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

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ABSTRACT

Extracellular xylanase production of *Bacillus cereus* BSA1 was optimized under submerged fermentation using Taguchi orthogonal array (OA). An L₁₈ layout of OA was constructed at three-levels of six factors, i.e. temperature, pH, and xylan, Na₂HPO₄, NH₄NO₃ 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; NH₄NO₃ 0.5, Na₂HPO₄, 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.

KEY WORDS

*Bacillus cereus* BSA1 optimization submerged fermentation xylanase

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-
Mandal et al.

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.

<table>
<thead>
<tr>
<th>Serial No.</th>
<th>Factor</th>
<th>Level 1</th>
<th>Level 2</th>
<th>Level 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Temperature (°C)</td>
<td>30</td>
<td>35</td>
<td>40</td>
</tr>
<tr>
<td>2.</td>
<td>pH</td>
<td>5</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>3.</td>
<td>Xylan concentration (g/100 ml)</td>
<td>0.3</td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>4.</td>
<td>Phosphate concentration (g/100 ml Na₂HPO₄)</td>
<td>0.05</td>
<td>0.1</td>
<td>0.5</td>
</tr>
<tr>
<td>5.</td>
<td>Nitrogen concentration (g/100 ml as NH₄NO₃)</td>
<td>0.1</td>
<td>0.3</td>
<td>0.5</td>
</tr>
<tr>
<td>6.</td>
<td>Na⁺ concentration (g/100 ml as NaCl)</td>
<td>0.05</td>
<td>0.5</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Figure 1. Individual factors performance at different levels.
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.

### 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%: NH₄NO₃ 0.5; NaCl 0.05; Na₂HPO₄ 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 × 10⁸/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.

### Table 2. L₁₈ (3⁶) orthogonal array of designed experiments.

<table>
<thead>
<tr>
<th>Serial. No.</th>
<th>1 (Temperature)</th>
<th>2 (pH)</th>
<th>3 (Xylan)</th>
<th>4 (Na₂HPO₄)</th>
<th>5 (NH₄NO₃)</th>
<th>6 (NaCl)</th>
<th>Xylanase activity (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
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<td>1</td>
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<td>1</td>
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<td>3</td>
<td>4.119</td>
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<td>1</td>
<td>3.95</td>
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<tr>
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<td>3</td>
<td>2</td>
<td>2</td>
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</tr>
<tr>
<td>11</td>
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<td>2</td>
<td>1</td>
<td>1</td>
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<td>2.673</td>
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<tr>
<td>12</td>
<td>1</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>4.90</td>
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<td>2</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>6.596</td>
</tr>
<tr>
<td>15</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>5.383</td>
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<td>3</td>
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<td>3</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>3.73</td>
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<td>17</td>
<td>3</td>
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<td>1</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>4.19</td>
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<td>2</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4.56</td>
</tr>
</tbody>
</table>

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).

Figure 2. Relative influence of significant factors and interaction.

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

**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 influential factors for xylanase biosynthesis like temperature, pH, and xylan, phosphate (Na₂HPO₄), nitrogen (NH₄NO₃) 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

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**Table 1. Main effects of selected factors in L₁₈ (3⁶) orthogonal array of designed experiments.**

<table>
<thead>
<tr>
<th>Serial. No.</th>
<th>Factors</th>
<th>Level 1</th>
<th>Level 2</th>
<th>Level 3</th>
<th>L₂-L₁</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Temperature</td>
<td>3.481</td>
<td>5.936</td>
<td>4.243</td>
<td>2.455</td>
</tr>
<tr>
<td>2</td>
<td>pH</td>
<td>4.238</td>
<td>4.742</td>
<td>4.679</td>
<td>0.503</td>
</tr>
<tr>
<td>3</td>
<td>Xylan</td>
<td>3.902</td>
<td>5.002</td>
<td>4.756</td>
<td>1.1</td>
</tr>
<tr>
<td>4</td>
<td>Na₂HPO₄</td>
<td>4.503</td>
<td>4.4</td>
<td>4.757</td>
<td>0.103</td>
</tr>
<tr>
<td>5</td>
<td>NH₄NO₃</td>
<td>4.905</td>
<td>4.4333</td>
<td>4.323</td>
<td>0.473</td>
</tr>
<tr>
<td>6</td>
<td>NaCl</td>
<td>4.832</td>
<td>4.507</td>
<td>4.321</td>
<td>0.326</td>
</tr>
</tbody>
</table>

**Table 2. Analysis of variance (ANOVA) in L₁₈ (3⁶) orthogonal array of designed experiments.**

<table>
<thead>
<tr>
<th>Serial. No.</th>
<th>Factors</th>
<th>DOF (f)</th>
<th>Sums of squares</th>
<th>Variance (V)</th>
<th>F Ratio (F)</th>
<th>Pure sum (S)</th>
<th>Percentage P (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Temperature</td>
<td>2</td>
<td>56.814</td>
<td>28.407</td>
<td>154.530</td>
<td>56.446</td>
<td>65.528</td>
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<tr>
<td>2</td>
<td>pH</td>
<td>2</td>
<td>2.715</td>
<td>1.357</td>
<td>7.384</td>
<td>2.347</td>
<td>2.725</td>
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<td>3</td>
<td>Xylan</td>
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<td>6.007</td>
<td>32.680</td>
<td>11.647</td>
<td>13.521</td>
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<td>Na₂HPO₄</td>
<td>2</td>
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<td>0.608</td>
<td>3.311</td>
<td>0.849</td>
<td>0.986</td>
</tr>
<tr>
<td>5</td>
<td>NH₄NO₃</td>
<td>2</td>
<td>3.437</td>
<td>1.718</td>
<td>9.351</td>
<td>3.070</td>
<td>3.564</td>
</tr>
<tr>
<td>6</td>
<td>NaCl</td>
<td>2</td>
<td>2.403</td>
<td>1.201</td>
<td>6.537</td>
<td>2.035</td>
<td>2.363</td>
</tr>
<tr>
<td>Other/error</td>
<td>41</td>
<td></td>
<td>7.535</td>
<td>0.183</td>
<td>-</td>
<td>-</td>
<td>11.313</td>
</tr>
<tr>
<td>Total</td>
<td>53</td>
<td></td>
<td>86.138</td>
<td></td>
<td></td>
<td></td>
<td>100.00</td>
</tr>
</tbody>
</table>

**Table 3. Estimated interaction of severity index for different factors in L₁₈ (3⁶) orthogonal array of designed experiments.**

<table>
<thead>
<tr>
<th>Serial. No.</th>
<th>Factors</th>
<th>Columns</th>
<th>SI (%)</th>
<th>Reserved column</th>
<th>Levels</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Xylan × NaCl</td>
<td>4 × 7</td>
<td>73.05</td>
<td>3</td>
<td>[2,1]</td>
</tr>
<tr>
<td>2</td>
<td>pH × NaCl</td>
<td>3 × 7</td>
<td>55.38</td>
<td>4</td>
<td>[2,1]</td>
</tr>
<tr>
<td>3</td>
<td>(NH₄)₂NO₃ × NaCl</td>
<td>6 × 7</td>
<td>53.43</td>
<td>1</td>
<td>[1,3]</td>
</tr>
<tr>
<td>4</td>
<td>Xylan × NH₄NO₃</td>
<td>4 × 6</td>
<td>52.40</td>
<td>2</td>
<td>[2,1]</td>
</tr>
<tr>
<td>5</td>
<td>Na₂HPO₄ × NH₄NO₃</td>
<td>5 × 6</td>
<td>50.60</td>
<td>3</td>
<td>[1,2]</td>
</tr>
<tr>
<td>6</td>
<td>Temperature × Na₂HPO₄</td>
<td>2 × 5</td>
<td>36.50</td>
<td>7</td>
<td>[2,3]</td>
</tr>
<tr>
<td>7</td>
<td>Xylan × Na₂HPO₄</td>
<td>4 × 5</td>
<td>30.29</td>
<td>1</td>
<td>[2,3]</td>
</tr>
<tr>
<td>8</td>
<td>pH × (NH₄)₂NO₃</td>
<td>3 × 6</td>
<td>26.88</td>
<td>5</td>
<td>[3,1]</td>
</tr>
<tr>
<td>9</td>
<td>pH × Na₂HPO₄</td>
<td>3 × 5</td>
<td>26.14</td>
<td>6</td>
<td>[2,3]</td>
</tr>
<tr>
<td>10</td>
<td>Temperature × pH</td>
<td>2 × 3</td>
<td>25.79</td>
<td>1</td>
<td>[2,2]</td>
</tr>
<tr>
<td>11</td>
<td>Na₂HPO₄ × NaCl</td>
<td>5 × 7</td>
<td>10.47</td>
<td>2</td>
<td>[3,1]</td>
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<td>Temperature × NaCl</td>
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<td>8.76</td>
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<td>[2,1]</td>
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<tr>
<td>13</td>
<td>Temperature × NH₄NO₃</td>
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<td>7.48</td>
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<td>[2,1]</td>
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<tr>
<td>14</td>
<td>Temperature × Xylan</td>
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<tr>
<td>15</td>
<td>pH × Xylan</td>
<td>54 × 4</td>
<td>0.49</td>
<td>7</td>
<td>[2,3]</td>
</tr>
</tbody>
</table>

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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 3, respectively, have the highest influence on xylanase yield. Other factors showed lower effect on the enzyme production.

Table 6. Optimum of culture conditions and their contribution.

<table>
<thead>
<tr>
<th>Serial No.</th>
<th>Factors</th>
<th>Values</th>
<th>Level</th>
<th>Contribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Temperature</td>
<td>35</td>
<td>2</td>
<td>1.382</td>
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<tr>
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<td>2</td>
<td>0.188</td>
</tr>
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<td>Xylan</td>
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<td>0.203</td>
</tr>
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<td>NH4NO3</td>
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<td>0.351</td>
</tr>
<tr>
<td>6</td>
<td>NaCl</td>
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<td>1</td>
<td>0.278</td>
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</tbody>
</table>


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.

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$_2$HPO$_4$, NH$_4$NO$_3$, 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.

**Acknowledgments**

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**References**


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