Production optimization of alkali-thermo tolerant crude endoglucanase from *Funalia leonina* by response surface methodology

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A remarkable yield of an alkali-thermo-tolerant endoglucanase (2.93 IU/ml) was obtained from a white rot fungus, *Funalia leonina* through fermentation process in solid state, optimized by response surface methodology. The three test variables, viz. pH of medium, incubation temperature and incubation time were optimized as they have significant effect on enzyme production. After solving the model equation, the optimum values of medium pH, incubation temperature and incubation period were found to be 5.8, 34°C and 10 days respectively, for maximum endoglucanase production. The consequence of culture preservation medium on enzyme production capacity was also studied and wheat bran agar medium was found the most suitable medium for culture preservation.

Keywords: Endoglucanase, *Funalia leonina*, preservation medium, response surface methodology.

ENDOGLUCANASE, an industrially important multirole enzyme, is mainly used in bioethanol production, starch processing, syrup production, detergents, pulp and paper industry, grain alcohol fermentation, extraction of vegetable and fruit juices, animal feed production and textile industry[1,2].

Production of extracellular enzymes in microbes is deeply influenced by various factors, such as pH of the medium, incubation temperature and period of incubation[3]. The initial pH of the medium plays an important role in enzymatic reactions and regulates the enzyme transport across cell membranes[4]. Enzyme-mediated reactions (external or periplasmic) are influenced by the pH of the cultivation system and ultimately have an effect on enzyme productivity[5]. The metabolic activities of microbes are highly dependent on incubation temperature. Microbial ribosomes and fluidity of membranes are intensively influenced by the cultivation temperature and finally affect the production of enzyme[5,6]. Incubation period (time) also plays a crucial role in enzyme synthesis of microbes. The log phase of microbial growth exhibits optimum level of primary metabolites (such as enzymes) and pre- and post-period of the log phase negatively affect the concentration and quantity of microbial product. Long incubation period results in reduction of endoglucanase synthesis. The possible reasons are fast exhaustion of available nutrients or proteolytic degradation of produced enzyme or feedback inhibition of cellulase biosynthesis by the end products, e.g. glucose and cellobiose[7,8].

In conventional one factor at a time approach of optimization, only one factor is changed at a time while others are kept constant. Due to the large number of experiments, determining the optimized values of variables is a highly laborious and time-consuming process. Moreover the obtained value/s may not be a true optimum value due to overlooking of variable interactions. On the other hand, using factorial design and regression analysis, response surface methodology (RSM) evaluates the true optimum values of variables using variable interaction strategy and builds the models for analysis of different variables to get an ultimate response[9,10]. In this communication, the production optimization of endoglucanase from *Funalia leonina*, using Box–Behnken design under RSM is reported. The effect of culture preservation medium on enzyme production capacity of test fungus has also been explored.

F. leonina (Klotzsch) Patouillard, 1900 was obtained from the culture collection centre of Forest Research Institute, Dehradun. The culture was propagated at 28°C on potato dextrose agar (PDA) plates and slants. After a growth of 5–7 days, the cultured plates and slants were stored at 4°C. Malt extracts agar (MEA), wheat bran agar (WBA) and wheat straw agar (WSA) media were also used for maintaining the culture.

For endoglucanase production wheat straw (powdered, 30–60 mesh size of particles) was used as a carbon source. Modified Reese and Mandel's medium with composition: 2.0 K_2HPO_4 g/l; 1.4 $(NH_4)_2SO_4$ g/l; 0.3 Urea g/l; 0.005 $FeSO_4 \cdot 7H_2O$ g/l; 0.002 $CoCl_2$ g/l; 0.0016 $MnSO_4 \cdot 7H_2O$ g/l and 0.0014 $ZnSO_4 \cdot 7H_2O$ g/l, (ref. 11) was used as basal mineral solution (BMS) for enzyme production.

A three-factor three-level Box–Behnken design under RSM was deployed to optimize the three physical parameters/factors (initial pH of medium, incubation temperature and incubation period) of enzyme production by test fungus. RSM is a combination of statistical design and empirical model (regression based). The interactions among test variables are mathematically expressed as a polynomial model[10]. For implementation in statistical design, a narrow range of test parameters was selected on the basis of single variable at a time approach-based experiments carried out earlier. Experiment designing, analysis of variance (ANOVA), regression and graphical analysis were carried out using a statistical software

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package, STATISTICA-7 (StatSoft Inc., Tulsa, USA). A summary of the physical parameters and their levels for the test fungus is given in Table 1.

On the basis of test variable interactions, an experimental model related to the response was given by STATISTICA-7. This software, on the basis of variables used in the experiment, provides an empirical model related to the response, measured according to experimental variables. For a three-factor, three-level (e.g. Box–Behnken) design, the equation is

$$R = X_0 + X_1A + X_2B + X_3C + X_{11}A^2 + X_{22}B^2 + X_{33}C^2 + X_{12}AB + X_{23}BC + X_{13}AC, \quad (1)$$

where R is the predicted response or dependent variable; X_0 the intercept, X_1, X_2, X_3 are linear coefficients, X_{11}, X_{22}, X_{33} are squared coefficients and $X_{12}AB$, $X_{23}BC$, $X_{13}AC$ are interaction coefficients.

After solving the model equation (regression equation), the optimum levels (values) of the selected test variables (factors or parameters) were obtained. In 250 ml Erlenmeyer flasks, a set of fifteen experiments was performed using solid state fermentation (SSF) for endoglucanase production. The pH of the medium containing the desired carbon source was adjusted by using 1 M NaOH and 1 M H_2SO_4 solutions. After sterilization of the media, flasks were inoculated with fresh culture (5 days old) of test fungus by using five disks of 5 mm diameter. Based on the design, the culture was incubated at given temperature for the given duration. The enzyme was harvested after completion of the experiments and assayed.

After optimization of test variables, mass production of endoglucanase from test fungus was carried out under SSF at optimized conditions. Erlenmeyer flasks of 1 litre capacity were used for this purpose. Twenty gram of carbon source was taken in each flask and 80 ml of BMS of desired initial pH was added. These flasks were then sterilized by autoclaving. After cooling, 20 disks of 5 mm diameter from 5-day-old culture of test fungus were inoculated in the flasks. The flasks were incubated at desired temperature for the incubation period and harvested.

Table 1. Summary of physical parameters of the Box–Behnken design for endoglucanase production by the test fungus *F. leonina*

Factor/parameter	Range studied	Variation interval	Coded value	Value of the factor
Initial pH of medium	4–9	2.5	–	4
			0	6.5
			+	9
Incubation temperature (°C)	25–45	10	–	25
			0	35
			+	45
Incubation period (days)	6–14	4	–	6
			0	10
			+	14

The activity of various enzyme components of crude enzyme was determined by standard methods. Endoglucanase activity as carboxymethylcellulase (CMCase) and filter paper degrading activity (FPase) were assayed using the method of Ghose[12]. A DNS method reported by Bailey *et al.*[13] was used to estimate xylanase activity. Activity of laccase was determined using 2′-amino bis 3-ethylbenzthiazoline-6-sulphonic acid (ABTS) in sodium acetate buffer (0.1 M) of 3.4 pH[7]. The activity of alpha amylase was assayed according to Abe *et al.*[14] and lipase activity measured by using titration method depicted by Mateos Diaz *et al.*[15].

These assays were carried out in triplicate and average values of the experimental results were shown as final activity. Endoglucanase, FPase and amylase activities were expressed in the form of international units (IU) equivalent to the amount of enzyme which liberates 1 μmol glucose/min (xylose/min for xylanase) or micromoles of glucose (xylose for xylanase) liberated in one minute from one ml of reaction concoction under typical test conditions. One unit of laccase enzyme activity was expressed as the enzyme amount that converts 1 μmol of substrate per unit time (i.e. minute). The lipolytic activity of the enzyme was defined as international unit per ml of the enzyme which represents fatty acid (1 μmol) released per unit time from substrate. Lowry's method was used to estimate protein concentration in crude enzyme using bovine serum albumin (BSA) as the standard[16].

The test fungus was grown and preserved on four different types of media named PDA, MEA, WBA and WSA. To reveal the effect of preservation medium on enzyme production, fungus (grown as well as preserved on PDA, MEA, WBA and WSA) was allowed to produce enzyme at optimized fermentation conditions.

Optimum level of pH for maximum endoglucanase activity of crude enzyme was estimated in pH range of 4 to 9. Buffer solutions of 0.05 molarity were used. For pH range of 4–6, sodium citrate buffer was used. Sodium phosphate buffer and glycine buffer were used for pH range of 6.5–8 and 8.5–9.0 respectively. Carboxymethylcellulose (2%) solution prepared in each buffer was used as substrate for the reaction. To assess the temperature effect on crude endoglucanase, endoglucanase assay was performed at 40–80°C at intervals of 5°C.

The results of experiments based on the Box–Behnken design for studying the effect of three variables on endoglucanase production along with the observed and model predicted response (endoglucanase activity) are given in Table 2.

The value of R^2 was obtained as 0.9618 by regression equation after implementation of ANOVA. R^2 value greater than 0.75 shows the correctness of implemented model and it has always between zero and one. R^2 closer to one shows model strength and better predictability of the response. It indicates the measurement of unevenness present in observed response values elucidated by test

variables and their interactions[17]. In the form of percentage, R^2 value shows response variability in per cent elucidated by the model.

Here, 96.18% R^2 value implied the sample variation for endoglucanase activity on the basis of test variable interactions. The model was unable to explain only 3.82% of total variation. This shows that quadratic model was satisfactorily adjusted to the data of experiments. The model F-value (13.99) indicates a good model fit of endoglucanase activity. Model's adjusted R^2 value is 0.8931 and it corrects the R^2 value in the model for the sample size and number. If the model has many terms and small sample size, the adjusted R^2 may be evidently smaller than R^2 (ref. 17). In this exercise the adjusted R^2 is close to R^2. The regression equation coefficients were calculated using STATISTICA 7.0 and the surface response regression or second order polynomial regression equation was obtained

$$R = -19.2432 + 0.8787\,A - 0.0707\,A^2 + 0.9674\,B - 0.0139\,B^2 + 0.5406\,C - 0.0254\,C^2 - 0.0010\,AB - 0.0015\,AC - 0.0004\,BC, \quad (2)$$

where R is the endoglucanase activity (IU/ml), A the initial pH of medium, B the incubation temperature and C is the incubation days.

Model's p-value was 0.004 which shows significant model terms. The p-value is a tool to check significance of coefficient and shows the interaction strength between test variables. Smaller p-value indicates the rejection of the null hypothesis[18].

The optimum array of selected test variables (e.g. initial pH of cultivation medium, incubation temperature and time) for maximum response (endoglucanase activity) is shown by the response contour plots (Figures 1–3). A number of amalgamations of the two test variables, maintaining other variables at zero levels, are represented by contour plots. The maximum response is depicted by the innermost circle of contour plots due to interactions of test factors.

Table 2. The design matrix for three test variables in coded values of units along with observed and predicted response values (endoglucanase activity)

Run no.	pH	Temperature	Incubation days	Observed response (activity IU/ml)	Predicted response (activity IU/ml)
1	0	0	0	2.81	2.76
2	–1	0	–1	2.27	2.09
3	0	0	0	2.80	2.76
4	0	–1	–1	1.02	1.05
5	0	+1	–1	0.87	0.81
6	+1	0	+1	1.53	1.71
7	+1	+1	0	0.70	0.55
8	+1	–1	0	1.11	0.87
9	–1	+1	0	0.81	1.05
10	–1	–1	0	1.12	1.27
11	+1	0	–1	1.46	1.67
12	0	+1	+1	0.89	0.86
13	0	0	0	2.68	2.76
14	–1	0	+1	2.40	2.19
15	0	–1	+1	1.10	1.16

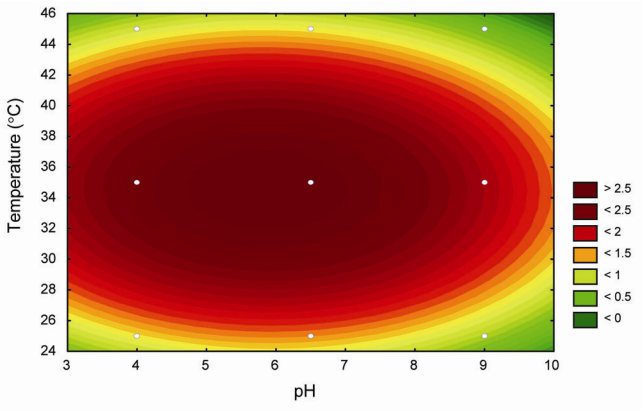

Figure 1. Contour plots representing the effect of pH and temperature on endoglucanase production by *F. leonina*.

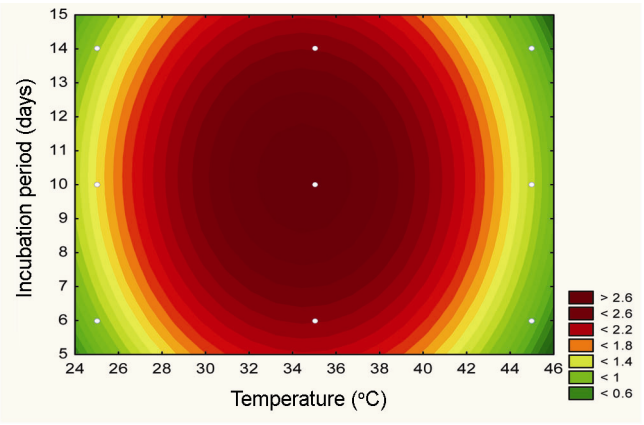

Figure 2. Contour plots representing the effect of incubation temperature and period on endoglucanase production by *F. leonina*.

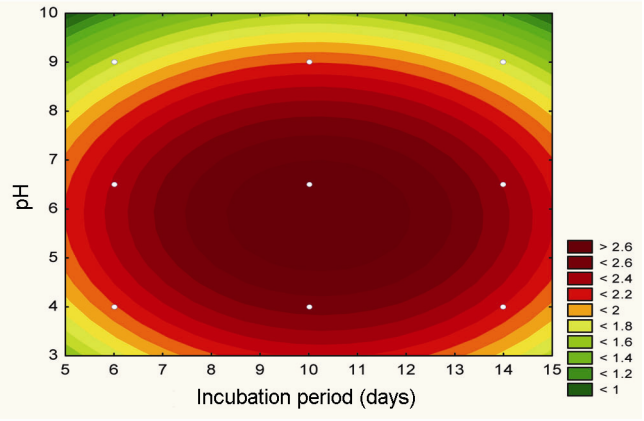

Figure 3. Contour plots representing the effect of incubation period and initial pH on endoglucanase production by the *F. leonina*.

On the basis of these response contour plots, the optimal ranges of initial pH of medium, incubation temperatures and incubation period for maximum production of the cellulase by *F. leonina* were found in the range of 4.8–7.0 pH, 33.5–37°C and 8–12 days respectively.

To reveal the optimum values of test factors, the response surface regression equation or second order polynomial equation was resolved through MATLAB 10 software. The optimum values of test variable *A* (initial pH), *B* (incubation temperature) and *C* (incubation time/days) predicted by the model were 5.8, 34°C and 10 days respectively for the maximum endoglucanase activity predicted by the model (2.76 IU/ml).

To validate the model, endoglucanase was produced by *F. leonina* using model predicted values. All trials were performed in triplicate and endoglucanase activity of crude enzyme preparation was obtained as 2.91 IU/ml. This experimental value of endoglucanase activity (2.91 IU/ml) is close to the model predicted value of endoglucanase (2.76 IU/ml) and verifies the validity of the model.

Based on deployed model values, mass production of enzyme was carried out using *F. leonina* in 1000 ml conical flask having 20 g of wheat straw (powdered 30–60 mesh size), 80 ml of basal mineral solution (initial pH of 5.8) and 20 disks of 5 mm diameter from 5 day old fungal culture kept at 34°C for 10 days. After enzyme harvesting, this crude enzyme was assayed for various enzyme activities using standard methods (Table 3). The total protein concentration of the crude enzyme was 4.36 mg/ml.

Table 3. Enzyme profile of crude enzyme preparation

Enzyme	Activity (IU/ml)
Endoglucanase	2.93 ± 0.04
FPase	0.88 ± 0.02
Xylanase	18.63 ± 0.12
Amylase	0.48 ± 0.03
Laccase	4.70 ± 0.43
Lipase	0.65 ± 0.02

Values of enzyme activities are presented as mean ± standard deviation ($n = 3$).

Table 4. Effect of culture preserving medium on enzyme production

Preserving media	Endoglucanase (IU/ml)
Wheat bran agar (WBA)	2.03 ± 0.04^{a}
Malt extract agar (MEA)	1.65 ± 0.03^{b}
Potato dextrose agar (PDA)	1.55 ± 0.03^{c}
Wheat straw agar (WSA)	1.98 ± 0.02^{a}

Endoglucanase activities are presented as mean ± standard deviation ($n = 3$). Duncan's multiple range test (DMRT) was used for comparison of all groups (at $p < 0.05$). Values having different superscripts are statistically different.

The *F. leonina* cultures grown and preserved on WBA and WSA plates have shown maximum endoglucanase activity, i.e. 2.03 and 1.98 IU/ml respectively (Table 4).

The reason for better response in WBA and WSA may be their complex nature when compared to MEA and PDA. This promotes secretion of hydrolytic enzymes which break down the complex molecule of wheat bran. PDA and MEA have easily available sugars which suppress the operons from producing responsible enzymes.

The pH profile of crude endoglucanase preparation of *F. leonina* revealed that the enzyme has slightly acidic pH optima. The maximum endoglucanase activity, 3.27 IU/ml, was obtained at pH 6.5, but it also showed activity over a pH range of 6.5–9.0. After pH 6.5 the endoglucanase activity was found to decrease and at pH 9.0, the enzyme showed 18% (0.59 IU/ml) of its maximum activity. Therefore, endoglucanase produced by the test fungus was found to be alkali-tolerant as it was active in the alkaline range. Duncan's multiple range test (DMRT) analysis revealed that each pH level significantly influenced the enzyme activity of the crude enzyme preparations.

The pH of medium directly affects the enzyme's structure and functions, as changes in concentration of hydrogen ions in the medium alter the amino acids' ionic behaviour and structure (three-dimensional) of the active sites[19]. Deviations from the optimal pH level lead to decline in enzyme activity rates[20–22].

The crude endoglucanase of *F. leonina* exhibited highest activity at 60°C (3.58 IU/ml). The endoglucanase activity increased from 40°C to 60°C and above 60°C its activity decreased (Table 5). The enzyme was tested up to 80°C and its activity was lost drastically at higher temperatures. At 70 and 80°C endoglucanase retained only 30 and 12% (1.08 and 0.42 IU/ml) of its maximum

Table 5. Effect of pH and temperature on the endoglucanase activity of the crude enzyme

pH	Activity (IU/ml)	Temperature (°C)	Activity (IU/ml)
4.0	0.89 ± 0.02^{j}	40	0.65 ± 0.03^{g}
4.5	1.69 ± 0.05^{h}	45	1.16 ± 0.02^{e}
5.0	2.15 ± 0.05^{f}	50	1.99 ± 0.04^{d}
5.5	2.42 ± 0.04^{d}	55	2.62 ± 0.03^{c}
6.0	3.08 ± 0.08^{b}	60	3.58 ± 0.04^{a}
6.5	3.27 ± 0.06^{a}	65	2.73 ± 0.05^{b}
7.0	2.82 ± 0.05^{c}	70	1.08 ± 0.05^{f}
7.5	2.31 ± 0.06^{e}	75	0.66 ± 0.03^{h}
8.0	1.90 ± 0.02^{g}	80	0.42 ± 0.02^{i}
8.5	1.29 ± 0.04^{i}		
9.0	0.59 ± 0.03^{k}		

Endoglucanase activities are presented as mean ± standard deviation ($n = 3$). DMRT was used for comparison of all groups (at $p < 0.05$). Values having different superscripts are statistically different. Endoglucanase assay conditions: temperature 50°C (for effect of pH), pH 6.5 (for effect of temperature) and incubation time 30 min (both type of experiments).

activity (3.58 IU/ml) and this is an indication of the thermal tolerance of the enzyme. The results of DMRT showed that enzyme activity of the crude enzyme was significantly influenced by each temperature level.

Temperature of the solution influences the enzyme activity due to changes in enzyme 3D structure by arrangement of proteins and their components. Towards optimum level of temperature, the rate of biochemical reactions increased due to increase in the kinetic energy of reacting molecules. At higher temperatures, enzymes lose their activity due to denaturation of proteins. The thermal tolerance or stability of enzymes seems to be achieved through amino acid substitution which results in the modification in their structure. Some factors like rearrangement of hydrogen bonds, interactions of ion pairs and other hydro-repulsive reactions may be a possible reason for high temperature resistance in enzymes[23]. Thus the present results show the alkali-thermo tolerant nature of the produced endoglucanase enzyme.

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