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Optimization of mutanase production by *Trichoderma harzianum*

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The present paper describes optimization of fermentation conditions in shaken flasks and scale-up of fermentor production up to 115 L. The response surface methodology (RSM) has been successfully applied in standardization of mutanase production by *Trichoderma harzianum* CCM F-340. The model was very well fitted to the experimental data and explained more than 96% of the whole variation of the response (adjusted R^2 = 0.962). In order to confirm the adequacy of the regression model based on the experimental data, validation cultures were grown in conditions created through optimization. The highest enzyme activity (0.747 U/mL) was reached in shaken flask cultures on Mandels' medium in a volume of 140 mL modified in terms of carbon (cell wall preparation from the polypore fungus *Laetiporus sulphureus* 8.08 g/L) and nitrogen (soybean peptone 1.38 g/L) sources, under culture conditions 30°C, pH 5.3, agitation 270 rpm. The scale-up of the culture in the bioreactors with a working volume of 5 and 115 L resulted in a slight decrease in the mutanase activity (0.734 and 0.682 U/mL, respectively). The validation experiment showed a 70.6% increase in the production of mutanase compared with the culture before optimization. The results proved that the cultures could be scaled-up successfully from shaken flasks to the bioreactor scale. Our results indicate that in optimal conditions, *T. harzianum* could be a highly effective extracellular mutanase source. This report is the first to deal with optimization of mutanase biosynthesis using a mathematical model and scale-up of enzyme production in controlled fermentors with a view to facilitate application thereof in industry.

Key words: Mutanase, *Trichoderma harzianum*, response surface methodology (RSM), bioreactors, submerged culture.

INTRODUCTION

Mutanases (α-(1→3)-glucan 3-glucanohydrolases) hydrolyze the α-(1→3)-glycosidic bonds of streptococcal mutan - water-insoluble, alkali-soluble α-glucan found in oral biofilms. Currently, the most promising application of mutan-

degrading enzymes is prevention of dental caries (Inoue et al., 1990; Pleszczyńska et al., 2011). Mutanases could be used as an active additive in preparations intended for oral hygiene, such as mouthwashes, toothpastes, and dental gels, and for washing and storage of prosthesis and prosthetic devices for removal of denture plaque located on their acrylic surfaces. As active ingredients, mutanases could become useful supplements to mechanical cleaning of teeth and dentures with a toothbrush, dental sticks, and dental floss.

In addition to their potential usefulness in dentistry as oral therapeutic agents, α-(1→3)-glucanases might be applicable in investigations of α-(1→3)-glycosidic linkages found in microbial cell-wall structures and glucans of certain higher plants. Mutanases obtained in a pure form are invaluable tools for studying the chemical structure of carbohydrates. Analyses of the products of mutan hydrolysis by these enzymes provided important structural information (Hare et al., 1978). Moreover, various preparations of mutanases have also been successfully used for obtaining fungal protoplasts (Balasubramanian et al., 2003).

The most common source of extracellular mutanases are filamentous fungi, such as *Trichoderma* spp., *Aspergillus* spp. and *Penicillum* spp., which exhibit higher enzyme activity than bacteria and yeast (Fuglsang et al., 2000; Guggenheim et al., 1972). However, to our knowledge, there are few reports on detailed characteristics of production of fungal mutanases. In our previous studies, we described the effects of nutrients and culture conditions on mutanase production by various *T. harzianum* strains in flask cultures and batch fermentation using "one-variable-at-a-time" experiments (Wiater et al., 2005b). The "one-variable-at-a-time" approach is time consuming and does not account for the interactions among the medium components. The statistical approach for medium optimization is believed to be a better alternative to the classical approach because of the utilization of fundamental principles of statistics, randomization, replication, and duplication (Singh et al., 2008). A combination of the factorial design and response surface optimization are used to identify the factors and their levels for obtaining an optimal response. In this report, we have applied the response surface methodology (RSM) to a biological model. This technique involves primary screening of variables by application of one of the screening methods, for instance the most popular Plackett-Burman design or the "one-variable-at-a-time" method or (Preetha et al., 2007; Xin et al., 2005) an experiment following the design for fitting the chosen model (Myers and Montgomery, 1995), as well as analysis of the experimental results and the response optimization process (Waśko et al., 2010). The most popular approach is based on the full factorial central composite design (CCD), which enables estimation of the coefficients in a second-order model.
The RSM is usually applied in engineering or industrial chemistry; however, this approach has limited use for biological systems.

In our previous pre-clinical and clinical studies, we demonstrated that mutanase produced by *T. harzianum* CCM F-340 degraded streptococcal biofilm *in vitro* and dental plaque on the teeth surface in humans quickly and effectively (Pleszczyńska et al., 2011; Wiater et al., 2008). However, the possibility of the practical application of mutanase is dependent on the costs and availability of the enzyme preparation. Thirty to forty percent of the production costs of industrial enzymes are estimated to be the cost of the growth medium (Joo et al., 2002). Therefore, it is important to optimize the conditions for cost-efficient enzyme production. Hence, our report is the first to deal with optimization of mutanase biosynthesis using a mathematical model and scale-up of enzyme production in controlled fermentors with a view to facilitate application thereof in industry.

The present paper describes production of mutanase from *T. harzianum* CCM F-340 using a sequential study of the factorial Plackett–Burman design followed by CCD. The factorial design of Plackett–Burman was used to screen the most significant factors affecting enzyme production. The CCD was used to identify the optimum levels of significant variables to generate an optimal response. This paper also describes the transfer of the results from the shaken flask culture to a laboratory 5 L fermenter, and finally to a 150 L pilot-scale fermenter.

MATERIALS AND METHODS

Microorganism

T. harzianum strain CCM F-340 (Czech Collection of Microorganisms, Brno, Czech Republic) was used as a starting culture for mutanase production.

Stationary culture conditions

Stock cultures of *T. harzianum* maintained at 4°C on potato dextrose agar slants were used for inoculation. Liquid medium A (pH 5.3), as described by Mandels et al. (1962), supported by 0.3% (unless otherwise stated) cell wall material from *Laetiporus sulphureus* (CWP) as a mutanase inducer, 0.05% peptone (unless otherwise stated), and 0.1% Tween 80 were used for mutanase production. Shaken cultures were conducted in 500 mL conical

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Table 1. Placket-Burman experimental design for evaluating factors influencing mutanase biosynthesis by *T. harzianum*.

Run	Bacto-peptone (g/L)		Soybean peptone (g/L)		CWP (g/L)		pH		Age of inoculums (h)		Volume of inoculums (%)		Volume of medium (mL)	
	C	A	C	A	C	A	C	A	C	A	C	A	C	A
1	-1	0	-1	0	-1	5	1	5.3	1	48	1	48	-1	15
2	1	0.5	-1	0	-1	5	-1	4	-1	12	-1	12	1	75
3	-1	0	1	0.5	-1	5	-1	4	1	48	1	48	1	75
4	1	0.5	1	0.5	-1	5	1	5.3	-1	12	-1	12	-1	15
5	-1	0	-1	0	1	10	1	5.3	-1	12	-1	12	1	75
6	1	0.5	-1	0	1	10	-1	4	1	48	1	48	-1	15
7	-1	0	1	0.5	1	10	-1	4	-1	12	-1	12	-1	15
8	1	0.5	1	0.5	1	10	1	5.3	1	48	1	48	1	75

C: Factors in coded values, A: factors in actual values.

flasks containing 15 - 200 mL of sterile medium depending on the experimental model. Unless otherwise stated, the flasks were seeded with conidia to a final concentration of about 2×10^5 conidia/mL and placed on an orbital rotary shaker (Inforce, Switzerland) at 270 rpm and 30°C for 3 days. Samples of the culture media were withdrawn periodically from shaken flasks and analyzed for mutanase activity.

L. sulphureus cell wall preparation (CWP)

The fruiting bodies of *L. sulphureus* (Bull.: Fr.) Murrill representing a combination of young and old basidiocarps were collected from deciduous trees growing in Lublin and its surroundings, Poland. The preparation of the cell wall from the *L. sulphureus* fruiting bodies was performed according to the procedure described by Wiater et al. (2008). The lyophilized fungal material was milled and the resulting powder was treated with water at 121°C for 1.5 h (×3). The wall material was removed by centrifugation (17001 × *g* for 30 min) and freeze-dried (Cell Wall Preparation, CWP).

Screening of important nutrient components using the Placket-Burman design

The Plackett-Burman design based on the first order model (Plackett and Burman, 1946) was chosen for primary screening of the seven factors at two levels; maximum (1) and minimum (-1). The experimental design and levels of each variable are presented in Table 1. The experiment was conducted in three replications in order to compute the variability of measurements within each unique combination of factor levels and estimate the pure error in the experiment. The results are presented in the form of a Pareto chart, where the absolute values of the ANOVA effect estimates are sorted from the largest to the smallest one. The *p*-value line shows the statistical significance of the influence of the factors on the feature studied (mutanase production).

Optimizing the concentration of the selected medium components with the use of the response surface methodology

A rotatable three-factor central composite design (CCD) with five replications of central points, axial points for α=1.68, and with nine replications of all combinations of the factors (including replications of the central points) was used to determine the unknown regression coefficient of the second-degree polynomial equation (Elibol, 2004):

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (1)$$

Where, Y is the predicted response variable (mutanase activity), β_0 is the interception, β_i are linear coefficients, β_{ii} are quadratic coefficients, and β_{ij} are coefficients of the interaction. X_i and X_j represent factors chosen on the basis of the results of Plackett-Burman design analysis. A total number of 20 experiments were employed for optimizing medium components and culture conditions for mutanase production (Table 2). The factorial points represent variance of the optimal design for a first-order model or a first-order two-factor interaction type model. The center points provide information about the existence of curvature in the system. If the curvature is found in the system, the axial points allow efficient estimation of the pure quadratic terms (Park et al., 2008). In the present study, the range of response values, Y, was very wide (Table 2). Generally, a ratio greater than 10 indicates higher likelihood that the transformation of the response value may improve the polynomial model. Therefore, the procedure described by Box and Cox (1964) was used in order to identify the power transformation for describing data in an appropriate form for statistical analysis. Application of the Box–Cox procedure indicated that the mutanase activity should be described by a square root transformation. The accuracy and general ability of the above polynomial model were evaluated using the adjusted coefficient of determination $Adj\text{-}R^2$ and the model *p*-value. The relationships between values predicted on the basis of this model and the chosen independent factors were represented in the form of 3D response surface plots. The calculations were performed using the Experimental Design unit of the STATISTICA software system (Stat Soft, 2007).

Validation of the experimental model and process scale-up

The statistical model was validated with respect to mutanase biosynthesis under the conditions predicted by the model in shaken flasks and in bioreactor conditions. The reference laboratory-scale fermentations were performed in a 5 L stirred tank bioreactor

Table 2. Experimental plan for optimization of mutanase biosynthesis using the central composite design.

Run	CWP (g/L)	Soybean peptone (g/L)	Volume of medium (mL)	Mutanase activity (U/mL)	Std Dev
1	3	0.2	80	0.358	0.014
2	3	0.2	200	0.341	0.018
3	3	1.8	80	0.019	0.008
4	3	1.8	200	0.021	0.006
5	9	0.2	80	0.049	0.009
6	9	0.2	200	0.030	0.008
7	9	1.8	80	0.654	0.021
8	9	1.8	200	0.643	0.009
9	0.96	1	140	0.041	0.005
10	11.04	1	140	0.173	0.008
11	6	0	140	0.060	0.011
12	6	2.344	140	0.009	0.006
13	6	1	39.2	0.562	0.011
14	6	1	240.8	0.584	0.019
15	6	1	140	0.558	0.029
16	6	1	140	0.580	0.025
17	6	1	140	0.560	0.025
18	6	1	140	0.587	0.017
19	6	1	140	0.578	0.020
20	6	1	140	0.554	0.050

Biostat B Plus (Sartorius Stedim, Germany). The larger scale process was performed in a pilot-scale bioreactor (BioFlo Pro, New Brunswick Scientific, USA) with a total volume of 150 L. The specifications of the bioreactors are summarized in Table 3. The fermentation temperature was controlled at 30°C. The pH of the medium was adjusted to 5.3 before autoclaving and was not controlled during fermentation. Dissolved oxygen (DO) was calibrated after autoclaving to 0 by nitrogen input and to 100% by air to its saturation point. During fermentation, dissolved oxygen was not controlled. Foaming was controlled by adding Antifoam 204 (Sigma Chemical Co., USA) before autoclaving at a concentration of 50 mg/L. Samples of the culture were withdrawn at 24 h intervals and examined for mutanase activity.

The main task of the microbial process scale-up is to transfer the biological processes effectively from the laboratory to production scale. Mutanase synthesis scale-up was performed on the basis of the working conditions determined for the 5 L bioreactor. However, the optima of aeration and agitation intensities in the 5 L bioreactor were set up based on the data presented by Bhattacharyya et al. (2008). They also demonstrated operation strategies for enzyme production by fungal strains. In the case of filamentous fungi, a constant impeller tip speed is often used as a scale-up criterion, because of the shear sensitivity of the microorganisms used (Amanullah et al., 2004). Here, the impeller tip speed (U_{Tip}) in the 5 L bioreactor has been calculated in a form involving the impeller agitation rate (N) and the diameter of the impeller (D):

$$U_{Tip} = \pi ND \tag{2}$$

An equal tip speed results when the small-scale stirrer speed N_1 is multiplied by the inverse geometric ratio of the impeller diameters D_1/D_2 to get the large-scale stirrer speed N_2:

$$N_2 = N_1\left(\frac{D_1}{D_2}\right) \tag{3}$$

However, the geometric dimensional similarities must be kept in the large-scale bioreactor in order to apply this scale-up criterion. The geometric dimensional similarity is expressed as follows:

$$\left(\frac{D_{T2}}{D_{T1}}\right) = \left(\frac{V_{L2}}{V_{L1}}\right)^{\frac{1}{3}} \tag{4}$$

where D_T is the tank diameter and V_L is the actual liquid volume. This assumes reasonably constant impeller geometry (impeller diameter (D_I) and number of impellers). As shown in Table 3, the geometric similarity parameter calculated for the actual working volume of the pilot scale bioreactor decreased as the scale increased. In order to bring the geometric similarity parameters of the larger bioreactors to that of the 5 L vessel, the actual working volumes of 150 L scale were decreased to 115 L. As a result, all the values of liquid height ratios for all the bioreactors used were comparable (Table 3).

Determination of the physical properties of fermentation broth

Viscosity measurements were carried out in a rotational rheometer Rheo Stress 1 (Haake, Germany) equipped with a temperature control unit DC 30 (Haake, Germany) and concentric cylinder geometry Z20 DIN Ti (Haake, Germany). Equipment control and

Table 3. Scale-up of bioreactors based on geometric similarity and constant tip speed

Parameter	Scale (L)	
	5	150
Nominal volume (m^3)	0.0066	0.15
Working volume according to the manufacturer (m^3)	0.005	0.120
Actual working volume (m^3)	0.005	0.115
Liquid height H_L (m)	0.249	0.721
Cross section area of bioreactor (m^2)	0.020	0.159
Bottom geometry	Spherical	Spherical
Tank diameter D_T (m)	0.160	0.450
Tank height H_T (m)	0.345	0.980
Impeller type	Rushton	Rushton
Impeller diameter D_I (m)	0.064	0.150
Number of impellers	2	2
H_T/D_T	2.156	2.178
D_I/D_T	0.400	0.333
H_L/D_T	1.555	1.602
Aeration rate (vvm)	1	1
Tip speed (m/s)	1.005	1.005
Stirrer speed (rpm)	300	128
Geometric similarity at V_L ($D_T/(V_L)^{1/3}$)	1	0.926

data handling was performed using RheoWin software version 3.40 (Haake, Germany). The shear stress was measured at the shear rate of 100 s^{-1}. The density of the fermentation broth was estimated by glass pycnometry at 30°C using water as reference.

Determination of the volumetric oxygen transfer coefficient (K_La)

The K_La was determined by the method described by Jin-Ho et al. (2007). In this method, the airflow into the bioreactor is temporarily stopped and the dissolved oxygen is stripped by nitrogen flux. After the DO (dissolved oxygen) concentration had been reduced to 0, air flowed into the fermenter at 1.0 vvm. The K_La could be then deduced, since it corresponded to the slope of the dissolved oxygen concentration rate as a function of the dissolved oxygen concentration:

$$\frac{dC_L}{dt} = K_L a\left(C^* - C_L\right) \quad (5)$$

Where, K_L and a are the oxygen transfer coefficient in the liquid phase and the specific surface of air bubbles. C_L and C^* are the dissolved oxygen concentration and its saturation value.

Mutanase assay

The standard mutanase assay mixture contained 0.5 mL of 0.2% (w/v) dextranase-pretreated mutan (DTM) in 0.2 M sodium acetate buffer (pH 5.5) and 0.5 mL of a suitably diluted enzyme solution. After 1 h incubation at 45 °C, the reducing sugars released were quantified by the Somogyi-Nelson method (Nelson, 1944; Somogyi, 1945). One unit of mutanase activity (U) was defined as the amount of enzyme hydrolyzing mutan to yield reducing sugars equivalent to 1 µmol of glucose/min and expressed as units per ml of culture (U/mL). 1 U corresponds to 16.67 nkat.

Preparation of dextranase-pretreated mutan (DTM)

Dextranase-pretreated mutan (DTM) was prepared (50 U of dextranase/mg of native mutan, pH 6.0, 37°C, 3 × 24 h) as a substrate for mutanase activity. Native mutan was synthesized from sucrose with the use of a mixture of crude glucosyltransferases of cariogenic *S. sobrinus*/*downei* CCUG 21020 as described previously (Wiater et al., 2005a). Dextranase of *Penicillium* sp. with an enzyme activity of 12.9 U/mg preparation was purchased from Sigma-Aldrich (St. Louis, USA). The linkage structure of the native and the dextranase-pretreated mutan determined by ^{1}H NMR showed that they were mixed-linkage α-(1→3) and α-(1→6) biopolymers with a greater proportion of α-(1→3) to α-(1→6) linkages, namely, 59.1 and 40.9 mol % for native mutan and 79.8 and 20.2 mol % for DTM, respectively.

RESULTS AND DISCUSSION

Placket-Burman design

The Pareto Chart illustrates the order of significance of the variables affecting mutanase production by *T. harzianum* (Figure 1). All the examined features, except for the age of the inoculum, significantly influenced mutanase production. The first three factors in the rank of the absolute value of estimators were chosen for the

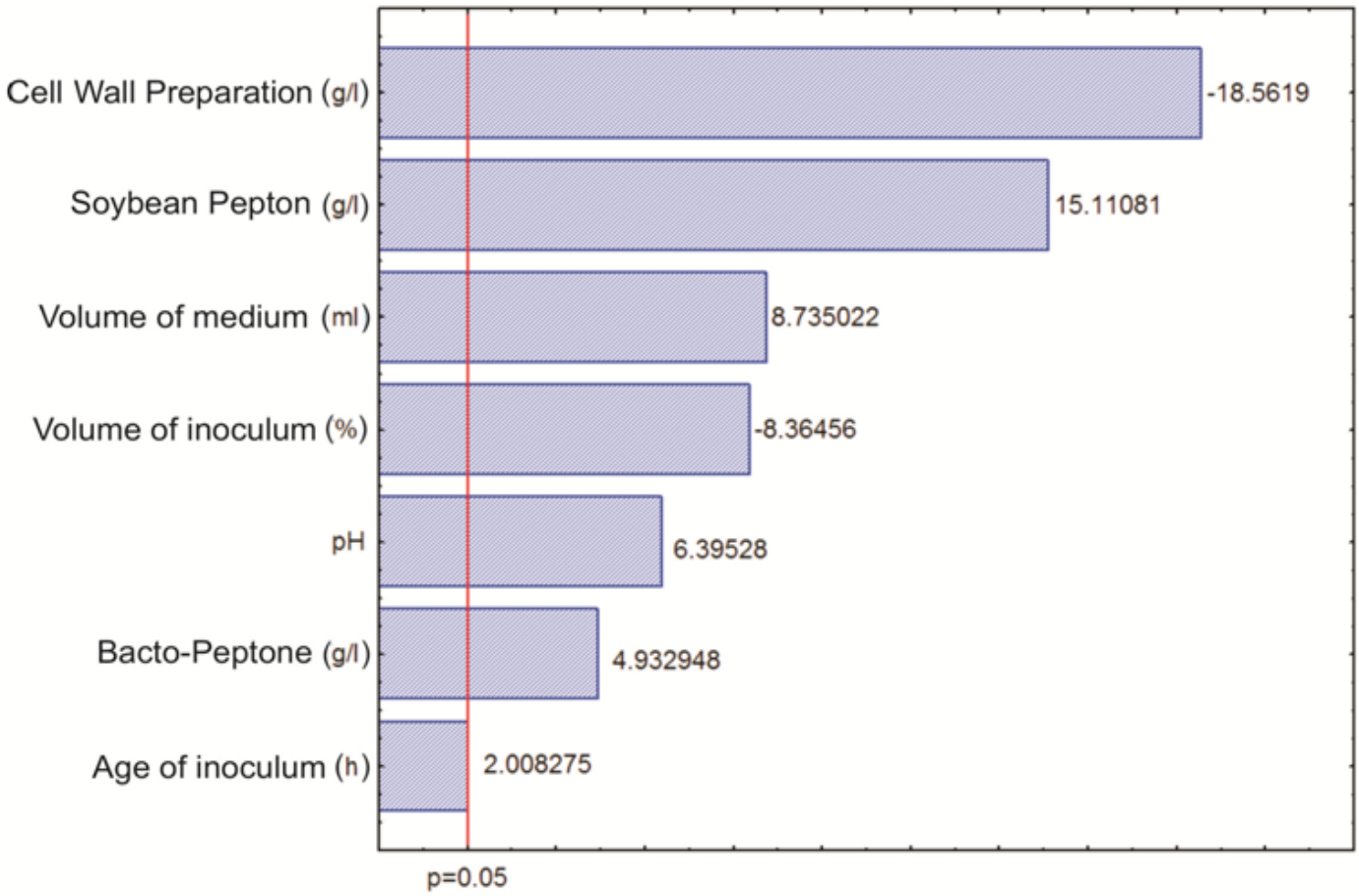

Figure 1. Pareto chart for the Plackett-Burman design for mutanase biosynthesis by *T. harzianum.*

further steps of the analysis (CWP, soybean peptone, and volume of the medium). The cell wall preparation from *L. sulphureus* (CWP) is rich in α-(1→3)-glucans; it effectively induces mutanase activity and fully substitutes other inducers when used as a sole carbon source in the culture medium (Wiater et al., 2008). CWP used as a mutanase inducer had the highest absolute value; however, its influence on the enzyme production was negative (-18.5619), which indicates that an excessive concentration of this substrate can reduce production of the enzyme. This finding is in agreement with our previous study, which indicated that an increase in the CWP concentration in the culture medium above 0.5 % markedly decreased the mutanolytic activity, and at the CWP dose of 2 %, the mutanase yield declined by 81 % in comparison with that obtained with 0.4% CWP (Wiater et al., 2008). Soybean peptone had the highest positive effect (15.1108) on mutanase production. It provided a source of nitrogen for fungal growth and enzyme production, which resulted in a higher yield than that reported for other organic nitrogen sources (Wiater et al., 2005b). An additional advantage of the use of this nitrogen source is the fact that soybean peptone is free of allergens and animal pathogens (Hemmer et al., 2011). This may prove significant for mutanase production on the industrial scale. The third important variable for mutanase production is the volume of the growth medium (8.735), which is connected with the aeration of the culture. *Trichoderma harzianum* is an aerobic filamentous fungus, which absolutely needs oxygen as a substrate.

Therefore, the ratio of the flask size and volume of the medium becomes a restricting element in the liquid state culture of the aerobic mycelium. Due to the morphology of the mycelium, oxygen transfer is not easily built up (Jin-Ho et al., 2007). Additionally, increasing the agitation rate can improve mycelium aeration.

The magnitude of the effects indicates the level of significance of the variables with respect to mutanase production. Consequently, based on the results of the experiment, statistically significant variables, i.e. CWP, soybean peptone, and the medium volume, which had a positive effect on mutanase production were further investigated with a central composite design to find the optimal range of these variables.

`**Table 4.** Analysis of variance for the current regression model.

Source of variation	SS	df	MS	*F*-value	*p*-value
Model	1.439	5	0.288	97.4	< 0.0001
X_1-CWP	0.059	1	0.059	20.1	0.0005
X_2-Soybean Peptone	0.019	1	0.019	6.5	0.0236
$X_1 \times X_2$	0.559	1	0.559	189.5	< 0.0001
X_1^2-CWP^2	0.279	1	0.279	94.5	< 0.0001
X_2^2-Soybean Peptone^2	0.565	1	0.565	191.5	< 0.0001
Residual	0.041	14	0.003		

SS - sum of squares, df – degree of freedom, MS - mean square.

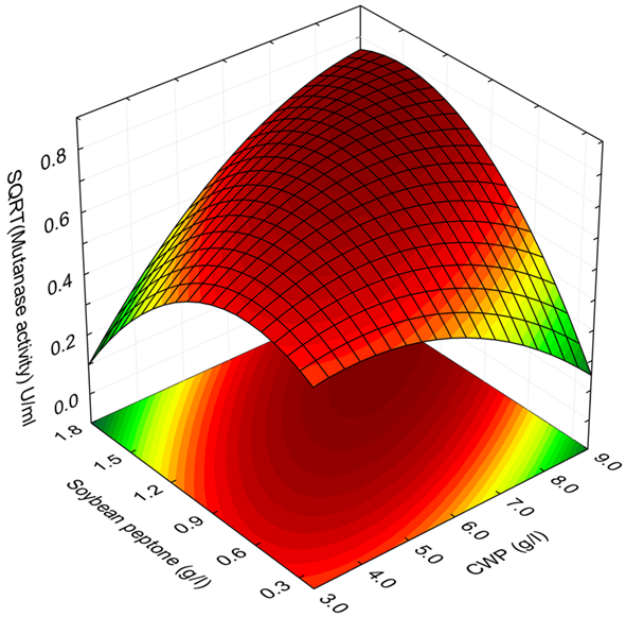

Figure 2. Three dimensional response surface plot for the effect of CWP (X_1), soybean peptone (X_2) on mutanase production by *T. harzianum.*

Central composite design

To examine the combined effect of the selected factors, a full-factorial central composite design of RSM (Response Surface Methodology) containing six central points and star points was applied to maximize the mutanase production. Table 2 presents the design with the level of variables and the values of mutanase activity observed. Run 7 shows maximum mutanase production reaching 0.654 U/mL (CWP - 0.9%, soybean peptone - 0.18%, medium volume - 80 mL). The regression analysis of mutanase activity showed that X_1 and X_2 in linear and quadratic terms was highly significant, giving an overall curvilinear effect. The changes in the medium volume (X_3) were found to be insignificant at the probability level of 95%; hence, they were removed from the model by the backward elimination procedure of ANOVA. Besides the above-mentioned factors, the interaction effect between the X_1 and X_2 was also found to be significant ($p < 0.0001$). Based on the results of CCD design analysis, the following quadratic regression function for the original factor values was determined:

$$\sqrt{Y} = 0.321 + 0.096X_1 - 0.0153X_1^2 + 0.124X_2 - 0.367X_2^2 + 0.110X_1X_2 \quad (6)$$

The model was also very well fitted to the experimental data and explained more than 96% of the whole variation of the response (adjusted R^2 = 0.962). The results of ANOVA indicate statistical significance of the model (Table 4).

The 3D response surface plots show graphical representations of the equation (6). The changes in the response value are represented by different colors from green, describing the minimum, to red, describing the maximum of the predicted value (Figure 2).

The final goal of the present study was to find operating conditions (CWP and soybean peptone) that maximize mutanase activity. In this work, the statistical CCD model made it possible to design a scale-up optimization experiment. It was evident that mutanase production was mainly influenced by the CWP concentration of 4 - 8 g/L and the soybean peptone concentration of 0.6 - 1.4 g/L. Using the model, the optimal condition for mutanase production was obtained when CWP was at the concentration of 8.08 g/L, soybean peptone 1.38 g/L, and medium volume 140 mL. Under optimum conditions, the maximum predicted response of mutanase activity was 0.631 U/mL.

Validation of the experimental model and process scale-up

In order to confirm the adequacy of the regression model set on the basis of the experimental data obtained,

validation cultures were grown in conditions created on the basis of optimization. The cultures were grown in shaken flasks and bioreactors with working volumes 5 and 115 L. The highest mutanase activity (0.747 U/mL) was observed in the flask cultures (data not shown). Compared with the mutanase activity from the culture in non-optimized conditions, a 5% increase was observed (Wiater et al., 2008). The activity obtained during the validation experiment was higher by 33% from the maximum value recorded during the tests within the central composite design. On the other hand, the maximum activity of the enzyme in the 5 L bioreactor was 0.734 U/mL. As shown in Figure 3, after the third day of the culture in the 5 L bioreactor, 90% of maximum mutanase activity was achieved and the highest value was recorded on the seventh day. These data indicate a 70.6% increase in mutanase production, in comparison with the enzyme activity from the culture in the 5 L bioreactor before the optimisation experiment (data not shown). The increase in the process scale to the volume of 115 L caused a slight decrease in mutanase activity. The highest activity, that is, 0.682 U/mL, recorded on the fourth day of the process, was approx. 7% lower than the activity of mutanase observed in the case of the 5 L bioreactor culture. However, all the above results at each scale showed that within the measurement variations for the biological system, the pH, viscosity, K_La, and mutanase activity across the scales appeared to be comparable. The presented data clearly indicate that the culture could be scaled-up successfully from shaken flasks to the bioreactor scale.

The comparison of process performance across the scales revealed that the shaken flask process exhibited slightly higher mutanase activity. One may speculate that this outcome may be to some extent determined by the negative impact of shearing forces generated as a result of agitation, which causes unfavourable changes in cell morphology. According to Amanullah et al. (2004), in fungal fermentation, engineering variables such as agitation conditions require attention due to their effect on the morphology, which in some cases can affect productivity. A decrease in productivity resulting from the impact of shearing forces on the cells was observed in the case of synthesis of penicillin by *Penicillium chrysogenum* (Smith and Lilly, 1990) and pigments by *Monascus* (Kim et al., 2002). In the case of studies presented by Kim et al. (2002), increasing the stirrer rotation speed from 350 to 550 rpm resulted in a 10-fold increase in pigment production efficiency. However, the pigment yields at 700 rpm were reduced to approximately 55% of the yield at 500 rpm. A study by Li et al. (2002) documented a similar effect in large-scale (80 m^3), fed-batch *Aspergillus oryzae* fermentations. It must be noted that fungal cultivation processes are challenging due to the presence of a variety of interconnected factors. Agitation conditions require attention also due to their effect on the oxygen transfer and dissolved oxygen tension, which in many cases can affect productivity. This hypothesis has also been substantiated by the investigations of Amanullah et al. (2002, 2004), and Cui et al. (1998). In general, under the conditions of strong agitation, small pellets and short mycelia are formed. If the cells in the culture broth exist in the form of mycelia with short branches or compact pellets, the viscosity of the culture broth can be maintained at low levels, resulting in high K_La values (Kim et al., 2002). Data presented in Figure 3 allow a conclusion that the limited oxygen transport related to the increased viscosity of the medium is a probable cause of minor differences in mutanase activity (Figure 4). The viscosity was inversely proportional to the volumetric mass transfer coefficient, K_La (Figure 3). The correlation coefficient between these parameters in the case of the 5 L and 115 L reactors amounted to -0.81 ($p=0.015$) and -0.79 ($p=0.021$) respectively. Air bubbles were more likely to be clumped together rather than dispersed, even with agitation, leading to the reduction of the K_La value. The presented results suggest that further studies should aim at optimization of conditions in terms of not only agitation speed but also bioreactor construction. The hydrodynamic conditions during fermentation are also important due to the medium composition and particularly due to the presence of Triton X100. This substance can be positive because it disperses CWP and is conducive to intense foam formation during aeration and agitation. During the study, we observed that a layer of foam containing both cells and CWP was formed even though a small amount of anti-foaming agent was added. This is an unfavourable phenomenon because it results in decreasing the inductor concentration in the medium, which causes a drop in mutanase production. Foam reduction can be performed with the use of mechanical foam breakers or addition of antifoams. However, excessive addition of chemical antifoams may markedly decrease the value of K_La and thus product formation (Martin et al., 1994). Hence, in the present study a small amount of anti-foaming agent was added. Probably, the mechanical foam breakers should be used in foam control to avoid the drawbacks associated with the use of chemical antifoams. Further work is still needed to resolve this problem.

Conclusion

Using the RSM optimization of mutanase production by *T. harzianum* strain CCM F-340, the highest enzyme activity was achieved at the Mandels medium modified in terms of carbon and nitrogen sources: CWP 8.08 g/L, and soybean peptone 1.38 g/L and under the following culture conditions: temperature 30°C, pH 5.3, agitation 270 rpm, and medium volume 140 mL. To sum up, mutanase activity in a shaken flask CCD experiment was

5% higher than before optimization. However, the validation experiment in bioreactor scale showed a 70.6% increase in production of mutanase compared to the culture in the bioreactor before optimization. This indicates that *T. harzianum* in optimal conditions in large culture volume could be a highly effective extracellular mutanase source. However, our experimentally observed mutanase activity can still be further improved *via* optimization of fermentation conditions. This involves development of a novel fermentation mode (that is, fed-batch vs. continuous) and the type of bioreactor construction (i.e. airlift bioreactor).

Conflict of Interests

The author(s) have not declared any conflict of interests.

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