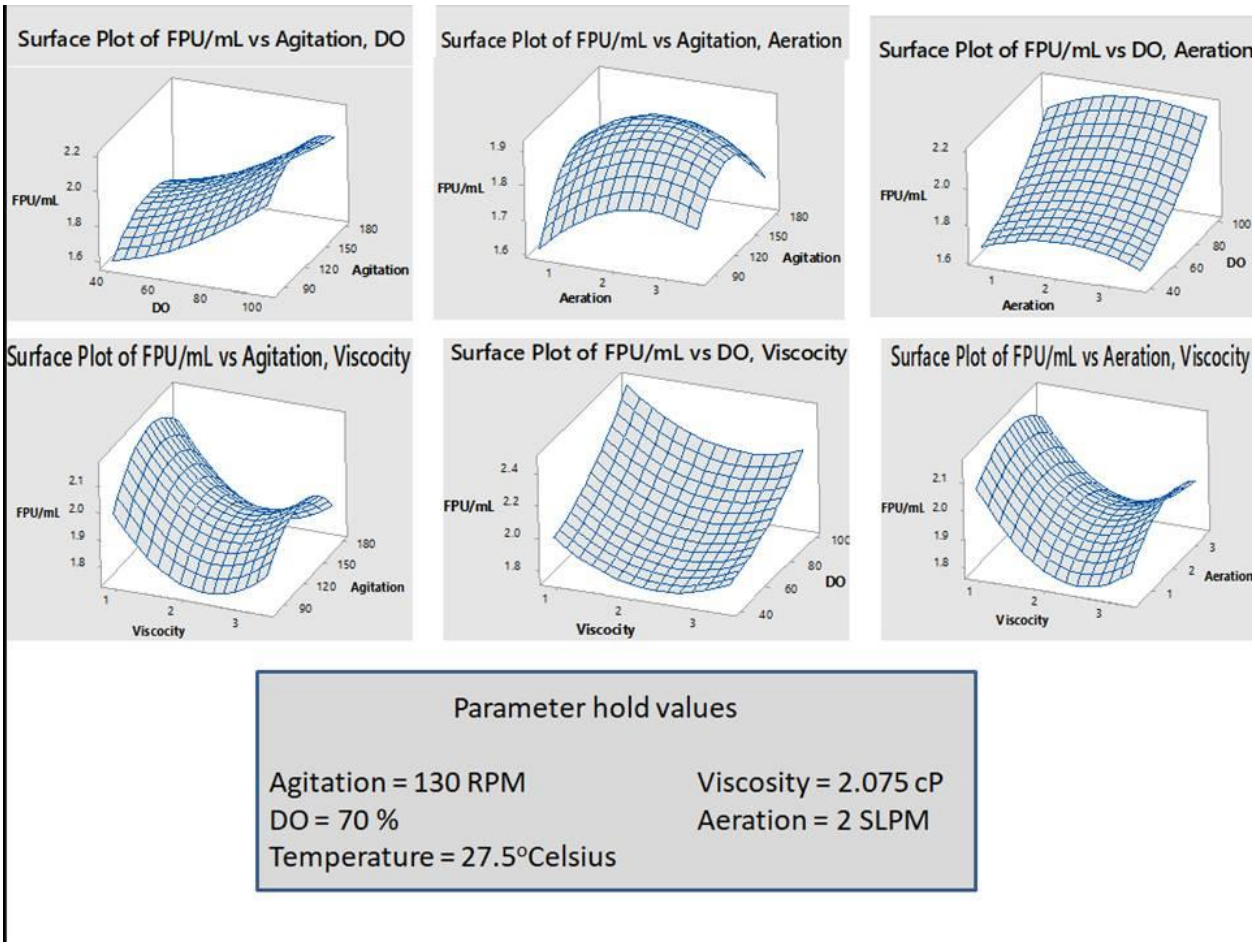

Fig. 2. Response surface plot for various combinations of the parameters chosen for optimization with FPU/mL cellulase activity as the response. Hold values are the model-suggested parameter values

The suggested optimum agitation was 130 rpm, which could yield 1.9 FPU/mL as shown in fig. 2. At values lower than 150 rpm, from 90-130 rpm, the FPU/mL gradually increases from 1.6 FPU/mL to reach 1.9 FPU/mL, at 130 rpm, as seen in fig. 2. This could be attributed to the fact that at lower agitation speeds, the mixing would be less [44], and the mixing of the sparged air into the broth would be reduced. At increased agitation rates above 130 rpm-180 rpm, the FPU values gradually decrease to reach 1.8 FPU/mL as shown in fig. 2. This could be attributed to breakage of mycelia by shear due to increased agitation rate [49] and decrease in dissolved oxygen concentration within the reactor that further contribute to decreased fungal growth rates. The mycelia would require mild agitation rates such as that suggested by the design, which would just suffice providing appropriate nutrient mixing and oxygen homogeneity within the culture broth [44].

The suggested optimum DO% to be maintained within the reactor was 70%, which yielded 1.7-2 FPU/mL, as seen in fig. 2. From 1.6 FPU/mL at 40% DO, values gradually increased until the optimum suggested value as seen in fig. 2. Reduced DO% contribute to lesser oxygen presence and hamper growth within the bioreactor, thereby decreasing enzyme production [34,35,44]. At values of DO% from 70% to 100%, the enzyme activities could reach a maximum of 2.2 FPU/mL, as seen in fig. 2. However, it is impractical to maintain 100% DO

within the reactor, owing to the decreased oxygen transfer rates in the reactor due to increasing viscosity; increased Oxygen Uptake Rates [34, 35 & 44] by the organism; and decreased mass transfer coefficients due to antifoam addition.

Computational Fluid Dynamics (CFD)-based simulation for Dissolved Oxygen concentration (DO%)

The Dissolved oxygen concentration (DO%) and viscosity were found to be the most significant parameters, which affect the SMF for cellulase production; the results of the experimental designs, as seen above, further elucidated the fact. As shown in fig.3, the oxygen mass transfer typically occurred in regions close to the impeller in highly viscous fluids, affecting the gas-liquid dispersion patterns [44]. The Dissolved oxygen concentration distribution for an unsteady state fluid flow and varying viscosities showed that, at the suggested optimum viscosity2.075cP, the DO% distribution varied between 63%-70% Dissolved Oxygen concentration as seen in the fig. 3. At lower viscosities of around 0.8cP, observed during the start of the fermentation, around 80%-99%DO% distribution was observed as shown in the fig. 3. However, it would be impractical to maintain the viscosity at 0.8 cP, owing to the reasons cited above. Similarly, at the end of the fermentation, the viscosity usually rises to 3.5 cP, decreasing the DO% distribution to 27%-62% (shown in fig. 3), which may decrease the fungal growth and cellulase production. Hence, during the course of the experimental run, it was decided to maintain the viscosity at the optimized and simulation-suggested 2 cP (approximately close to the model suggested value). At higher rates of agitation, the hyphae break and release the accumulated cellulase (from the hyphal tips) into the media. Under controlled agitation, the mycelia remain intact.

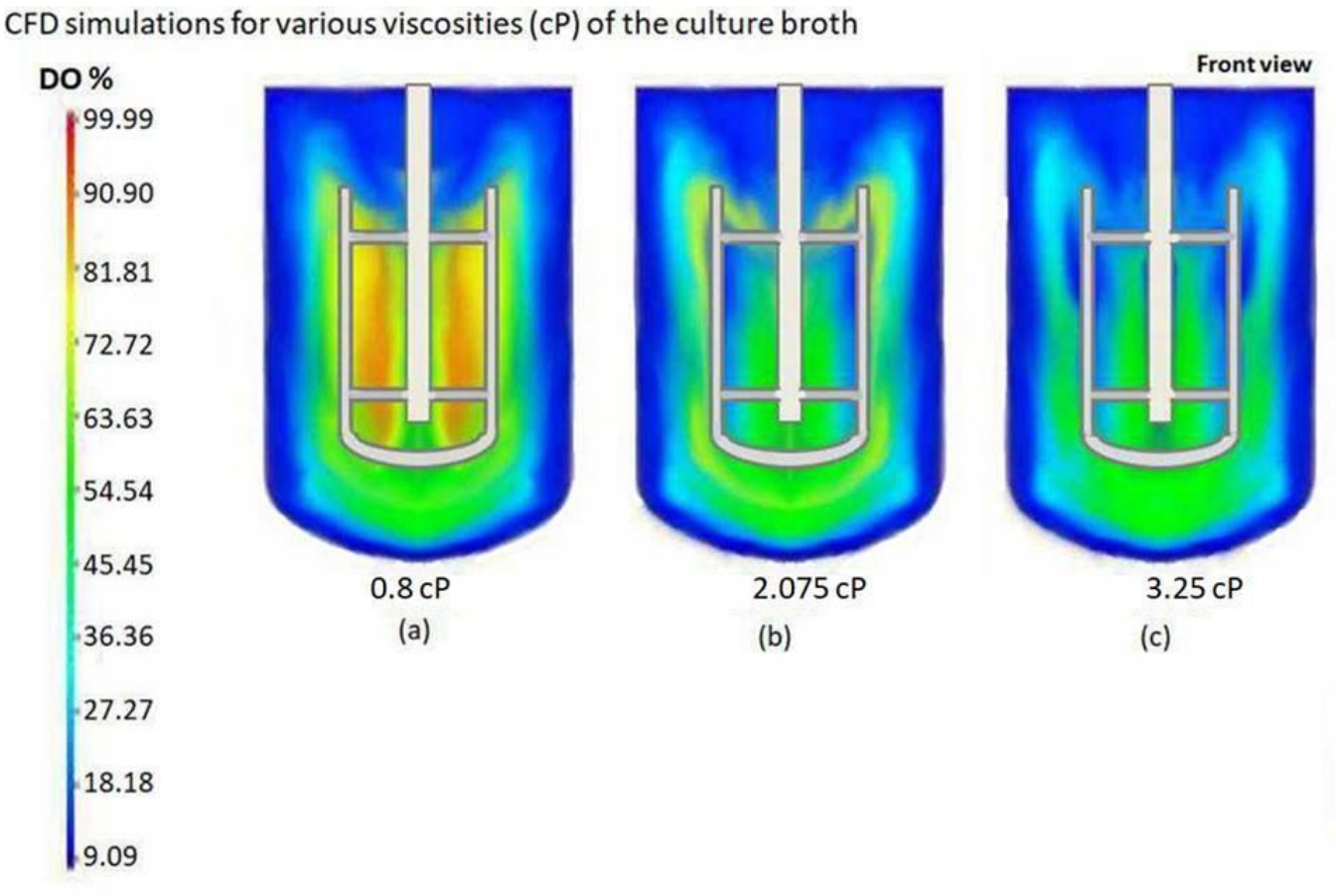

Fig. 3. Computation Fluid Dynamic simulation of DO % for various broth viscosities

SMF for cellulase production based on the experimental design and CFD simulation

An SMF was carried out in a bioreactor using the suggested optimum values of temperature, viscosity, agitation, aeration, and DO%. It was observed that problems related to foaming; increased viscosity; and higher agitation shear rates were eliminated. The batch cellulase production was halted after reaching complete utilization of cellulose at 168 h as shown in Fig. 4 (a), with 3.8 g/L fungal biomass concentration. Catabolite repression may be observed [51] as the glucose accumulated to 3.2 g/L (HPLC data not shown) at the end of 168 h as shown in fig. 4 (b) (after complete depletion of cellulose). The DO % decreases from 70%,after 36-72 h (shown in fig.5 (b)) due to the viscosity increase to 2.2 cP (shown in fig. 5 (a), and the biomass increase of 2.8 g/L (as shown in fig. 4 (a)). DO% gradually decreased to a value of 55%, which may be due to the reduced Oxygen Transfer Rate of 190 ±0.02 ppm oxygen/day and an increased in oxygen uptake rate of 0.9 ± 0.04 ppm oxygen/day within the reactor [29]. However, 100 mL of minimal media was fed every 24 h after 72 h to maintain the viscosity at 2 cP as seen in fig. 5 (a). The culture volume increased to 1.4 L at the end of the fermentation due to the addition of minimal media. The biomass yield coefficient ($Y_{x/s}$) was 0.32 ± 0.01 g biomass /g substrate; $Y_{x/O2}$-1.03 ± 0.01g biomass/ ppm oxygen; the maximum specific growth rate was 0.03± 0.002 g biomass / hour; and the maintenance coefficient was 0.04 ± 0.001 g biomass/ g substrate/hour [52]. The mass transfer coefficient (K_La) of the reactor decreased from 498/hour to 50/hour during the operation due to the increase in viscosity, and variations in OTR and OUR [29]. The critical oxygen concentration required was 0.9 ± 0.04 ppm oxygen/day. The results (enzyme activities) obtained from the SMF varied around 12.2% from the model-predicted results of the statistical design. An algorithm based model usually predicts moderate values, which are generated on the basis of numerical effect or responses; the practical ambience varies greatly in comparison to an algorithm's prediction [53]. The enzyme activities obtained were, 1.85 FPU/mL, 12.48 CMCase/mL, 743.5 Xylanase/mL, and 3165.3 Beta glucosidase/mL.

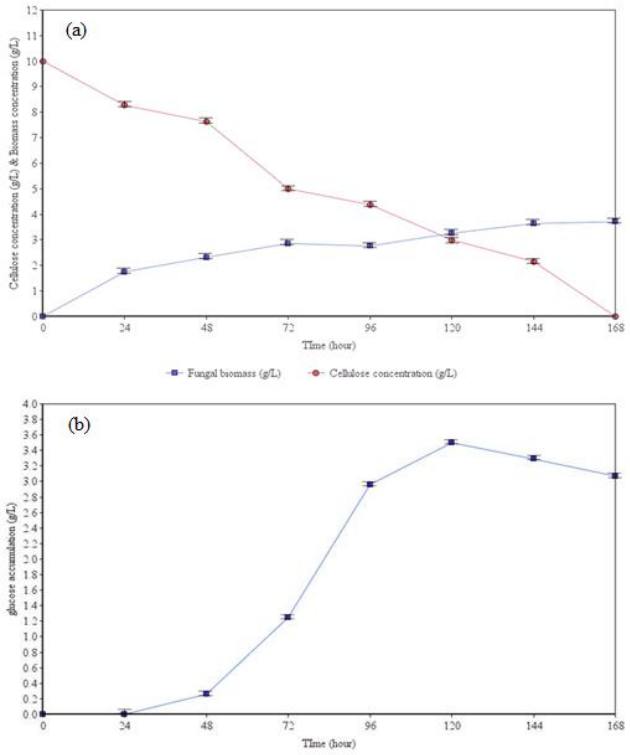

Fig. 4. (a) Cellulose consumption (g/L) and fungal biomass (g/L) formation in the cellulase production process (SMF). (b) Glucose accumulation (g/L) throughout the course of the cellulase production process

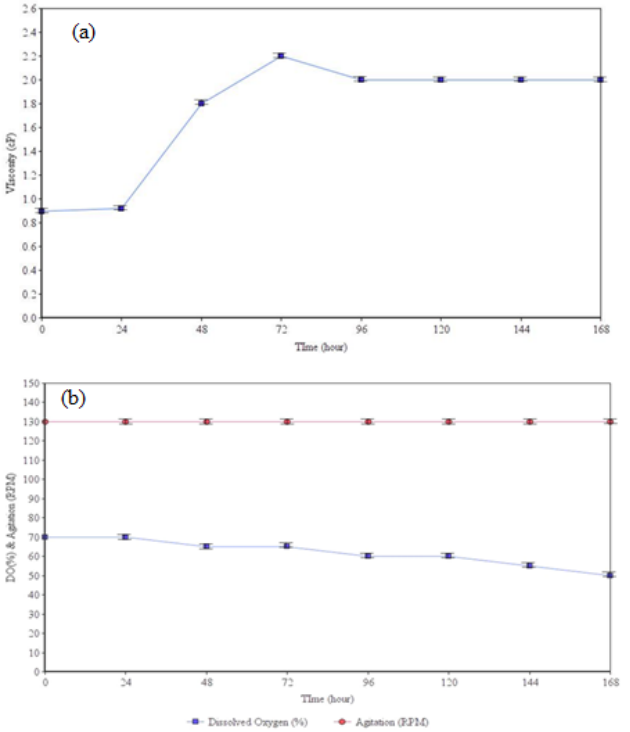

Fig. 5. (a) Variations in viscosity (cP) throughout the course of the cellulase production process. (b) Variations in Dissolved oxygen (%) and agitation (rpm) throughout the course of the cellulase production process

Conclusions

A statistical optimization design and Computational Fluid Dynamic (CFD) simulation suggested certain optimum reactor's parameter values. Once the values were known, an SMF was performed using the suggested values. The hypothesis was not proven to a greater extent as the experimental validation using the simulated parameters (as stated by the model) did not yield enhanced enzyme activities, as opposed to that predicted by the model. The variation between the predicted and actual experimental values was 12.2%. However, the optimum values contributed to an unmonitored operation of the reactor to yield consistent enzyme activities of cellulases (though not enhanced). Such an unmonitored process may find wider applications in large-scale operations. This work is one of the few statistical optimization techniques that have been performed for process variables for a cellulase production process, which employs a novel substrate mixture. Such a design based approach may be applied for the production of cellulases with better enzyme activities by biofuel researchers and industries alike.

Contribution of the authors

The first and the second authors have equally contributed towards this research work.

Conflict of interest

The authors declare no conflict of interests.

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