**Journal of Industrial Microbiology & Biotechnology (1998) 21**, 178–183

http://www.stockton-press.co.uk/jim

# pH and thermal stability studies of carboxymethyl cellulase from intergeneric fusants of *Trichoderma reesei/Saccharomyces cerevisiae*

R Srinivas and T Panda

*Biotechnology Research Centre, Indian Institute of Technology, Madras, Chennai, 600 036 India*

**The combined effect of pH and temperature on carboxymethyl cellulase from two intergeneric fusants (M 14 and M 62) of *Trichoderma reesei* QM 9414/*Saccharomyces cerevisiae* NCIM 3288 was studied using response surface methodology. A central composite design for two variables was employed for the optimization studies. This study was compared with similar studies carried out with *Trichoderma reesei* QM 9414. The optimal pH and temperature for the enzymes derived from these organisms were: for the fusant M 14—pH 5.7 and 41.7°C, for the fusant M 62—pH 5.3 and 43°C, and for *Trichoderma reesei* QM 9414—pH 4.31 and 38.3°C.**

**Keywords:** carboxymethyl cellulase; intergeneric fusants; pH and thermal stability; response surface methodology

## Introduction

Endoglucanases are a part of the cellulase complex involved in cellulose degradation. They attack cellulose fibres at random and cleave them at more amorphous regions which, in turn, create sites for other enzymes like exoglucanases and $\beta$-glucosidases. They cause rapid change in the degree of polymerization with a slow increase in reducing power [14]. The synergistic action of endoglucanase (EG), cellobiohydrolase (CBH) and $\beta$-glucosidase with the help of xylanases and hemicellulases results in the complete degradation of cellulosic materials [10]. Endoglucanases are also called carboxymethyl cellulases (CMCase) as they are more active on substituted celluloses like carboxymethyl cellulose. For the direct conversion of cellulose to ethanol, a chemofusion method has been adopted using *Trichoderma reesei* and *Saccharomyces cerevisiae* [1]. The fusion was a success and endoglucanase was the key enzyme in this process. However, the ethanol yield was low compared to other processes [11]. We are interested in increasing the yield of ethanol using intergeneric fusants. If one can increase biosynthesis of the key endoglucanase, more cellulose will be degraded to glucose which will be finally converted to ethanol. To attempt this task, one needs detailed information on endoglucanase synthesis, its localization, distribution and its characteristics [12]. A novel approach has been considered to reduce the combined denaturing effect of pH and temperature on carboxymethyl cellulase using response surface methodology [7]. For this study, two representative fusants (M 14 and M 62) were selected which exhibit desirable properties. Similar studies were conducted with endoglucanase from *Trichoderma reesei* which serves as the control. The objectives of enzyme technology are to attain the capacity to design and to tailor the enzyme characteristics to suit the requirements of the process [5]. Enzyme inactivation plays an important role in biotechnological processes. The desirable features of the enzyme, which include good catalytic ability and stability, have to be enhanced. The activity of the enzyme is a measure of its ability to catalyze a process, while the stability of the enzyme is judged by its residual activity after the enzyme inactivation process. Both of these properties are affected to a large extent by pH, temperature, activators, and inhibitors. In this communication, the combined effect of pH and temperature on carboxymethyl cellulase from fusants was studied, and a suitable combination was determined using response surface methodology.

Correspondence: Professor T Panda, Biotechnology Research Centre, Indian Institute of Technology, Madras, Chennai, 600 036 India
Received 5 May 1997; accepted 17 July 1998

## Materials and methods

### Organisms

The intergeneric fusants of *Trichoderma reesei/ Saccharomyces cerevisiae* were developed in the author's laboratory [1]. *Trichoderma reesei* QM 9414 was obtained from the National Chemical Laboratory, Pune, India.

### Culture maintenance

*Trichoderma reesei* was maintained on malt-agar slants containing (kg $m^{-3}$): malt-extract 30, agar 25. The fusant of *Trichoderma reesei/Saccharomyces cerevisiae* was maintained on slants containing (kg $m^{-3}$): glucose monohydrate, 5.0; $KH_2PO_4$, 2.0; $NaH_2PO_4$, 6.9; $MgSO_4 \cdot 7H_2O$, 0.3; Tween 80, 0.2; urea, 0.3 and trace elements (mg $L^{-1}$): $FeSO_4 \cdot 7H_2O$, 5.0; $MnSO_4 \cdot 6H_2O$, 1.6; $ZnSO_4 \cdot H_2O$, 2.0; $CaCl_2 \cdot 2H_2O$, 2.0. Initial pH was adjusted to 5.0 with 1 M NaOH. Freshly prepared slants were incubated at 30°C for 24 h and stored at 4°C.

### Growth medium

The medium proposed by Anjani Kumari and Panda [1] was used for growth of the fusant. The initial pH was adjusted to 5.0 with 1 M NaOH. One hundred cc of the medium was dispensed in 500-cc Erlenmeyer flasks. Spores

**Table 1** Coded values of the independent variables in the central composite design for two variables

| Variable parameter | Level | | | | |
|---|---|---|---|---|---|
| | −1.414 | −1 | 0 | +1 | +1.414 |
| $x_1$, temp °C | 36 | 40 | 50 | 60 | 64 |
| $x_2$, pH | 3.3 | 3.8 | 4.8 | 5.8 | 6.2 |

from 24-h-old slants were suspended in 10 cc sterile water and added to the medium aseptically. The culture was grown for 24 h at 30°C on a temperature-regulated orbital shaker maintained at 160 rpm. A 24-h-old culture was used as the inoculum for enzyme production. The growth medium for *T. reesei* contained (kg m$^{-3}$): glucose, 5.0; $(NH_4)_2SO_4$, 1.4; $KH_2PO_4$, 2.0; $NaH_2PO_4$, 6.9; $MgSO_4 \cdot 7H_2O$, 0.3; citric acid monohydrate, 10.5; Tween 80, 0.2; peptone, 1.0; urea, 0.3. The medium was supplemented with the following minerals (mg L$^{-1}$): $FeSO_4 \cdot 7H_2O$, 5.0; $MnSO_4$, 1.6; $ZnSO_4 \cdot 7H_2O$, 1.4; $CaCl_2 \cdot 2H_2O$, 2.0. The initial pH was adjusted to 5.0 with 1 M NaOH. Studies were conducted in shake flask cultures. Spores from 5-day-old slants were suspended in 10 cc of sterile water and added to the medium aseptically. The culture was grown at 30°C for 36 h on a temperature-controlled orbital shaker maintained at 160 rpm.

### *Carboxymethyl cellulase production*

The medium described by Srinivas *et al* [11] was used for enzyme production. A 24-h-old seed culture of the fusant and 36-h-old seed culture of *Trichoderma reesei* was used as the inoculum for the enzyme production. The initial pH was adjusted to 5.0 and cultures were grown at 30°C on an orbital shaker maintained at 160 rpm. The cultures were harvested on the 4th, 6th and 8th days for the fusants M 14, M 62 and *Trichoderma reesei* (WT) respectively. The supernatant was stored at 4°C.

### *Assay of endoglucanase*

Two per cent sodium carboxymethyl cellulose prepared in 50-mM citrate buffer (pH 4.8) was used as the substrate. A reaction mixture containing 0.5 cc of substrate and 0.5 cc of enzyme preparation was incubated at 50°C for 30 min [6]. Liberated glucose was estimated using the DNS method [9]. One unit (U) of enzyme activity is defined as the amount of enzyme that releases 1 $\mu$m of glucose from the substrate in 1 min per ml of the enzyme preparation at 50°C and pH 4.8.

### *Activity staining for endoglucanase*

Activity staining for endoglucanase was done using 10% polyacrylamide gel containing 0.1% carboxymethyl cellulose in the separating gel. PAGE was performed under native conditions according to the method of Laemmli [8]. The activity bands were visualized after staining the gel with Congo Red (1 kg m$^{-3}$) followed by destaining with 1 M NaCl for 15 min [13].

### *pH and thermal stability*

The pH and temperature were chosen as the independent variables in a central composite design [2]. Using this method, a total number of treatment combinations was $2^k + k + n_o$ where $k$ is the number of variables and $n_0$ is the number of repetitions of the experiment at the centre point. For calculations, the variable $X_i$ was coded as $x_i$ according to the following Equation:

$$x_i = (X_i - X_o)/\Delta X$$
$$i = 1,2,3\ldots k \quad (1)$$

where $x_i$ is coded (dimensionless) value of the variable $X_i$; $X_o$ = the value of $X_i$ at the centre point and $\Delta X$ = step change.

The behaviour of the system was explained by the following polynomial equation:

$$y = \beta_o + \Sigma\beta_i x_i + \Sigma\beta_{ii} x_i^2 + \Sigma\beta_{ij} x_i x_j, \quad (2)$$

**Table 2** Experimental and predicted activities of carboxymethyl cellulase from fusant M 14 after 55 min of incubation at defined pH and temperature

| Observation order | Temperature $x_1$ (≡$X_1$ °C) | pH $x_2$ (≡$X_2$) | Carboxymethyl cellulase (U) | | | |
|---|---|---|---|---|---|---|
| | | | Set 1 | Set 2 | Experimental | Predicted |
| 1 | −1 (≡40) | −1 (≡3.8) | 0.0216 | 0.0210 | 0.021 | 0.035 |
| 2 | 1 (≡60) | −1 (≡3.8) | 0 | 0 | 0 | −0.001 |
| 3 | −1 (≡40) | 1 (≡5.8) | 0.1255 | 0.1265 | 0.126 | 0.130 |
| 4 | 1 (≡60) | 1 (≡5.8) | 0.056 | 0.058 | 0.057 | 0.046 |
| 5 | 0 (≡50) | 0 (≡4.8) | 0.107 | 0.107 | 0.107 | 0.102 |
| 6 | 0 (≡50) | 0 (≡4.8) | 0.102 | 0.100 | 0.101 | 0.102 |
| 7 | 0 (≡50) | 0 (≡4.8) | 0.104 | 0.102 | 0.103 | 0.102 |
| 8 | −1.414 (≡36) | 0 (≡4.8) | 0.1065 | 0.1075 | 0.107 | 0.095 |
| 9 | 1.414 (≡64) | 0 (≡4.8) | 0 | 0 | 0 | 0.010 |
| 10 | 0 (≡50) | −1.414 (≡3.3) | 0.010 | 0.010 | 0.010 | 0.002 |
| 11 | 0 (≡50) | 1.414 (≡6.2) | 0.0962 | 0.0958 | 0.096 | 0.102 |
| 12 | 0 (≡50) | 0 (≡4.8) | 0.1054 | 0.1046 | 0.105 | 0.102 |
| 13 | 0 (≡50) | 0 (≡4.8) | 0.095 | 0.097 | 0.096 | 0.102 |
| 14 | 0 (≡50) | 0 (≡4.8) | 0.0989 | 0.0991 | 0.099 | 0.102 |

$R = 0.9858$; $R^2 = 0.9718$.

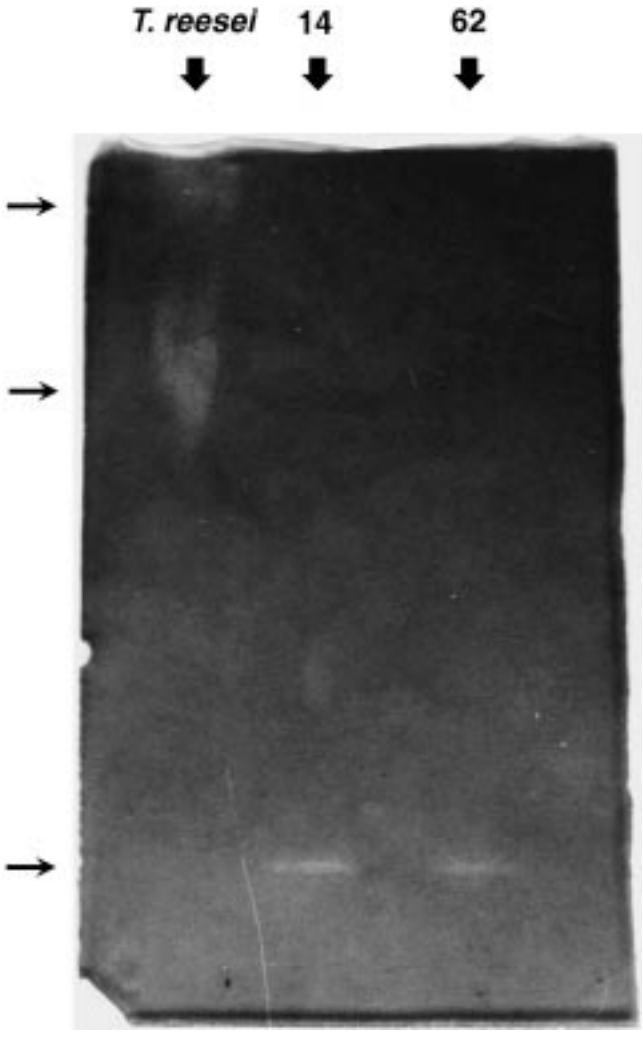


**Figure 1** Staining pattern of endoglucanase from the fusants and *Trichoderma reesei* (WT).

where $y$ is the predicted response, $\beta_o$ is offset term, $\beta_i$ the linear effect, $\beta_{ii}$ the squared effect, and $\beta_{ij}$ the interaction effect.

The above equation was solved using Design Expert (Stat-Ease, Minneapolis, MN, USA) to estimate the response of the variable. Experiments were performed in duplicate. To obtain optimum values of the variables the regression equation was maximized using a package, Matlab. Enzyme activity was taken as the dependent variable. A $2^2$-factorial experimental design with four axial points ($\alpha = 1.414$) and six replicates at the centre point with a total number of 14 experiments was employed. The coded values are given in Table 1. The pH of the enzyme solution was adjusted with 1 M HCl or 1 M NaOH according to the experimental plan given in Table 2 and then the enzyme solution was incubated at the defined temperature for 60 min. Samples were collected every 5 min and assayed for endoglucanase.

## Results and discussion

### *Activity staining*

There are three main cellulase components in the *Trichoderma* cellulolytic system, namely endoglucanases, exoglucanases, and $\beta$-glucosidases [4]. Endoglucanases and exoglucanases act synergistically to attack native cellulose. Endoglucanases are more active on substituted celluloses like carboxymethyl cellulose which was used as substrate in the present study. We observed reduced activity of endoglucanase from the fusants compared with *Trichoderma reesei*, which might have occurred by changes in the genes responsible for the synthesis of one of the endoglucanases during recombination. After activity staining, only one Eg was present in the intergeneric fusants (both M 14 and M 62) of *Trichoderma reesei/Saccharomyces cerevisiae* whereas there are two endoglucanases present in *Trichoderma reesei* (WT) using filter paper as the inducer (Figure 1). This suggests that there is a prominent genetic alteration in the intergeneric fusants during the fusion. The staining pattern also suggests that endoglucanases from the fusants differ in molecular weight. Further analysis of these molecules is in progress. At this juncture it becomes necessary to consider a method which reduces the denaturation effect of pH and temperature on Egs from the intergeneric

**Table 3** Experimental and predicted activities of carboxymethyl cellulase from fusant M 62 after 55 min of incubation at defined pH and temperature

| Observation order | Temperature $x_1$ ($\equiv X_1$ °C) | pH $x_2$ ($\equiv X_2$) | Carboxymethyl cellulase (U) | | | |
|---|---|---|---|---|---|---|
| | | | Set 1 | Set 2 | Average | Predicted |
| 1 | −1 (≡40) | −1 (≡3.8) | 0.093 | 0.089 | 0.091 | 0.106 |
| 2 | 1 (≡60) | −1 (≡3.8) | 0 | 0 | 0 | −0.020 |
| 3 | −1 (≡40) | 1 (≡5.8) | 0.168 | 0.168 | 0.168 | 0.184 |
| 4 | 1 (≡60) | 1 (≡5.8) | 0.125 | 0.127 | 0.126 | 0.107 |
| 5 | 0 (≡50) | 0 (≡4.8) | 0.170 | 0.172 | 0.171 | 0.170 |
| 6 | 0 (≡50) | 0 (≡4.8) | 0.168 | 0.166 | 0.167 | 0.170 |
| 7 | 0 (≡50) | 0 (≡4.8) | 0.162 | 0.162 | 0.162 | 0.170 |
| 8 | −1.414 (≡36) | 0 (≡4.8) | 0.193 | 0.195 | 0.194 | 0.171 |
| 9 | 1.414 (≡64) | 0 (≡4.8) | 0 | 0 | 0 | 0.027 |
| 10 | 0 (≡50) | −1.414 (≡3.3) | 0 | 0 | 0 | 0.003 |
| 11 | 0 (≡50) | 1.414 (≡6.2) | 0.1465 | 0.1475 | 0.147 | 0.148 |
| 12 | 0 (≡50) | 0 (≡4.8) | 0.167 | 0.171 | 0.169 | 0.163 |
| 13 | 0 (≡50) | 0 (≡4.8) | 0.165 | 0.165 | 0.165 | 0.163 |
| 14 | 0 (≡50) | 0 (≡4.8) | 0.164 | 0.162 | 0.163 | 0.163 |

$R = 0.9795$; $R^2 = 0.9595$.

**Table 4** Experimental and predicted activities of carboxymethyl cellulase from *Trichoderma reesei* (WT) after 55 min of incubation at defined pH and temperature

| Observation order | Temperature $x_1$ (=$X_1$ °C) | pH $x_2$ (=$X_2$) | Carboxymethyl cellulase (U) | | | |
|---|---|---|---|---|---|---|
| | | | Set 1 | Set 2 | Average | Predicted |
| 1 | −1 (≡40) | −1 (≡3.8) | 1.283 | 1.277 | 1.280 | 1.315 |
| 2 | 1 (≡60) | −1 (≡3.8) | 0.776 | 0.772 | 0.774 | 0.570 |
| 3 | −1 (≡40) | 1 (≡5.8) | 0.803 | 0.807 | 0.805 | 0.909 |
| 4 | 1 (≡60) | 1 (≡5.8) | 0.984 | 0.982 | 0.983 | 0.847 |
| 5 | 0 (≡50) | 0 (≡4.8) | 1.203 | 1.210 | 1.207 | 1.274 |
| 6 | 0 (≡50) | 0 (≡4.8) | 1.209 | 1.211 | 1.210 | 1.274 |
| 7 | 0 (≡50) | 0 (≡4.8) | 1.199 | 1.207 | 1.203 | 1.274 |
| 8 | −1.414 (≡36) | 0 (≡4.8) | 1.308 | 1.300 | 1.304 | 1.185 |
| 9 | 1.414 (≡64) | 0 (≡4.8) | 0.392 | 0.396 | 0.394 | 0.614 |
| 10 | 0 (≡50) | −1.414 (≡3.3) | 0.608 | 0.598 | 0.603 | 0.701 |
| 11 | 0 (≡50) | 1.414 (≡6.2) | 0.602 | 0.614 | 0.608 | 0.610 |
| 12 | 0 (≡50) | 0 (≡4.8) | 1.209 | 1.205 | 1.207 | 1.140 |
| 13 | 0 (≡50) | 0 (≡4.8) | 1.200 | 1.202 | 1.201 | 1.140 |
| 14 | 0 (≡50) | 0 (≡4.8) | 1.211 | 1.217 | 1.214 | 1.140 |

$R = 0.9256$; $R^2 = 0.8567$.

**Table 5** ANOVA for the effect of pH and temperature on the stability of the carboxymethyl cellulase from fusant M 14

| Source | Sum of squares | Degrees of freedom | Mean square | F | $P > F$ |
|---|---|---|---|---|---|
| Model | 0.026383 | 5 | 0.005277 | | |
| Error | 0.000764 | 7 | 0.000109 | 48.32 | 0.0001 |
| Corrected total | 0.027147 | 12 | | | |

**Table 6** ANOVA for the effect of pH and temperature on the stability of the carboxymethyl cellulase from fusant M 62

| Source | Sum of squares | Degrees of freedom | Mean square | F | $P > F$ |
|---|---|---|---|---|---|
| Model | 0.062498 | 5 | 0.0125 | | |
| Error | 0.002647 | 7 | 0.00378 | 33.05 | 0.0001 |
| Corrected total | 0.065145 | 12 | | | |

**Table 7** ANOVA for the effect of pH and temperature on the stability of the carboxymethyl cellulase from *Trichoderma reesei* (WT)

| Source | Sum of squares | Degrees of freedom | Mean square | F | $P > F$ |
|---|---|---|---|---|---|
| Model | 0.962657 | 5 | 0.192531 | | |
| Error | 0.171347 | 7 | 0.024478 | 7.865 | 0.0086 |
| Corrected total | 0.134004 | 12 | | | |

fusants. This study becomes vital to develop the fusants for higher ethanol yield.

### *pH and thermal stability*

The most important physical parameters that can affect enzyme stability are pH and temperature [3]. Experiments were performed at different combinations of pH and temperature. A suitable combination was determined using statistical experimental design to enhance the stability of the enzyme [7]. The details of the experimental plan are given in Tables 2–4 for the respective organisms. Using the results of the experiments, the following Equations (3–5) were obtained for the fusants M 14, M 62 and *Trichoderma reesei* (WT) respectively, indicating activity as a function of pH and temperature.

$$y = 0.101834 - 0.030167\,x_1 + 0.035456\,x_2 - 0.024733\,x_1^2 - 0.024983\,x_2^2 - 0.012\,x_1x_2 \quad \text{(for fusant M 14)} \tag{3}$$

$$y = 0.166166 - 0.050922\,x_1 + 0.051365\,x_2 - 0.031837\,x_1^2 - 0.04359\,x_2^2 + 0.01225\,x_1x_2 \quad \text{(for fusant M 62)} \tag{4}$$

$$y = 1.206990 - 0.201873\, x_1 - 0.03271\, x_2 - 0.120688\, x_1^2 - 0.242474\, x_2^2 + 0.171\, x_1 x_2 \quad \text{(for } T.\ reesei\text{)} \quad (5)$$

where $x_1$ = temperature (°C) and $x_2$ = pH.

The coefficients of determination ($R^2$) are 0.9718, 0.9595 and 0.8567 for fusant 14, fusant 62 and *T. reesei*. $R^2$ is a measure of total variation of the observed values of activity about the mean explained by the fitted model, which is often expressed in percentage. This implies that 97.18%, 95.95% and 85.67% of total variation in the activities of carboxymethyl cellulase from fusant M 14, fusant M 62 and *T. reesei* are explained by the fitted models (Equations 3–5). The coefficient of correlation ($R$) is 0.9858, 0.9795 and 0.9256 respectively for carboxymethyl cellulase from fusant M 14, fusant M 62 and *T. reesei*. It explains the correlation between the experimental and predicted values from the model. The correlation coefficient is large in all three cases, which indicates that quadratic models fit excellently with the experimental values of activity (Tables 2–4). The predicted values of the activity obtained by the equation and actual values of the activity which is determined experimentally (in duplicates) at each point for fusants M 14, M 62 and *T. reesei* are shown in Tables 2–4. A summary of the ANOVA is given in Tables 5–7. The F values: 48.32, 33.05, 7.87 for fusant M 14, fusant M 62 and *T. reesei* (WT), respectively, are greater than $F_{5,7}$ within a rejection region having an $\alpha$-level that is $P < 0.0001$, 0.0001 and 0.0086 respectively. Lack of fit can be detected at the $\alpha$-level and of significance if the values of F exceed the tabulated value, $F_{5,7}$ [7]. The isoresponse contour plots (Figures 2–4) explain the effect of pH and temperature on the activity of carboxymethyl cellulase from different organisms studied. The stationary point is the point at which the slope of the response surface is zero when taken in all directions. It was observed that all the contour plots are elliptical and the stationary point is attained at the centre by moving along the major and

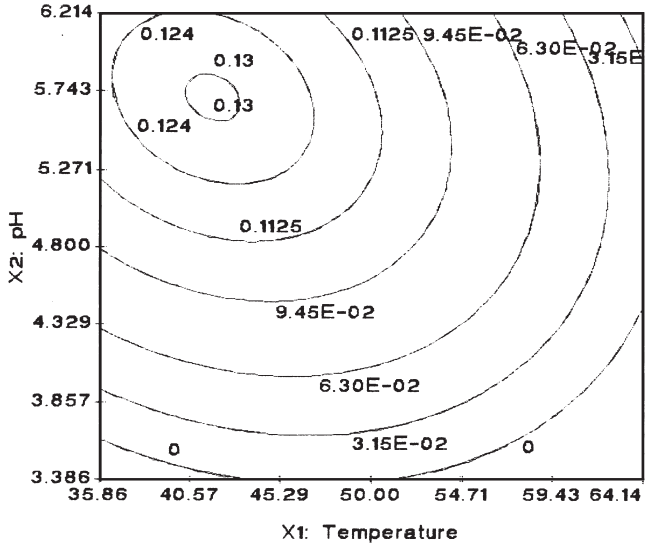


**Figure 2** Isoresponse contour plot of the activity of carboxymethyl cellulase from fusant M 14: effect of pH and temperature.

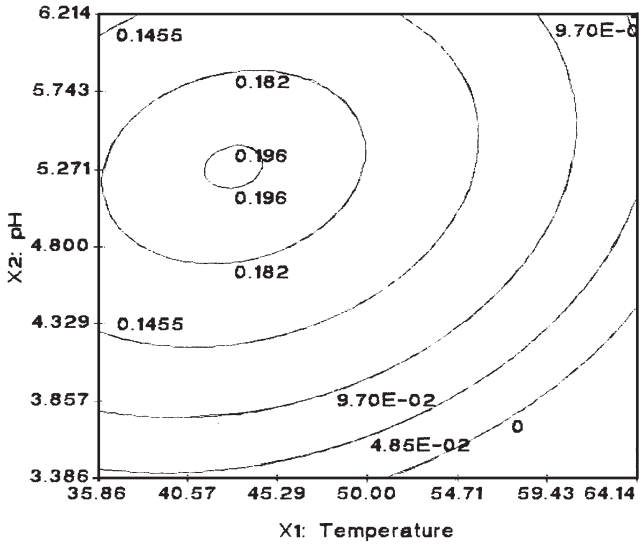


**Figure 3** Isoresponse contour plot of the activity of carboxymethyl cellulase from fusant M 62: effect of pH and temperature.

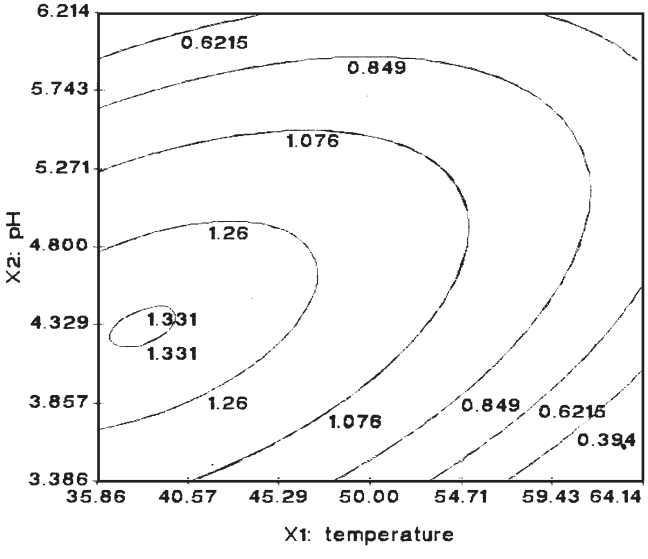


**Figure 4** Isoresponse contour plot of the activity of carboxymethyl cellulase from *Trichoderma reesei* QM 9414: effect of pH and temperature.

**Table 8** Actual and predicted values of carboxymethyl cellulase activities obtained after analysis of experimental data

| Organism | Optimum pH and temperature (°C) | Carboxymethyl cellulase activity (U) | |
|---|---|---|---|
| | | Experimental | Predicted |
| Fusant M 14 | 5.7, 41.7 | 0.134 | 0.131 |
| Fusant M 62 | 5.3, 43 | 0.256 | 0.251 |
| *T. reesei* | 4.31, 38.3 | 1.504 | 1.147 |

minor axis of the ellipse. The values of the temperature and pH combinations where the enzyme is more stable are obtained after maximization of the regression equation. The actual calculated values are 41.73°C and 5.7 for fusant M 14, 43°C and 5.3 for fusant M 62, and 38.3°C and 4.3 for *Trichoderma reesei* QM 9414. The values obtained were verified experimentally which resulted in minimum deactivation (Table 8). The above results show that endoglucanase from both the fusants are stable at higher pH and temperature when compared to endoglucanase from *Trichoderma reesei* (WT).

One can proceed further to study the thermal stability of the enzyme at higher temperatures and extreme pH values. This is very important because improved knowledge of enzyme deactivation and its thermodynamic characterization is necessary to enhance the feasibility of the biotechnological process as rapid inactivation may constrain the efficiency of the process. This is presently under investigation.

## References

1 Anjani Kumari J and T Panda. 1994. Intergeneric hybridization between *Trichoderma reesei* QM 9414 and *Saccharomyces cerevisiae* NCIM 3288 for the direct conversion of cellulose to ethanol. Enzyme Microb Technol 16: 870–882.
2 Box GEP and JS Hunter. 1957. Multifactor experimental design for exploring response surfaces. Ann Math Stat 28: 195–241.
3 Chitnis A and A Sadana. 1989. pH dependent enzyme deactivation models. Biotechnol Bioeng 34: 804–818.
4 Coughlan MP. 1985. The properties of fungal and bacterial cellulases with comment on their production and application. Biotechnol Genet Eng Rev 3: 39–109.
5 Foster RL (ed). 1980. Nature of Enzymology. Croom Helm, London, pp 93–105.
6 Ghose TK. 1984. Measurement of cellulases. Commission Biotechnology. International Commission of Pure and Applied Biotechnology, pp 1–102.
7 Khuri AI and A Cornell. 1987. Response surfaces designs and analyses. In: Statistics: Text Books and Monographs (DB Cowen, RG Cornell, AM Kshirsagar, WJ Kennedy and EG Schilling, eds), Marcel Dekker, New York.
8 Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685.
9 Miller GL. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugars. Anal Chem 31: 426–429.
10 Molony AP, SI McCare, TM Wood and MP Coughlan. 1985. Isolation and characterization of the 1, 4-$\beta$-d-glucan glucanohydrolases of *Talaromyces emersonii*. Biochem J 225: 365–374.
11 Srinivas D, K Jagannadha Rao, K Théodore and T Panda. 1995. Direct conversion of cellulosic materials to ethanol by the intergeneric fusants of *Trichoderma reesei* QM 9414/*Saccharomyces cerevisiae* NCIM 3288. Enzyme Microb Technol 17: 418–423.
12 Srinivas R and T Panda. 1997. Localization of carboxymethyl cellulase in the intergeneric fusants of *Trichoderma reesei* QM 9414/*Saccharomyces cerevisiae* NCIM 3288. Bioproc Eng 18: 71–73.
13 Wolfgang HS, K Bronnenmeier, F Grabnitz and WL Staudenbauer. 1987. Activity staining of cellulases in polyacrylamide gels containing mixed linkage $\beta$-glucans. Anal Biochem 164: 72–77.
14 Wood TM and KM Bhat. 1988. Methods in Enzymology (Biomass–Part A) (WA Wood and ST Kellog, eds), Vol 160, pp 87–91, Academic Press, New York.