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Research Article

Optimization of carboxymethyl cellulase production from vegetable waste by using response surface methodology

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Keywords

Vegetable waste, carboxymethyl cellulase, solid state fermentation, *Trichoderma atroviride*, response surface methodology.

Abstract

Use of vegetable waste as raw material for carboxymethyl cellulase production has revealed to be an attractive alternative for reduction of environmental impact. Box-Behnken design was performed to evaluate the effects of temperature, pH, incubation time and spore suspension on production of carboxymethyl cellulase from vegetable waste by *Trichoderma atroviride* in solid state fermentation. Statistical analysis of results showed that linear and quadric terms of these four variables had significant effects and evident interactions existing between pH and temperature concentration were found to contribute to the response at a significant level. Under these conditions, temperature of 32.5ºC, pH of 5.5, spore suspension of 1.75 ml and incubation time is 5.5th day; the model predicted maximum carboxymethyl cellulase activity. Carboxymethyl cellulase production from vegetable waste enjoyed the advantages of simple process, low cost and short fermentation time, which should be further studied to make it applicable for industrial purpose.

Introduction

Agricultural, food and forestry industries produce large volumes of waste annually worldwide which cause a serious disposal problem. This is especially problematic in countries where the economy is largely based on agriculture and where farming practice is very intensive. India accounts for 50 MT of vegetable waste, which is about 30 % of its total agricultural production (Verma *et al.,* 2011). Hence, utilization of these wastes generated at different levels of delivery starting from the agricultural farm, post-harvest handling, storage, processing, and from distribution to consumption would be economically highly beneficial. Such wastes can either be used directly as an untreated material for microbial growth or be used by appropriate treatment with different enzymes for bioenergy production (Singh *et al*., 2012).

This makes wastes very appropriate for their exploitation as raw materials in the production of industrially relevant compounds under solid-state fermentation (SSF) conditions. Utilization of microbes or enzymes for digestion of discarded vegetable biomass into a desired form of fuel is presently one of the most accepted waste management strategies.

In this context, solid state fermentation (SSF) has emerged as a promising technology for the enzyme production from cheaply available agro-industrial residues waste (Ali *et al*., 2013). The use of agro wastes not only helps to overcome the problem of solid waste management but allows the development of biotechnological processes from cheap natural resources and is considered as suitable substrates for the production of enzymes, especially cellulase and xylanase under solid state fermentation (Vimalashanmugam and Viruthagiri, 2014). The interest in SSF comes from its simplicity and closeness to the natural growth conditions of many micro-organisms, especially fungi

The cost of enzymes play an important role in determining the economics of an enzymatic hydrolysis and it can be reduced by finding optimum conditions for their production (Lynd *et al.,* 2002). Reducing costs of enzyme production by

optimizing the fermentation medium is the basic research for industrial application. The first step in achieving this goal is establishment of a suitable enzyme production technology. Temperature, Initial pH, Incubation time and spore suspension are key operating parameters that affect enzyme fermentation process. A better understanding of these operating parameters on enzyme production will facilitate improvement of the process. The conventional approach for optimization of a multivariable system is usually one variable at a time. However, such approach needs to carry out numerous sequential experimental runs and cannot explain the interactions between variables. (Dey *et al*., 2001).

For this, statistical optimization is preferable because it is helpful in evaluating interactions among the possible influencing parameters with limited number of experiments (Francis *et al*., 2003). It involves a specific design of experiments, which minimizes error in determining effect of parameters, and the results are achieved in an economical manner. Response surface methodology (RSM) is one such scientific approach that is useful for developing, improving and optimizing processes and is used to analyze the effects of several independent variables on the system response, main objective being the determination of optimum operational conditions within the operating specifications (Kumar *et al*., 2011; Huang *et al*., 2013). The optimization of culture medium and culture conditions for improvement of production of carboxymethyl cellulase (CMCase) has been reported by many scholars (Irfan *et al*., 2012; Romdhane *et al*., 2010). In this study, response surface methodology (RSM) was used to determine the effects of several variables and to optimize CMCase production conditions from vegetable waste using *Trichoderma atroviride*.

Materials and Methods

Microorganism and inoculum preparation

T. atroviride was originally isolated from soil samples collected from different regions of Haryana. The strain was grown in a potato dextrose agar medium (PDA), in erlenmeyer flasks, at 28 ºC for 4 days, using an inoculum of 10^6 spores/ml. The strain was subcultured every 15 days, and preserved at 4 ºC.

Inoculum preparation

The fungal culture was grown on PDA slants and the spores were harvested aseptically from 5-day –old slants. Sterile distilled water containing 0.1% (w/v) tween 80 was added to each fungal slant and then used as inocula.

Enzyme Production under solid state fermentation

Fermentation was carried out in Erlenmeyer flasks (250 ml) with 5 g of vegetable waste powder, supplemented with nutrients' concentration in media as follows (g/L): NH_4NO_3 2.0, KH_2PO_4 2.0, $MgSO_4 \cdot 7H_2O$ 1.0, $CaCl_2$ 0.3 and trace elements (mg/L): $FeSO_4.7H_2O$ 5.0, $MnSO_4.7H_2O$ 1.6, $ZnSO_4.7H_2O$ 3.45, $CoCl_2.6H_2O$ 2.0. Thoroughly mixed substrate was autoclaved (121^0C for 20 min) and allowed to cool at room temperature. The substrate was inoculated with 2 ml of fungal spore suspension and incubated at 30^0C. Crude enzyme was extracted from fermented substrate by adding citrate buffer (50 ml) containing to flasks after fixed time. Flasks were kept on a rotary shaker at 180 rpm for 1 hr for proper mixing, and centrifuged at 10,000g for 10 min at 4^0C. The supernatant was collected and used as crude enzyme extract for enzyme estimation.

Enzyme assays

Carboxymethyl cellulase (CMCase) was carried out by mixing 0.5 ml enzyme sample with 0.5 ml of 1% Carboxymethylcellulose (CMC) in 0.05M sodium citrate buffer (pH 4.8) at 50 °C for 30 min. Reducing sugar was determined using 3, 5-dinitrosalicylic acid (DNS) reagent with glucose as a standard (Miller, 1959). The CMCase expressed as U/g. One unit (U) of enzyme activity is defined as the amount of enzyme required to liberate 1µmol of product per 30 min.

Optimization of significant variables using Box-Behnken design

The interactive effects of four significant factors pH, incubation temperature, incubation time and spore suspension on the response for CMCase production were determined statistically using RSM. Box-Behnken design was used to optimize the fermentation conditions for all variable factors.

Statistical Analysis

The statistical analysis of the model was represented in the form of analysis of variance (ANOVA). The software Design-Expert (Version 9, Stat-Ease) was used for experimental design, data analysis and quadratic model building. Each run was performed in triplicate and the average of CMCase yield obtained was taken as the experimental values of the dependent variable or response. The response surface graphs were obtained to understand the effect of variables individually and in combination, and to determine their optimum levels for maximum CMCase production.

Results and Discussion

Box- Behnken design from the statistical software package Design-Expert (version 9, Stat-Ease) was used to determine the suitable process conditions for CMCase production by *Trichoderma atroviride* using vegetable waste. A total of four independent variables; incubation time (A), incubation temperature (B), pH (C) and spore suspension (D) were studied in 29 experiments and presented along with the mean predicted and observed responses in Table 1.

Table 1. Observed and predicted responses for the experiments performed using BBD design for vegetable waste.

		Factor 1	Factor 2	Factor 3	Factor 4	Observed Response	Predicted Response
Std	Run	A:Incubation Day	B:Temperature	C:pH	D:Spore Suspension	CMCase	CMCase
			C		Ml	U/g	U/g
16	1	5.5	40	8	1.75	45	43.98
11	2	3	32.5	5.5	3	89.08	84.91
25	3	5.5	32.5	5.5	1.75	97.88	99.71
8	4	5.5	32.5	8	3	82.62	83.14
9	5	3	32.5	5.5	0.5	86.12	82.65
7	6	5.5	32.5	3	3	70.46	72.80
29	7	5.5	32.5	5.5	1.75	112.89	99.71
28	8	5.5	32.5	5.5	1.75	94.87	99.71
21	9	5.5	25	5.5	0.5	56.56	58.24
15	10	5.5	25	8	1.75	50.71	50.30
13	11	5.5	25	3	1.75	49.41	42.35
1	12	3	25	5.5	1.75	51.34	52.40
14	13	5.5	40	3	1.75	40.91	33.24
17	14	3	32.5	3	1.75	60.12	65.72
4	15	8	40	5.5	1.75	46.19	47.86
19	16	3	32.5	8	1.75	75.16	75.92
6	17	5.5	32.5	8	0.5	80.19	80.57
12	18	8	32.5	5.5	3	92.01	87.40
10	19	8	32.5	5.5	0.5	90.41	86.50
22	20	5.5	40	5.5	0.5	47.92	51.04
27	21	5.5	32.5	5.5	1.75	99.04	99.71
24	22	5.5	40	5.5	3	48.42	52.10
18	23	8	32.5	3	1.75	65.15	69.75
5	24	5.5	32.5	3	0.5	70.02	72.22
26	25	5.5	32.5	5.5	1.75	93.86	99.71
3	26	3	40	5.5	1.75	45.49	45.72
20	27	8	32.5	8	1.75	78.48	78.24
23	28	5.5	25	5.5	3	58.09	60.33
2	29	8	25	5.5	1.75	54.12	56.61

From multiple regression analysis, it was observed that the second-order polynomial equation can explain CMCase production regardless of the significance of the coefficients:

$$Y = 99.71 + 1.59A - 3.86B + 4.67C + 0.79D - 0.52AB - 0.43AC - 0.34AD + 0.70BC - 0.26BD + 0.50CD - 9.56A^2 - 39.50B^2 - 17.74C^2 - 4.78D^2$$

Where Y is the predicted response (CMCase yield), and A, B, C and D are the coded variables for incubation day, temperature, pH, spore suspension respectively.

The statistical significance of the regression model was checked by F-test. Generally the 'F' value with a low probability 'P' value indicates high significance of the regression model (Rene *et al.,* 2007). The ANOVA result for carboxymethyl cellulase activity shows the F value to be 22.18, which implies that the terms in model have a significant effect on the response. There was only 0.01% chance that a "Model F-Value" this large could occur due to noise.

From the ANOVA summary (Table 2), the model was found to be statistically significant ($P < 0.01$) at the 99% confidence level. The coefficient estimate and the corresponding Prob>F values (Table 2) suggested that among independent variables studied, temperature and pH as well as the squared terms of these two variables had a significant effect on cellulase production by *Trichoderma atroviride.* The goodness of fit was manifested by the determination coefficient (R^2). In this case the R^2 value of 0.9569 indicated that the response model can explain 95.69% of the total variations. The value of the adjusted determination coefficient ($AdjR^2$) was also high enough (0.9137) to indicate the significance of the model as shown in table 3.

Table 2: Analysis of variance (ANOVA) for response surface quadratic model for the production of carboxymethyl cellulase.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	11450.52	14	817.89	22.18	< 0.0001
A-Incubation Day	30.24	1	30.24	0.82	0.3804
B-Temperature	178.64	1	178.64	4.85	0.0450
C-pH	262.17	1	262.17	7.11	0.0184
D-Spore Suspension	7.46	1	7.46	0.20	0.6598
AB	1.08	1	1.08	0.029	0.8665
AC	0.73	1	0.73	0.020	0.8900
AD	0.46	1	0.46	0.013	0.9124
BC	1.95	1	1.95	0.053	0.8216
BD	0.27	1	0.27	7.194E-003	0.9336
CD	0.99	1	0.99	0.027	0.8722
A^2	592.96	1	592.96	16.08	0.0013
B^2	10120.46	1	10120.46	274.50	< 0.0001
C^2	2041.60	1	2041.60	55.38	< 0.0001
D^2	148.35	1	148.35	4.02	0.0646
Residual	516.15	14	36.87		
Lack of Fit	281.00	10	28.10	0.48	0.8433
Pure Error	235.16	4	58.79		
Cor Total	11966.67	28			

Table 3. Statistical information for ANOVA

Sources	Response Value
R-Squared	0.9569
Adj R-Squared	0.9137
Pred R-Squared	0.8340
Adeq Precision	15.222
Std. Dev.	6.07
Mean	70.09
C.V. %	8.66
PRESS	1985.97

A model can be considered reasonably reproducible if CV is not greater than 10%. Here, a lower value of CV (8.66) indicated a greater reliability of the experiments performed. The adequate precision value of 15.22 also indicates adequate signal and suggests that the model can navigate the design space.

A normal probability plot and a dot diagram of these residuals are shown in Fig. 1. The data points on this plot lie reasonably close to a straight line and supports to the conclusion that experimental variables have significant effects and the underlying assumptions of the analysis are satisfied.

Response Surface Graph and Optimization Conditions

The relationship between independent and dependent variables was graphically represented by 3D response surface generated by the model (Figures 2–5). Response surface plots as a function of two factors at a time, maintaining all other factors at fixed levels are more helpful in understanding both the main and the interactive effects of two factors.

Fig. 2 depicts the three-dimensional response surface graphical representation showing the effect of incubation temperature and pH on the CMCase production.

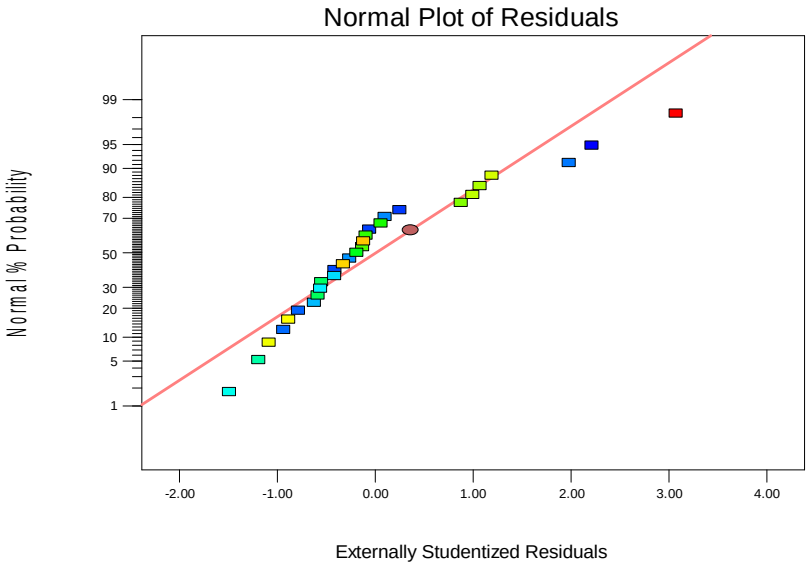

Figure 1. Normal probability versus residual error

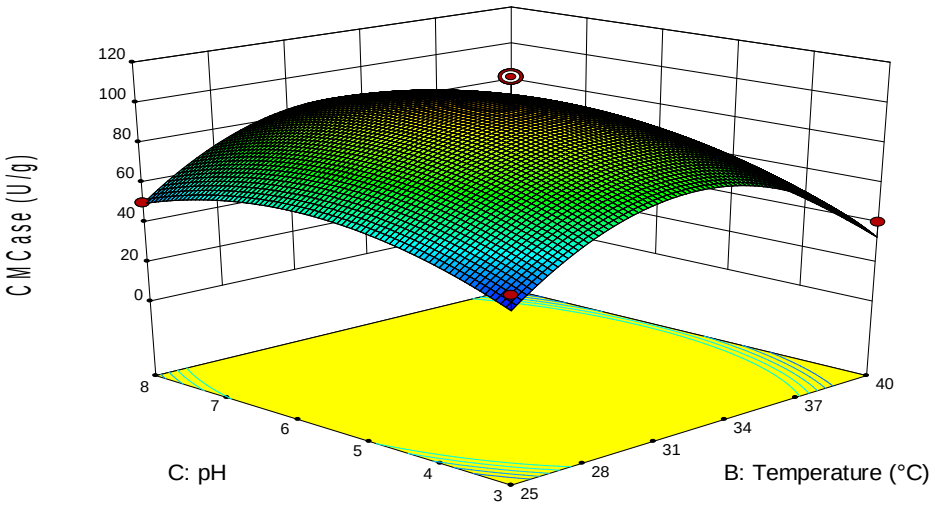

Figure 2. Three-dimensional response surface plot for CMCase production showing the interactive effects of temperature and pH for vegetable waste.

The convex response surface predicted that the CMCase production increased to the peak with the increase of temperature and pH up to 32.5 ºC and 5.5, respectively; then declined with the further increase of these two parameters. CMCase activity decreased considerably even for slight increase in temperature (above 32.5°C) due to the fact that variations of a few degrees around the optimal temperature can notably modify the growth and the metabolism of the microorganism.

This result demonstrated that the highest enzyme activity (112.89 U/g) as shown in Table 1 was obtained when the pH and temperature were 5.5 and 32.5°C, respectively. The same trend was observed in figure 4 and 5 in which it is clear that increase in temperature upto 32.5°C aided in the production of CMCase. This result is considerably similar to what was reported by Shafique *et al.* (2009) who indicated that the optimum temperature for maximum cellulase production for *T. reesei* was 30 ± 2°C. Our results are also correlated with Jaradat *et al.* (2008), who reported the cellulase enzyme was active over a pH range of 4–7 with maximum activity at pH 6 and at temperature 30°C.

The results on interaction effects of incubation day and pH also found a similar trend with the CMCase production increasing with increase in pH to a certain level initially and either decreasing or remaining stable thereafter for all the incubation day as shown in fig. 3. Maximum CMCase activity from vegetable waste was achieved with the optimum incubation period of 5.5th day at 5.5 pH (Bhatti *et al*., 2006). Further increase in the incubation time results in the reduction of enzyme activity Thus, it was demonstrated that prolonged processing time had an adverse effect on the progress of the solid state fungal growth. This may be due to depletion of macro and micronutrients in the fermentation medium with the lapse in time, which stressed the fungal physiology resulting in the inactivation of secretary machinery of the enzymes (Simões *et al*., 2009; Aishwarya *et al*., 2011). This agrees with previous study done by Fatma *et al*., (2010) who reported maximum cellulase production was obtained at 5th day by *Trichoderma reesei* F-418 cultivated on alkali treated rice straw using solid state fermentation (SSF) technique. In the similar study, Bai *et al*. (2013) also reported that *Trichoderma viride* produces maximum CMCase and xylanase at pH 5 after 5th day of fermentation period.

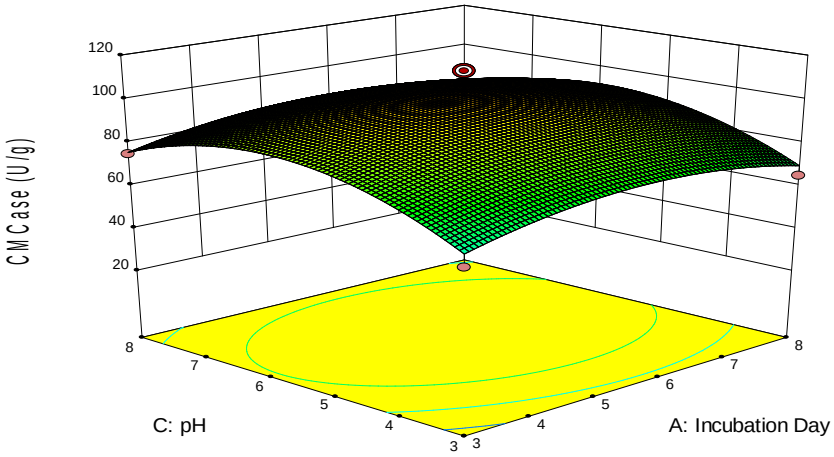

Figure 3. Three-dimensional response surface plot for CMCase production showing the interactive effects of incubation day and pH for vegetable waste.

The effects of other two pairs viz. temperature- spore suspension and temperature- incubation day on the CMCase production formed the stationary ridge systems as shown in Fig. 4 and Fig. 5, respectively. The results illustrated by Figs. 4 and 5 clearly show that, within the range of experiment (Temperature: 25-40°C), any changes in temperature had a difference in the CMCase production, while the change in incubation day and spore suspension had a less significant effect on the CMCase production. The maximum CMCase production was at 1.75ml of inoculum size at 5.5th day. The decrease in enzyme production with further increase in inoculum might be due to depletion of nutrients by the enhanced biomass, which resulted dwindle in metabolic activity (Kashyap *et al.*, 2002). Similar results were shown by Munawar (2011) who observed that highest cellulase production by using 2 ml inoculums volume of *Trichoderma ressei* using *Antigonum leptopus* leaves as substrate.

These data indicated that the *Trichoderma atroviride* is a more efficient CMCase production. From the response surface graphs it could be concluded that the optimum values of pH, temperature, spore suspension and incubation time for the maximum production of CMCase (112.89 U/g) by *T.atroviride* from vegetable waste were in 5.5, 32.5°C, 1.75 ml and 5.5th day respectively.

Model validation and confirmation

Chauhan and Gupta (2004) have emphasized on the acceptance of any model with $R^2 > 0.75$. The fitting of the experimental data to the regression model was checked and suitably explained by the value of determination coefficient (R^2 = 0.956). Besides, the relationship between the experimental values and predicted values (Fig.6) showed that the plotted points cluster around the diagonal line, indicating good fitness of the model. This showed that the model was useful to optimize the experimental conditions.

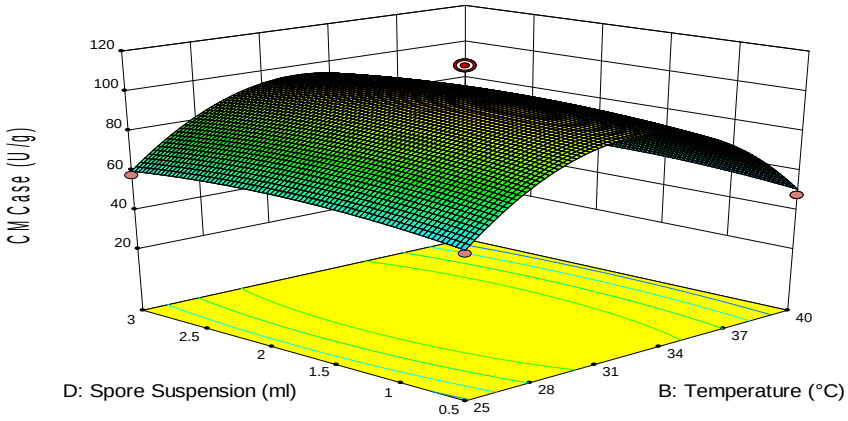

Figure 4. Three-dimensional response surface plot for CMCase production showing the interactive effects of temperature and spore suspension for vegetable waste.

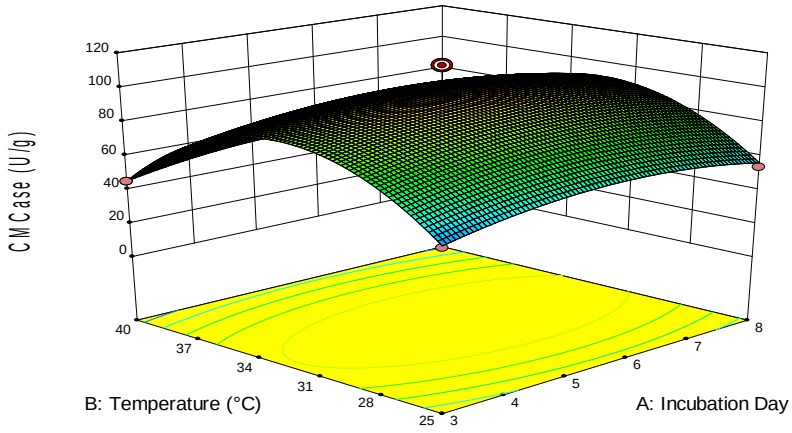

Figure 5. Three-dimensional response surface plot for CMCase production showing the interactive effects of incubation days and temperatue for vegetable waste.

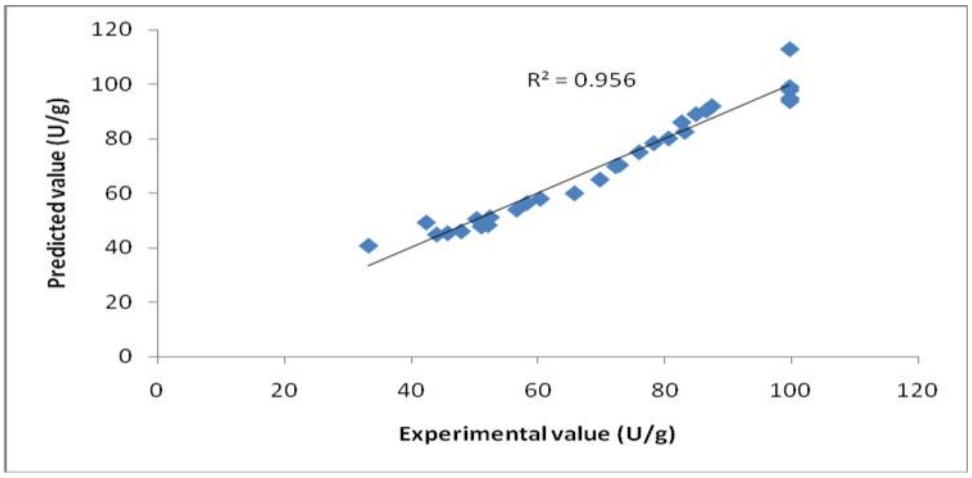

Figure 6. Parity plot showing the distribution of experimental and predicted values of optimization of CMCase production.

Conclusion

Large quantities of vegetable wastes are generated all over the world. The environmental pollution problems associated with conventional disposal methods have been an impulse for the search for alternative, environment-friendly methods of handling biowastes. These biological wastes can be used as support-substrates in SSF to produce industrially relevant metabolites, such as enzymes, organic acids, flavour and aroma compounds and polysaccharides, with a great economical advantage. The results highlighted that *T. atroviride* has potential to be an indigenous source of CMCase production cultured on cheap vegetable wastes as sole carbon source in solid state fermentation.

The optimum pH and temperature of the crude extract were 5.5 and 32.5°C, respectively. The maximum CMCase activity detected was of 112.89U/g, on the 5.5th day of cultivation, when a mineral medium was added with 1.75 ml spore suspension of *T.atroviride*. These results were close to the CMCase production (99.71U/g) predicted by the regression model, which proved the validity of the model. The findings of present study might be applied in a large scale for production of CMCase with cost-effective using vegetable waste as a cheap substrate.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

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