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ARTICLE

Parametric optimization of submerged fermentation conditions for xylanase production by *Bacillus cereus* BSA1 through Taguchi Methodology

Asish Mandal[1], Sanjay Kar[2], Tapan Dutta[3], Bikas Ranjan Pati[4], Keshab Chandra Mondal[4], Pradeep Kumar Das Mohapatra[4]*

[1]Post Graduate Department of Botany, Ramananda College, Bishnupur, Bankura, West Bengal, India
[2]Post Graduate Department of Botany, Midnapore College, Midnapore -721 102, West Bengal, India
[3]Teachers' Training Department, Panskura Banamali College, Panskura -721152 , Purba Medinipur, West Bengal, India
[4]Department of Microbiology, Vidyasagar University, Medinipur – 721102, West Bengal, India

ABSTRACT Extracellular xylanase production of *Bacillus cereus* BSA1 was optimized under submerged fermentation using Taguchi orthogonal array (OA). An L_{18} layout of OA was constructed at three-levels of six factors, *i.e.* temperature, pH, and xylan, Na_2HPO_4, NH_4NO_3 and NaCl concentrations, influencing the xylanase synthesis. The enzyme production was studied in 18 parallel batch systems using different levels of each factor. The results were processed with Qualitek-4 software using 'bigger is better' quality character, and combination of 35 °C; pH 6.0; and xylan 0.5; NH_4NO_3 0.5, Na_2HPO_4, 0.1; NaCl 0.05 concentrations (in w/v %) with a predictive xylanase production of 7.404 U/ml was obtained. Fermentation experiment was performed for further validating the statistical output, and it resulted 10.24% in the xylanase yield (from 6.44 U/ml to 7.10 U/ml) as compared to one variable at a time (OVAT) design. Interaction effects of the factors individually and in combination can be evaluated by using Taguchi method design of experiment.
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Introduction

Biodegradation of xylan, requires various xylanolytic enzymes of which endo-β-1, 4- xylanases (EC 3.2.1.8) depolymerize the xylan backbones into short xylooligosaccharides (Beg et al. 2001; Pandey et al. 2014). Besides, a set of accessory enzymes including α-L-arabinofuranosidases, α-D-glucuronidases, and acetyl xylan esterases are also needed for proper degradation (Sukhumsirichart et al. 2014). Extracellular xylanases derived from microorganisms have tremendous industrial interest. The most important fields of the applications are the biobleaching and biopulping, where the xylanases facilitate the release of lignin from paper pulp and thereby reduce the bleaching agents such as chlorine (Beg et al. 2001; Polizeli et al. 2005). Microbial xylanases are important in the biofuel preparation from agro-wastes, and they are also widely applied in food, feed (Subramaniyan et al. 1997) and agro fiber industries (Kanimozhi and Nagalakshmi 2014). Along with cellulase and pectinase it occupies about 20% of global enzyme market (Polizeli et al 2005). The global market of industrial enzymes is increasing rapidly. It was only 1 million US dollars in 1970 and became 4.5 billion dollars in 2012 and is thought to reach 7.1 billion by 2018 (Kalim et al. 2015). In certain industrial processes, bacterial xylanases are more preferred than fungal enzymes because of their thermo- and alkali-tolerance. (Beg et al. 2001). *Bacillus* species are excellent sources of xylanases having high activity under alkaline pH and high temperature conditions (Subramaniyan and Prema 2002).

The production cost of industrial enzymes is highly influenced by the cost of the growth medium (Katapodis et al. 2007), therefore, it is important to optimize the composition of the growth medium for high-yield enzyme production. The conventional optimization procedures including one factor at a time (OVAT) design, require time consuming experimental work and cannot provide information about the mutual interactions of the parameters (Rao et al. 2008; Das Mohapatra et al. 2009). On the contrary, statistical design of experiments helps to investigate the influence of controlled factor in multivariate system. Taguchi orthogonal array (OA) design of experiment (DOE) involves the study of any given system by a set of independent variables (fac-

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*Corresponding author. E-mail: pkdmvu@gmail.com

tors) over specific levels of interest (Taguchi 1986; Roy 1990; Mitra 1998). This approach also establishes the relationship between variables and operational conditions (Roy 2001). In this methodology, the design is made by selecting the best conditions that produce consistent performance (Roy 2001) and the results from small-scale experiments are valid to scale up the performance (Phadke and Dehnad 1988). ANOVA (analysis of variance) analysis of experimental data gives statistical relationship of the output. Taguchi methodology has successfully been applied to optimize the production of many industrial enzymes such as alkaline protease (Laxman et al. 2005), laccase (Prasad et al. 2005), tannase (Das Mohapatra et al. 2009), acid amylase and L-asparaginase, (Prakasan et al.

Table 1. Selected culture condition factors and assigned levels for the orthogonal design.

Serial No.	Factor	Level 1	Level 2	Level 3
1.	Temperature (°C)	30	35	40
2.	pH	5	6	8
3.	Xylan concentration (g/100 ml)	0.3	0.5	1.0
4.	Phosphate concentration (g/100 ml Na_2HPO_4)	0.05	0.1	0.5
5.	Nitrogen concentration (g/100 ml as NH_4NO_3)	0.1	0.3	0.5
6.	Na^+ concentration (g/100 ml as NaCl)	0.05	0.5	1.0

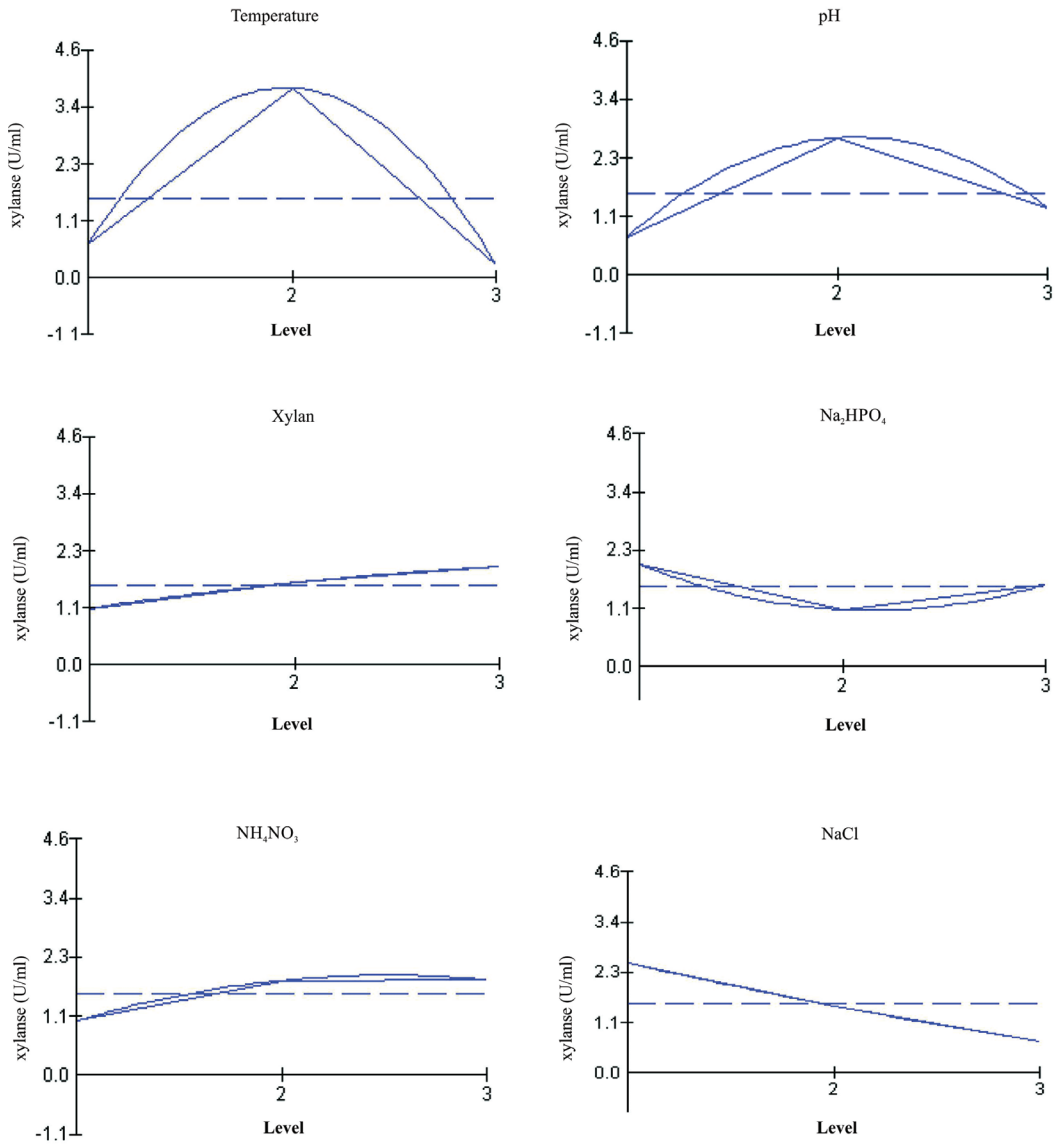

Figure 1. Individual factors performance at different levels.

Table 2. L_{18} (3^6) orthogonal array of designed experiments.

Serial. No.	1 (Temperature)	2 (pH)	3 (Xylan)	4 (Na_2HPO_4)	5 (NH_4NO_3)	6 (NaCl)	Xylanase activity (U/ml)
1	1	1	1	1	1	1	2.939
2	1	2	2	2	2	2	3.206
3	1	3	3	3	3	3	3.156
4	2	1	1	2	3	3	4.276
5	2	2	2	3	3	1	6.876
6	2	3	3	1	1	2	6.13
7	3	1	2	1	2	3	4.119
8	3	2	3	2	1	3	4.91
9	3	3	1	3	2	1	3.95
10	1	1	3	3	2	2	4.013
11	1	2	1	1	3	3	2.673
12	1	3	2	2	1	1	4.90
13	2	1	2	3	1	3	6.356
14	2	2	3	1	2	1	6.596
15	2	3	1	2	3	2	5.383
16	3	1	3	2	3	1	3.73
17	3	2	1	3	1	2	4.19
18	3	3	2	1	2	3	4.56

2007). This method is superior over the other similar statistical approaches, including the Response Surface Methodology (RSM), because much less time is required to conduct the experiment (Aggarwal et al. 2008). Additionally, Benyounis and Olabi (2008) reported that Taguchi method could improve the reliability at low cost as compared to RSM and Artificial Neural Networks (ANNs).

This study presents the statistical optimization of submerged culture conditions for xylanase production by *Bacillus cereus* BSA1 using Taguchi method. Multifactorial optimization of bacterial xylanase production has not been reported until to date.

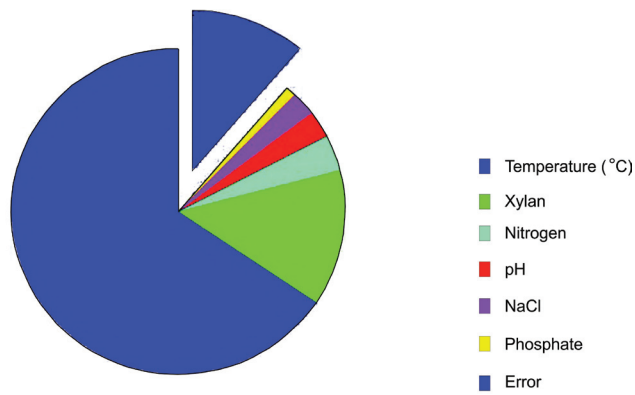

Figure 2. Relative influence of significant factors and interaction.

Materials and Methods

Microorganisms and culture conditions

Bacillus cereus BSA1 has been used in this study for enzyme production (Mandal et al. 2008). Unless otherwise stated, xylanase production was performed in 250-ml Erlenmeyer flasks containing 50 ml of liquid medium (composition in w/v%: NH_4NO_3 0.5; NaCl 0.05; Na_2HPO_4 0.05; xylan 0.5.) The culture medium was inoculated with 1% (v/v) freshly prepared inoculums (bacterial count in the inoculum was about 4.96 × 10^8/ml) and was fermented in a rotary shaker (120 rpm) at 35 °C for 84 h. After centrifugation (5000 × g for 10 min) the cell-free supernatant was used as crude enzyme extract. The experiments were done in triplicate.

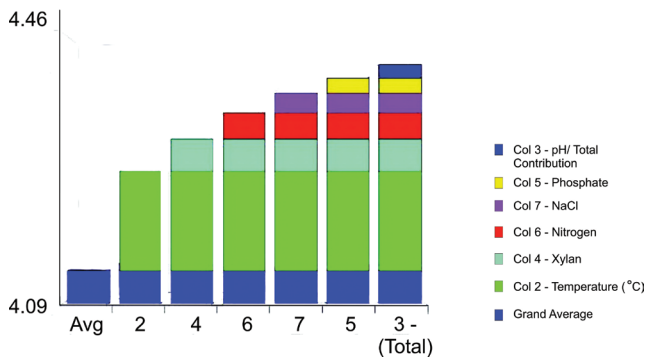

Figure 3. Optimum performance with the contributions of major factors.

Table 3. Main effects of selected factors in L_{18} (3^6) orthogonal array of designed experiments.

Serial. No.	Factors	Level 1	Level 2	Level 3	L_2-L_1
1	Temperature	3.481	5.936	4.243	2.455
2	pH	4.238	4.742	4.679	0.503
3	Xylan	3.902	5.002	4.756	1.1
4	Na_2HPO_4	4,503	4.4	4.757	-0.103
5	NH_4NO_3	4.905	4.4333	4.323	-0.473
6	NaCl	4.832	4.507	4.321	-0.326

Assay of xylanase activity

Xylanase activity was estimated by determining the released reducing sugar from the birch wood xylan (Fluka) with 3,5-dinitrosalicylic acid (DNS; Miller 1959). The reaction mixture contained 0.4 ml phosphate buffer (0.2 M, pH 7.0), 0.4 ml of 1% (w/v) xylan and 0.2 ml crude enzyme solution. After 30 minutes of incubation at 55 °C, 1 ml of 3% (w/v) DNS reagent was added to stop the reaction. The solution was boiled in a water bath for 15 min, then, absorbance was measured at 540 nm (Systronics Digital Spectrophotometer 105, India) against a blank without enzyme. Xylanase activity was determined by using a calibration curve for D-xylose (Sigma). One unit of xylanase activity (U) was defined as the amount of enzyme required to release 1 μmol of xylose per minute under the assay conditions.

Taguchi design

The Taguchi experimental design of Das Mohaaptra et al. (2009) was followed during the optimization procedures. Six most influensive factors for xylanase biosynthesis like temperature, pH, and xylan, phosphate (Na_2HPO_4), nitrogen (NH_4NO_3) and metal ion (Na^+) concentrations as identified through SmF (Mandal et al. 2008), and their effective levels were chosen (Table 1). The three levels of the factors were set as low, intermediate and high. In the next step, the orthogonal matrix of experiment was designed (Table 2).

Software

Qualitek-4 software (Nutek Inc., MI, USA) compatible for

Table 4. Analysis of variance (ANOVA) in L_{18} (3^6) orthogonal array of designed experiments.

Serial No.	Factors	DOF (f)	Sums of squares (S)	Variance (V)	*F* Ratio (F)	Pure sum (S')	Percentage P (%)
1	Temperature	2	56.814	28.407	154.530	56.446	65.528
2	pH	2	2.715	1.357	7.384	2.347	2.725
3	Xylan	2	12.015	6.007	32.680	11.647	13.521
4	Na_2HPO_4	2	1.217	0.608	3.311	0.849	0.986
5	NH_4NO_3	2	3.437	1.718	9.351	3.070	3.564
6	NaCl	2	2.403	1.201	6.537	2.035	2.363
	Other/error	41	7.535	0.183	-	-	11.313
	Total	53	86.138				100.00

Table 5. Estimated interaction of severity index for different factors in L_{18} (3^6) orthogonal array of designed experiments.

Serial. No.	Factors	Columns	SI (%)	Reserved column	Levels
1	Xylan × NaCl	4 × 7	73.05	3	[2,1]
2	pH × NaCl	3 × 7	55.38	4	[2,1]
3	$(NH_4)_2NO_3$ × NaCl	6 × 7	53.43	1	[1,3]
4	Xylan × NH_4NO_3	4 × 6	52.40	2	[2,1]
5	Na_2HPO_4 × NH_4NO_3	5 × 6	50.60	3	[1,2]
6	Temperature × Na_2HPO_4	2 × 5	36.50	7	[2,3]
7	Xylan × Na_2HPO_4	4 × 5	30.29	1	[2,3]
8	pH × $(NH_4)_2NO_3$	3 × 6	26.88	5	[3,1]
9	pH × Na_2HPO_4	3 × 5	26.14	6	[2,3]
10	Temperature × pH	2 × 3	25.79	1	[2,2]
11	Na_2HPO_4 × NaCl	5 × 7	10.47	2	[3,1]
12	Temperature × NaCl	2 × 7	8.76	5	[2,1]
13	Temperature × NH_4NO_3	2 × 6	7.48	4	[2,1]
14	Temperature × Xylan	2 × 4	7.1	6	[2,2]
15	pH × Xylan	54 × 4	0.49	7	[2,3]

Table 6. Optimum of culture conditions and their contribution.

Serial No.	Factors	Values	Level	Contribution
1	Temperature	35	2	1.382
2	pH	6	2	0.188
3	Xylan	0.5	2	0.449
4	Na_2HPO_4	0.5	3	0.203
5	NH_4NO_3	0.1	1	0.351
6	NaCl	0.05	1	0.278

Current grand average of performance: 4.553 U/ml. Total contribution from all factors: 2.850 U/ml. Expected result at optimum condition: 7.404 U/ml.

automatic design of Taguchi experiments was used. This software is equipped to use L-4 to L-64 arrays along with selection of 2 to 63 factors with two, three and four levels to each factor. The automatic design option allows Qualitek-4 to select the array used and assign factors to the appropriate columns.

Results and Discussion

Water pollution is an acute problem of the pulp and paper industries due to the chlorinated and other absorbable organic compounds released during bleaching process (Polizeli et al. 2005). This problem can be avoided by the application of microbial xylanolytic enzymes during the pre-bleaching processes. Microbial xylanases are frequently used in other biotechnological processes as well, therefore, it is very important to scale up of their enzyme production in the fermentation process.

The statistical approaches help to establish an optimized condition more easily and can make it feasible in industrial exploitation. Therefore, Taguchi OA was employed to study the effect of interaction of most essential parameters for enhancing xylanase production by *B. cereus* BSA1. The variations of xylanase production at different selected levels were represented in Figure 1. Among the tested six factors, temperature in level 2, xylan and nitrogen concentration at level 2 and level 3, respectively, have the highest influence on xylanase yield. Other factors showed lower effect on the enzyme production. The difference between level 2 and level 1 (L_2-L_1) of each factor (Table 3) indicates the relative influence of it on xylanase synthesis (Taguchi 1986). Increased level of NaCl caused decrease in enzyme production (Table 3). Figure 2 shows that the enzyme yield was highly influenced by temperature. Optimum performance with the major factors contribution on the xylanase production is represented in Figure 3. The percentage of contribution of each factor with interactions was again tested by ANOVA (Table 4). From the calculated ratios (F), it can also be revealed that temperature is the most significantly influencing factor for the xylanase production (Table 4). The next significant factors for the enzyme production in order to their relative influences were xylan > nitrogen > pH > Na^+ > phosphate. The importance of temperature and pH in biosynthesis of exo-enzymes has been reported by many authors (Frost and Moss 1987; Kim and Dordick 1997; Tunga et al. 1999; Dhillon et al. 2000)

In the complex environment of the fermentation any individual factor may interact with any or all of the other factors creating a large number of variations. The interaction between two factors gives a better insight into the overall process analysis and it is possible to calculate through Taguchi DOE by determining the severity index (SI). Severity index of the factors is represented in Table 5. In this table, the 'column' represents the locations to which the interacting factors are assigned. 'Reserved column' is used to study the interaction effect. 'Levels' indicated the factor levels desirable for the optimum conditions. Xylan and NaCl showed the highest severity index (73.05%). On the contrary, the lowest SI (0.49%) was observed in between pH and xylan (Table 5). An individual factor might show better effect but in combination with other, the effect became relatively low, which may due to the interactive effect of different factors (Prasad et al. 2005).

Optimum condition of each factor and their performance in terms of contribution for achieving, higher xylanase yield were summarized in Table 6. The highest xylanase activity can be achieved with the following optimized culture conditions: 35 °C; pH 6.0; xylan, 0.5% (w/v); Na_2HPO_4 0.5% (w/v); NH_4NO_3 0.1% (w/v), and NaCl 0.05%. The expected result at optimum condition was 7.404 U/ml, with total contribution from all the factors being 2.850 U/ml with grand average performance of 4.553 U/ml. To validate the proposed experimental methodology, fermentation was performed for xylanase production by employing the optimum level of each individual factor of Taguchi prediction. The obtained enzyme yield was 7.10 U/ml, which was 10.24% higher than that observed during OVAT optimization (the enzyme yield was 6.44 U/ml in that studies). This increase can be attributed to the interaction among the factors (Sharma et al. 2007). The experimental result was very close to the predicted value (7.404 U/ml) and thus the statistical evaluation was validated. In a previous study, the xylanase yield of *Trichoderma* strain has also been increased by using Taguchi methodology (Azin et al. 2007).

Conclusions

Xylanase production of *B. cereus* BSA1 was successfully improved through optimization of its culture conditions with Taguchi method. Using six factors (temperature, pH, con-

centration of xylan, Na_2HPO_4, NH_4NO_3 and NaCl,) at three levels, this analysis established the participation as well as the interactions of the factors. This is the first report on the optimization of both physical and chemical conditions of the fermentation environment for bacterial xylanase production using Taguchi methodology.

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