# Optimization of a culture medium for xylanase production by *Bacillus* sp. using statistical experimental designs

P.L. Pham, P. Taillandier*, M. Delmas and P. Strehaiano

**The concentrations of oat spelt xylan, casein hydrolysate and $NH_4Cl$ in the culture medium for production of xylanase from *Bacillus* sp. I-1018 were optimized by means of response surface methods. The path of steepest ascent was used to approach the optimal region of the medium composition. The optimum composition of the nutrient medium was then easily determined by using a central composite design and was found to be 3.16 g/l of xylan, 1.94 g/l casein hydrolysate, 0.8 g/l of $NH_4Cl$. The xylanase production was increased by 135% when the strain was grown in the optimized medium compared to initial medium.**

*Key words*: *Bacillus* sp. I-1018, central composite design, full factorial design, path of steepest ascent, xylanase.

Endo-1,4-$\beta$-xylanase is an enzyme which hydrolyses xylan, a main component of hemicellulose. The markedly increased interest in xylanases during the last few years is mainly due to the potential use of this enzyme in the pulp and paper industry (Viikari *et al.* 1994; Pham *et al.* 1995). Xylanases are produced by numerous microorganisms among which the fungi are the most potent producers. Of bacteria, *Bacillus* sp. I-1018 (Samain *et al.* 1992) has been reported to be thermophilic and produced thermostable xylanases.

Several different strategies to enhance enzyme production in various microorganisms have been successfully applied (Auden *et al.* 1967; Poonsuk & Doelle 1987; Linko & Zkong 1991). Among them, optimization of the culture medium has proved its efficiency. However, little information is available on complete optimization of culture media. In general, medium optimization by the traditional 'one-factor-at-a-time' technique was used (Gokhade *et al.* 1991). This method is not only time-consuming but also often leads to an incomplete understanding of the behaviour of the system, resulting in confusion and a lack of predictive ability. Some of this confusion may be avoided with the application of properly designed experiments and adequate multifactor models. Recently, a number of statistical experimental design methods have been employed in enzyme production (Gomes *et al.* 1989; Chen *et al.* 1992; Purkarthofer *et al.* 1993; Gomes *et al.* 1994).

The aim of this work was to combine different statistical experimental designs to optimize a culture medium for the production of xylanase by *Bacillus* sp. I-1018.

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## Materials and Methods

*Organism*

*Bacillus* sp. I 1018 from CNCM (Collection Nationale de Cultures de Microorganismes, Institut Pasteur, France) was used in this study. The strain was stored at 18 °C in culture medium with 10% (v/v) glycerol. The medium is the same as in inoculum cultivation cited in the following section.

*Shake Flask Culture*

All the cultures were grown in 250 ml Erlenmeyer flasks containing 50 ml of medium with various concentrations of oat spelt xylan (Sigma), casein hydrolysate and $NH_4Cl$ according to the experimental design. Each medium contained a fixed amount of $MgSO_4 \cdot 7H_2O$ (0.25 g/l), $K_2HPO_4$ (2.3 g/l), $NaH_2PO_4$ (0.6 g/l), 10 ml each of trace element and vitamin solution; the trace element solution contained (in g/l): nitrilotriacetic acid (NTA) 12.8, $FeCl_3 \cdot 6H_2O$ 1.35, $MnCl_2 \cdot 4H_2O$ 0.1 $CoCl_2 \cdot 6H_2O$ 0.024,

$CaCl_2 \cdot 2H_2O$ 0.1, $ZnCl_2$ 0.1, $CuCl_2 \cdot 2H_2O$ 0.025, $H_3BO_3$ 0.01, $Na_2MoO_4 \cdot 2H_2O$ 0.024, NaCl 1.0, $NiCl_2 \cdot 6H_2O$ 0.12, $Na_2SeO_3 \cdot 5H_2O$ 0.026; the vitamin solution contained (in mg $l^{-1}$) biotin 2, folic acid 2.0, pyridoxine HCl 10.0, thiamine HCl 5.0, riboflavin 5.0, nicotinic acid 5.0, DL calcium panthotenate 5.0, vitamin $B_{12}$ 0.1, *p* aminobenzoic acid 5.0, lipoic acid 5.0. All experiments were repeated in triplicate. The pH of the medium was adjusted to 7.3 with 1M NaOH prior to autoclaving (121 °C, 15 min). Inocula were grown for 18 h at 50 °C, 250 rev/min on the same medium as in shake flask cultures except for xylan, casein and $NH_4Cl$ concentrations which were 5, 1.5 and 0.8 g/l, respectively. Aliquots of 50 ml of enzyme production medium were placed in 250 ml conical flasks and seeded with inoculum to an initial concentration of about $3 \times 10^6$ bacteria/ml. The experiments were carried out in a water bath with a magnetic agitator at 50 °C and 250 rev/min.

*Analytical Methods*

Growth was monitored by measuring the optical density of the culture at 660 nm in a glass chamber 2 mm thick using an Hitachi U 2000 Spectrophotometer. A 'Petit Salumbéni' haemocytometer was used for direct cell counting. The number of cells per grid square was counted using a microscope (× 400 magnification). The cultures were centrifuged at 14,000 rev/min for 15 min at 17 °C and the supernatant fluids were stored at 18 °C until assay. Reducing sugars were determined according to the DNS method (Miller 1959).

*Enzyme Assay*

In this study, 'xylanase' refers to the total enzymatic activity present in the culture filtrate that contributes to the release of reducing sugars from purified birchwood xylan. $\beta$ Xylanase was assayed as described by the method of Bailey *et al*. (1992) using 1% birchwood xylan (Sigma, lot 113H0900) as substrate. The enzyme activities were determined at 60 °C and pH 5.8 using Britton Robinson universal buffer. Xylanase was expressed in nkatal (1 nkat is the amount of enzyme that can catalyse the release of 1 nmol of product in 1 s under specified conditions). Overall cellulolytic activity was assayed as Filter Paper Units (FPase) and endocellulase activity was assayed as carboxymethylcellulase (CMCase) according to the IUPAC standard instruction (Ghose *et al*. 1987) using filter paper (Whatman No. 1) and carboxymethylcellulose.

*Experimental Design*

*Full Factorial Design (FFD).* In the first phase, a FFD was used to approach the optimal region. It is known that the FFD method estimates the main effects of factors and their interactions simultaneously (Khuri & Cornell 1987). In addition, FFD is efficient in determining the path of steepest ascent to approach the neighborhood of the optimum response. It is therefore particularly adapted to the initial stages. For a $2^3$ FFD with three factors at two levels, eight experimental runs are required.

*Central Composite Design (CCD).* The CCD for investigating a number ($k$) of factors consists of two parts: (1) a basic two level ($2^k$) factorial design from which linear and interaction effects can be determined; (2) an interposed secondary arrangement of a centre with $2k$ extended points provides the necessary information for estimating curvilinear effects.

Upon completion of the experiments, a second order equation is then fitted to the data by a standard multiple regression procedure. This results in an empirical model which relates the response measured to the independent factors of the experiment. For a two factor system the model is (equation 1):

$$y \quad b_0 + b_1 x_1 + b_2 x_2 + b_{12} x_1 x_2 + b_{11} x_1^2 + b_{22} x_2^2 \qquad (1)$$

where $y$, predicted yield; $b_0$, intercept; $b_1$, $b_2$, linear coefficients; $b_{11}$, $b_{22}$, quadratic coefficients.

Evaluation of this surface indicates where optimum conditions exist within the experimental area covered, or in what direction further experiments are necessary to achieve better results.

STATGRAPHIC, version 4.0, was used for the regression analysis of the experimental data obtained. The significance of the regression coefficients was tested by a Student $t$ test. This test, based on the hypothesis that the true parameter is zero, was employed in the multiple regression to elucidate the significance of the factors. If the $t$ value is greater than $t_{1-\alpha, \lambda}$ for a significant level $\alpha$, with $\lambda$ degree of freedom, the term contributes a significant effect to the response.

The levels of significance were given as *** $P < 0.01$, ** $P < 0.05$, and * $P < 0.10$. The quality of the fit of the polynomial model equation was expressed by the coefficient of determination $R^2$ and its statistical significance was determined by a Fisher $F$ test. Differentiation calculation was then employed for predicting the optimum point.

## Results and Discussion

The first step in the process of seeking optimum conditions is to identify the input variables that have the greatest influence on the response. From preliminary experiments (data not shown) made in order to examine the effects of different medium components on the xylanase production, we chose the three factors which played the most important role in the xylanase synthesis. These were the concentrations of oat spelt xylan, casein hydrolysate and $NH_4Cl$.

In another study, a series of experiments to determine the ranges of concentrations of the three nutrients was conducted by using the 'one factor at a time' method. The results shown in Figure 1 indicate that the optimum for xylanase synthesis should be near the following concentrations (g/l): oat spelt xylan 5; casein 1.5; $NH_4Cl$ 0.8. Xylanase activities were determined after 30, 48 and 72 h of incubation.

*Phase 1 $2^3$ FFD Experiment*

The application of the Box Wilson method with three factors at two levels involves eight combinations. Xylanase yields were determined after 30 h, 48 h and 72 h of incubation. Because the results obtained after 48 h and 72 h did not markedly differ from 30 h, they are not presented here. Table 1 shows the values of variables at different levels of the FFD.

The experimental design and the results of the FFD are illustrated in Table 2.

The values of the regression coefficients were calculated and an equation of the first order (equation 2) can be written:

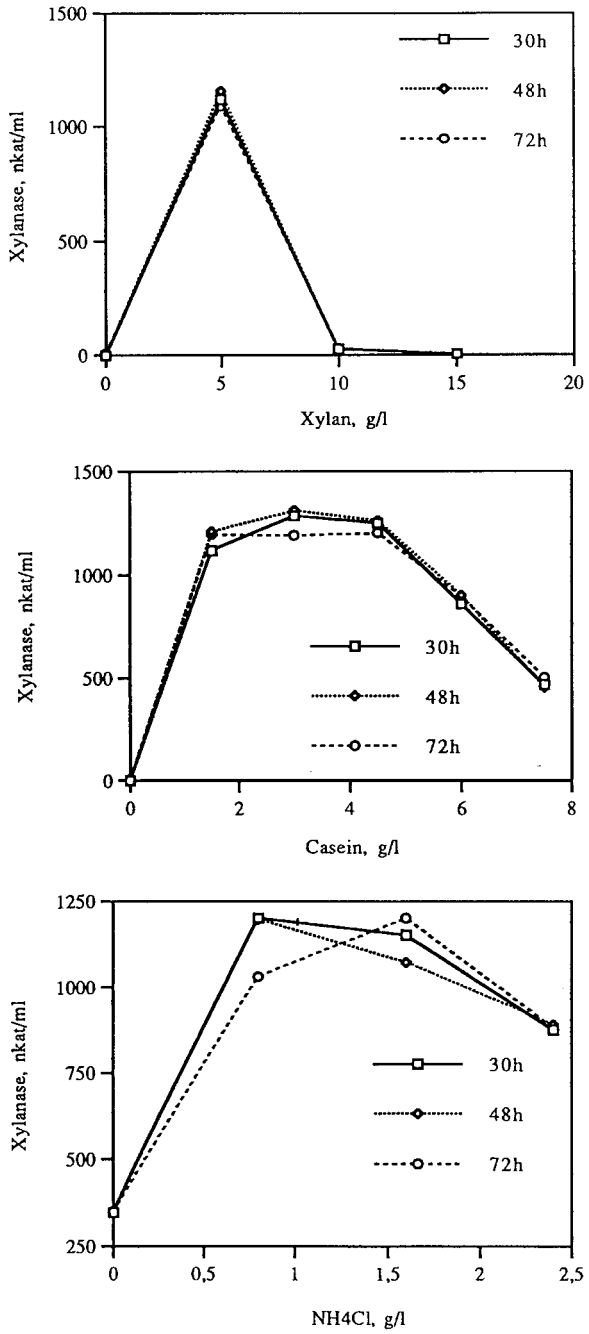


**Figure 1.** The effect of different concentrations of three medium components on xylanase production.

$$y = 941.34 - 829.54x_1 + 186.21x_2 + 43.21x_3 - 88.11x_1x_2 - 60.91x_1x_3 + 4.73x_2x_3 \quad (2)$$

Analysis of variance (ANOVA) in Table 3 showed that xylan and casein hydrolysate proved to be the two most important components of the medium for xylanase formation. Although the coefficient $b_3$ for $NH_4Cl$ was not significantly different from zero ($\alpha = 0.05$), it was not omitted from this model in order to avoid overlooking a factor that could be important. The factors xylan and casein hydrolysate were found to be significant at the probability level of $\alpha = 0.05$. Increasing or decreasing the concentrations of the respective factors, according to the signs of its main effects, should have a positive consequence for the formation of xylanase by *Bacillus* sp. I-1018. The coefficient of determination $R^2$ of the model was calculated to be 0.99. This indicates that the model explains 99% of the variability in the data. The statistical significance of the model equation was also confirmed by an *F*-test, which was 129.02. The model was found to be adequate to the data at a probability level of $\alpha = 0.05$.

*Phase 2 the Path of Steepest Ascent*

Based on the first-order model equation obtained, the path of steepest ascent was determined to find the proper direction of changing variables: decreasing the concentration of xylan and increasing the concentration of

**Table 1. Concentration of variables at different levels of the FFD.**

| Factors (g/l) | Xylan ($x_1$) | Casein ($x_2$) | $NH_4Cl$ ($x_3$) |
|---|---|---|---|
| Lower level ( 1) | 2.5 | 1 | 0.3 |
| Base level (0) | 5 | 1.5 | 0.8 |
| Upper level (+1) | 7.5 | 2 | 1.3 |

**Table 2. Experimental design and results of the FFD.**

| Trial | Code level | | | XA (nkat/ml) | |
|---|---|---|---|---|---|
| | $x_1$ | $x_2$ | $x_3$ | Observed | Predicted |
| 1 | 1 | 1 | 1 | 1428 | 1397.2 |
| 2 | 1 | 1 | 1 | 5.3 | 36.1 |
| 3 | 1 | 1 | 1 | 1905.5 | 1936.34 |
| 4 | 1 | 1 | 1 | 253.7 | 222.86 |
| 5 | 1 | 1 | 1 | 1565.1 | 1595.94 |
| 6 | 1 | 1 | 1 | 22.1 | 8.73 |
| 7 | 1 | 1 | 1 | 2184.9 | 2154.1 |
| 8 | 1 | 1 | 1 | 166.1 | 196.9 |
| 9 | 0 | 0 | 0 | 925.4 | 941.3 |
| 10 | 0 | 0 | 0 | 942.6 | 941.3 |
| 11 | 0 | 0 | 0 | 938.4 | 941.3 |

**Table 3. Results of the regression analysis of the FFD.**

| Term | Coefficient | *t* value | Significance level |
|---|---|---|---|
| Intercept | 947.33 | 30.52 | 0.0208** |
| $x_1$ | 829.54 | 26.9 | 0.0237** |
| $x_2$ | 186.21 | 6.04 | 0.1045* |
| $x_3$ | 43.21 | 1.40 | 0.3946 |
| $x_1x_2$ | 88.11 | 2.85 | 0.2143 |
| $x_1x_3$ | 60.91 | 1.97 | 0.2983 |
| $x_2x_3$ | 4.73 | 0.15 | 0.9030 |

$R^2$ = 0.9987; $R^2$ (adj. for d.f) = 0.9910; *F* ratio = 129.024.

**Table 4. Design of experiments to obtain the ascent and corresponding xylanase yields.**

| Maximum number | $x_1$ | $x_2$ | XA (nkat/ml) |
|---|---|---|---|
| 1 | 5 | 1.5 | 1004 |
| 2 | 4.5 | 1.6 | 1254 |
| 3 | 4 | 1.7 | 1570 |
| 4 | 3.5 | 1.8 | 2103 |
| 5 | 3 | 1.9 | 2380 |
| 6 | 2.5 | 2.0 | 1992 |
| 7 | 2 | 2.1 | 99 |

casein to improve xylanase yield. The concentration of $NH_4Cl$ was fixed at 0.8 g/l. Table 4 illustrates how the new variables should be oriented.

The design of the experiment of ascent illustrated in Table 4 showed that xylan was decreased serially by 0.5 g/l, casein increased by 0.1 g/l. It is clearly seen that the yield plateau has been reached at medium 5. This medium was chosen for the experiments described below.

*Phase 3 Central Composite Design Experiment*

As seen above, by determining the path of steepest ascent, the neighbourhood of the optimum response seems to be approached. The optimal concentration of media components was determined using a CCD with the two variable carbon and nitrogen sources. The levels of the two variable factors and experimental results are presented in Table 5. The fitted equation for estimation of xylanase yield had the following form:

$$y = 2388.04 + 133.67x_1 + 114.7x_2 + 4.23x_1x_2 - 206.39x_1^2 - 143.55x_2^2 \quad (3)$$

**Table 5. Second-order central composite design and experimental results.**

| Run No. | Xylan (g/l ($x_1$)) | Casein (g/l ($x_2$)) | XA (nkat/ml) | |
|---|---|---|---|---|
| | | | Observed | Predicted |
| 1 | 2.5 ( 1) | 1.8 ( 1) | 1756.5 | 1793.9 |
| 2 | 3.5 (+1) | 1.8 ( 1) | 2061.8 | 2052.8 |
| 3 | 2.5 ( 1) | 2.0 (+1) | 1992.5 | 2014.9 |
| 4 | 3.5 (+1) | 2.0 (+1) | 2314.7 | 2290.7 |
| 5 | 2.3 ( 1.414) | 1.9 (0) | 1825.9 | 1786.4 |
| 6 | 3.7 (+1.414) | 1.9 (0) | 2138.3 | 2164.4 |
| 7 | 3 (0) | 1.75 ( 1.414) | 1956.2 | 1938.8 |
| 8 | 3 (0) | 2.05 (+1.414) | 2259.3 | 2263.2 |
| 9 | 3 (0) | 1.9 (0) | 2441 | 2388.0 |
| 10 | 3 (0) | 1.9 (0) | 2325 | 2388.0 |
| 11 | 3 (0) | 1.9 (0) | 2517 | 2388.0 |
| 12 | 3 (0) | 1.9 (0) | 2341 | 2388.0 |
| 13 | 3 (0) | 1.9 (0) | 2315 | 2388.0 |

From equation (3), it was shown that the signs of $b_{11}$ and $b_{22}$ were both negative, so the parabola would be open downward (and suggested to have a maximum point). The coefficient $b_{12}$ was found not to be significantly different from zero at a significance level of $\alpha = 0.05$ (Table 6); nevertheless, this coefficient was not dismissed as unnecessary in the regression equation, since there was no reason for making the hypothesis $b_{12} = 0$.

We then had to determine the model adequacy. First, the statistical significance of the models was determined by the *F*-test. The test was made by the comparison of two variances: the pure error variance $s_p^2$ and the lack of fit variance $s_{lf}^2$. After calculation of these values, we obtained for the ratio $F = s_p^2/s_{lf}^2$ the value $F = 24.97$. Since this value was more than the critical value of $F$ at the level of 0.05, $F_{0.05}(5,7) = 3.97$, the obtained model was adequate.

The results of the regression analysis are shown in Table 6.

Second, the goodness of fit of the polynomial model was given by the coefficient of determination $R^2$ (adjusted for df), which was calculated to be 0.91, indicating that 91% of the variability in the response could be explained by the model. This indicates that equation (3) provided a suitable model for the response surface of the experiment of enzyme production. Figure 2 shows a three-dimensional diagram of the calculated response surface. It is reconfirmed that the fitted surface has a true maximum. This can also be seen in Figure 3, where the model equation is shown as a surface plot. The contour plot of the same equation shows a rather broad plateau region in which the activities change relatively little when the nutrient concentrations are varied. This indicated that the optimal solution can accommodate small errors or variability in the experimental factors.

From equations derived by differentiation of equation (3), we can obtain the maximum point of the model, which was 3.16 g of xylan/l; 1.94 g of casein/l. The model predicted a maximum response of 2.433.1 nkat/ml for this point. To confirm the results, experimental rechecking was done by shake flask experiments using a medium representing this maximum point and a value of $2.401.2 \pm 146.2$ nkat/ml$^{-1}$ ($N = 3$) was obtained. The good correlation between these two results verifies the

**Table 6. Analysis of variance for the full regression of CCD.**

| Term | Coefficient | *t* value | Significance level |
|---|---|---|---|
| Intercept | 2388.04 | 74.55 | 0.0000*** |
| $x_1$ | 133.67 | 5.27 | 0.0012*** |
| $x_2$ | 114.70 | 4.53 | 0.0027*** |
| $x_1x_2$ | 4.23 | 0.12 | 0.91 |
| $x_1^2$ | 206.39 | 7.59 | 0.0001*** |
| $x_2^2$ | 143.55 | 5.28 | 0.0011*** |

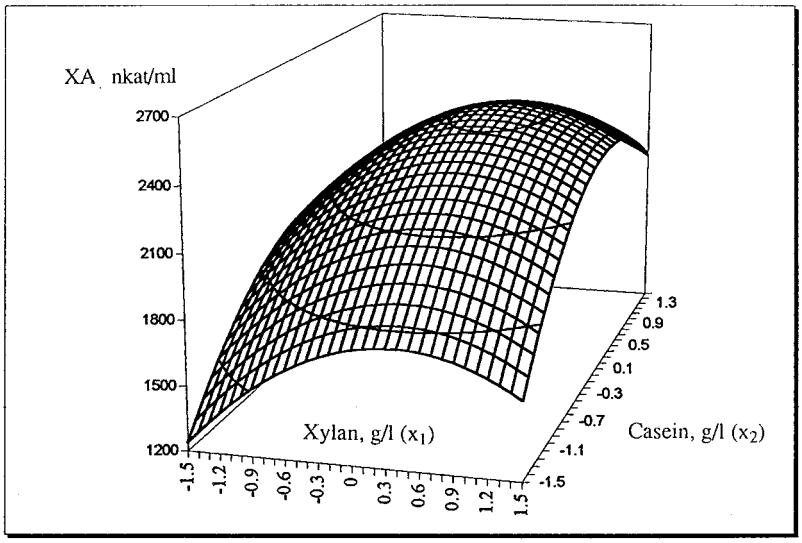


**Figure 2.** Surface plot of the central composite design experiment.

validity of the response model and the existence of an optimal point. The crude xylanase in the culture filtrate produced on xylan was analysed for xylanolytic and other enzyme activities and soluble protein (Table 7). No cellulase activity was detectable which makes this organism desirable for application in biobleaching.

In the conventional medium proposed by the Pasteur Institute, the carbon source was oat spelt xylan, the nitrogen sources were yeast extract and $NH_4Cl$. In a preliminary study we searched for the best carbon and nitrogen sources, which appeared to be, respectively, oat spelt xylan and casein hydrolysate; $NH_4Cl$ was found to be necessary. Statistical methods for medium optimization proved to be a powerful and useful tool for biotechnology. Xylanase production by *Bacillus* sp. I-1018 could be increased by 135% when the strain was grown on the medium that was developed by means of statistical design compared with conventional medium composition obtained from the common 'one-factor-at-a-time' method. Indeed, for the medium we proposed the

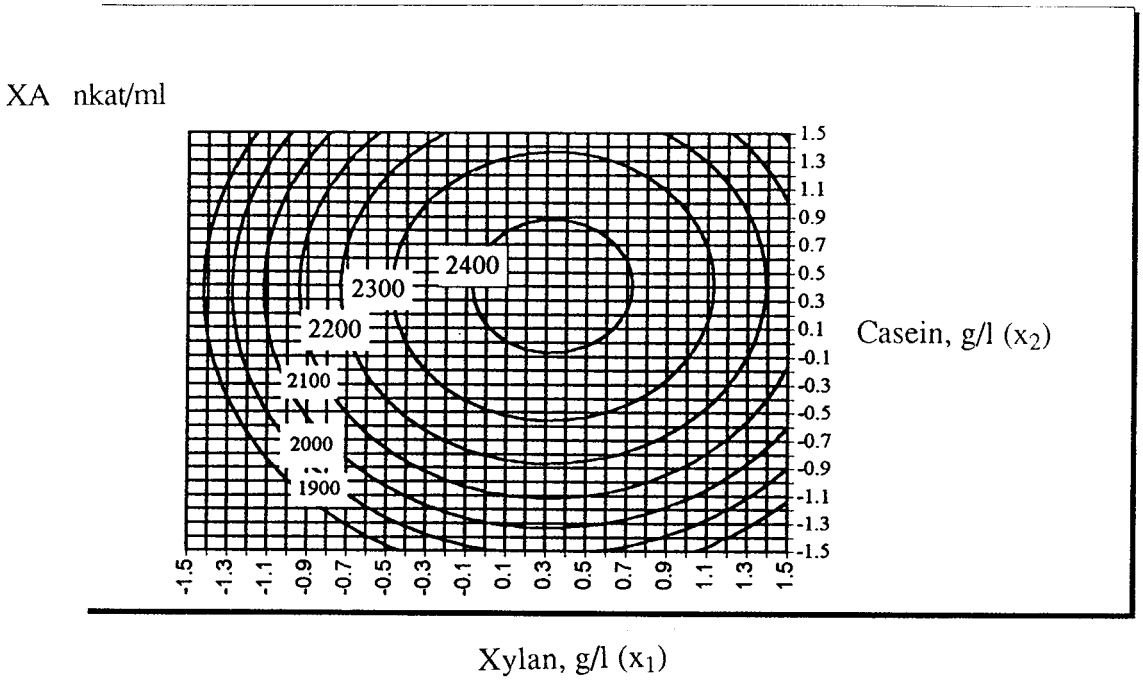


**Figure 3.** Contour plot of the calculated response surface.

**Table 7. Properties and other enzyme activities of xylanase produced by *Bacillus* sp. I-1018 on xylan.**

| | |
|---|---|
| $\beta$ Xylanase | 2,300 2,500 nkat/ml |
| CMC ase | Not detectable |
| FPase | Not detectable |
| Soluble protein | 127.2 mg/l |
| Reducing sugars | 0.04 g/l |
| Specific xylanase activity | Approximately 18867 nkat/mg protein |

concentration of the important components were optimized: they were all decreased especially the xylan concentration reduced from 5 to 3.16 g/l. This is interesting from an economic point of view when considering the price of this substrate.

Since *bacilli* are aerobic organisms, the next step for enhancing xylanase production should be the study of aeration conditions in bioreactors with the proposed medium.

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