Significant medium components for enhanced Jasmonic acid production by Lasiodiplodia theobromae using Plackett-Burman Design

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Abstract
A Plackett-Burman design and statistical screening was employed to optimize medium components for jasmonic acid production by Lasiodiplodia theobromae. The seven medium components malt extract, sucrose, NaNO₃, yeast extract, FeSO₄ 7H₂O, MnSO₄ · H₂O, and MgSO₄ 7H₂O were screened using a Plackett-Burman design to optimize medium component for jasmonic acid production using L. theobromae. When sucrose was used alone as a carbon source with these medium components, the jasmonic acid production was found to be 80 mg/l. Increase in jasmonic acid production (225.3 mg/l) was observed with augmentation of malt extract and changing other medium components concentration based on statistical screening. It was found that malt extract, sucrose, NaNO₃, MnSO₄ 7H₂O, and MgSO₄ 7H₂O were significant components influencing jasmonic acid production. Yeast extract and FeSO₄ 7H₂O also showed significantly negative effect on the jasmonic acid production. Therefore significantly positive factors could be increased and significantly negative factors could be decreased for higher jasmonic acid production.

Keywords
Jasmonic acid, Lasiodiplodia theobromae, Plackett-Burman design, Significant factors.

Introduction
Jasmonic acid (JA) (3-oxo-2-(2’-cis-pentenyl) cyclopantane-1-acetate) and methyl jasmonates are of importance in perfumery industries and being used world wide in various products like, toilet soaps, chewing gum, even in smoking preparations (1). JA and its derivatives are also very important secondary metabolites in the plant defense system (2, 3). JA was first isolated from the cultural filtrate of Lasiodiplodia theobromae (the synonym of Botryodiplodia theobromae) and its role was found as a plant growth regulator (4). In spite of high demand, very few fermentation studies were carried out for JA production using L. theobromae (5-7). This is the first report on screening of medium components for JA production using statistical experiment design.

The relevant literature and “prior art” serve only as starting point for the development of fermentation. By manipulating nutritional requirements, physical parameters and genetic makeup of the producing strain increase in productivity of microbial metabolite can be achieved. A fermentation improvement program may begin by measuring product yield as a response to factors like strength of medium components. Nutritional requirement can be manipulated either by the conventional or
statistical approach. Conventional methods involve changing one independent variable at a time (OVAT) while others are kept at fixed level. However, statistical methods offer several advantages over conventional methods being rapid and reliable, shortlists significant nutrients, helps understanding the interaction among the nutrients at various concentrations and reduces total number of experiments tremendously resulting in saving time and cost of glassware, chemicals and manpower (8, 9). Screening is the first phase of an experimental study to eliminate non-significant factors so that efforts may be concentrated upon most significant ingredients selected for further optimization.

The selection of the medium components for this study was carried out based on the literature survey and our preliminary laboratory work. A systematic experiment was then carried out by setting the independent variables according to a Plackett-Burman design (10) at two levels and JA produced was measured in each batch, followed by statistical analysis in order to interpret the significant medium components. Such approach is a useful screening process employed to identify the contribution of each medium component to the response of the system and thus allows for a reduction in the number of variables that need to be considered (11).

The Plackett-Burman design is highly recommended when more than five factors have to be investigated. These designs are very useful for economically detecting large main effects, assuming all interactions are negligible when compared with the few important main effects. Two level factorial Plackett-Burman design, was selected because it screens up to \( v \) variable in just \( v+1 \) experimental run (10, 12). These experimental designs are available in multiple of four runs (13) and advantageous over multifactorial design as it is difficult and involves large number of variable to be screened in terms of number of experiments (2\( v \), where \( v \) is the total number of variable). Higher-order linear full factorial and quadratic Box-Behnken designs would have required 66 and 52 experimental batches, respectively, which would have been prohibitively uneconomical (14). In this study, the aim was to screen the effects among seven independent variables (Table 1) in an economical manner (13). Due to orthogonal nature of the Plackett-Burman Design, it gives only pure effect of each variable not confounded with interaction among variables (15).

**Materials and Methods**

**Microorganism**

*L. theobromae* (MTCC 3068) strain was obtained from Microbial Type Culture Collection (MTCC) Institute of Microbial Technology, Chandigarh, India. This strain was maintained on potato dextrose agar (Hi-Media, Mumbai, India) slants. After inoculation, slants were incubated at 30°C for three to four days for obtaining growth and later stored at 4°C. Strain was sub-cultured every month.

**Chemicals and solvents**

(±) JA, a racemic mixture, was purchased from Sigma, Bangalore, India and used as a standard reference compound for quantification of JA. All dehydrated media components were purchased from Hi-media, Mumbai, India. All solvents (99.9%) were purchased from Qualigens, Mumbai, India. Ammonia (25% w/v) was used fresh.

**Cultural technique**

Sample of stock culture was transferred from working slants to potato dextrose agar plates and incubated for three days at 30°C. The basal medium contained (g/l) KH\(_2\)PO\(_4\), 2.0; KCl, 0.3; ZnSO\(_4\) \(7\)H\(_2\)O, 0.03; CuSO\(_4\) \(7\)H\(_2\)O, 0.003; and Na\(_2\)MoO\(_4\) \(2\)H\(_2\)O, 0.003. The pH was maintained 5.5 (6) through out the experiment and the
concentrations of other medium components malt extract, sucrose, \(\text{NaNO}_3\), yeast extract, \(\text{FeSO}_4 \cdot 7\text{H}_2\text{O}\); \(\text{MnSO}_4 \cdot 7\text{H}_2\text{O}\) and \(\text{MgSO}_4 \cdot 7\text{H}_2\text{O}\) were as per the combination given in Table 1 and 2. Agar plug (eight mm diameter) was cut with sterile cork borer and used for inoculation of 100 ml of the broth culture in 250 ml Erlenmeyer flask and incubated for eight days at 30°C in static condition.

**Screening of important nutrient components**

Total seven medium components (independent variables, \(v=7\)) were selected for the study with each variable represented at two levels, high (+) and low (-) concentrations also four dummy variables were kept at two concentrations. The input factor and their corresponding upper and lower level values are shown in Table 1. A two level design only minima and maxima of input variables were used to form a design space. The resulting twelve experimental cases for seven input parameters (\(v+1+4\) dummy variables) are shown in Table 2 with the number of positive signs and negative signs. Each column contained equal number of positive and negative signs. Thus each row represented a trial batch and each column represented an independent (assigned) or dummy (unassigned) variable. The effect of each variable was determined by the following equation:

\[
E_{i(i)} = \left( JAi^+ - JAi^- \right) / N \quad - (1)
\]

Where \(E_{i(i)}\) is the concentration effect of the tested variable. \(JAi^+\) and \(JAi^-\) are the jasmonic acid production from the trials where the variable (\(xi\)) measured was present at high and low concentrations, respectively; and \(N\) is the number of trial divided by two. Experimental error was determined by calculating the variance among the dummy variables as follows:

\[
V_{eff} = (E_d)^2 / n \quad - (2)
\]

Where \(V_{eff}\) is the variance of the concentration effect, \(E_d\) is the concentration effect for the dummy variable and \(n\) is the number of dummy variables. The standard error (SE) of the concentration effect was calculated by taking square root of the variance of an effect and the significance level (\(p\)-value) of each concentration effect was determined using student’s \(t\)-test:

\[
t_{(xi)} = E_{(xi)} / SE \quad - (3)
\]

Where, \(E_{(xi)}\) is the effect of variable \(xi\) (16).

**Extraction and measurement of JA**

Cultural filtrate of \(L. \text{theobromae}\) after acidification to pH 3 with 6 M HCl, was extracted with equal volume of ethyl acetate. Extract was concentrated to 100 times. JA measurement was carried out using high performance thin layer chromatography (HPTLC). Concentrated extract was loaded on silica gel 60 F\(_{254}\) aluminum foils (Merck, Germany) along with standard JA using Linomate-5 spray on applicator (Camag, Switzerland) of HPTLC under the flow of \(N_2\). Foils were ran with iso-propanol: ammonia: water (10:1:1 (v/v)) (17). After running, foils were dried in air and scanned with Scanner-3 (Camag, Switzerland) and quantified with the help of winCATS software ver. 1.2.2 by measuring density of the JA band separated on the TLC foils (18).

**Statistical analysis of data**

Yields of JA obtained in experiments were calculated for determination of variable significance using Microsoft Excel regression coefficient and statistical \(t\)-values for equal unpaired samples. Ingredient with highest \(t\)-value was considered as best nutrient and thus selected for further optimization studies. A main effect with a positive sign indicates that the high concentration of this variable is near to optimum and a negative sign indicates that the low
concentration of this variable is nearer to optimum.

**Results and Discussion**

Selection of appropriate carbon source, nitrogen source and other nutrient is one of the most critical stages for the development of an efficient and economic fermentation process. In this study a Plackett-Burman design was employed to evaluate the main effect of the medium components for the JA production by *L. theobromae* at optimized physical parameters.

Table 1 represents the independent variables and the respective high and low concentrations of seven factors used in the optimization study. Malt extract was selected as it contains up to 3.4% of lipids in its dry matter with linoleic acid as their major constituent (50–60% of total lipids) (19). Sucrose was used previously in our study as well as elsewhere as main carbon source for same fungi and found significant, therefore included in the study (6). NaNO₃ was also selected based on our study for the screening of the carbon and nitrogen source for the JA production (data not shown). NaNO₃ was reported to be preferred nitrogen source either with glucose (1) or fructose (6) for JA production using *L. theobromae* (Table 3). The main effects of the components in the medium for JA production are presented in the Fig 1. The malt extract showed the maximum positive effect on JA production, followed by MnSO₄ 7H₂O, MgSO₄ 7H₂O, Sucrose and NaNO₃. The effect of FeSO₄ 7H₂O and yeast extract were negative which suggested that these components are required in the medium for JA production but in lower concentration than the low level.

Malt extract was found better medium component than yeast extract. As shown in Table 2, in batches 6 and 10, where yeast extract was supplied in higher concentration but malt extract was not given, the yield of JA was reduced drastically and in batches 7, 8 and 9, high concentration of malt extract and low concentration of yeast extract resulted in higher production of JA. Low concentration of yeast extract may be required to meet requirement of vitamins for general metabolism as well as for some enzymes of JA pathway (for example, 12-oxo-phytodienoic acid reductase) (21). Supplementation of one or more vitamins of “B” group either as separate or in form of yeast extract improves the JA production (1). Yeast extract serve as dual-purpose nitrogen source as well as vitamin source but it is an expensive component
for designing an economical medium (15). From the results of the Plackett-Burman design it could be concluded that yeast extract is probably required for the growth but not for the JA production and should be supplied in low concentration.

Sucrose can not replace the malt extract, as the highest JA (225 mg/l) was produced in batch 2 does contain low level of malt extract and when only sucrose was used in previous experiment, it produced only 80 mg/l of JA (7). Moreover, malt extract is useful as it contain linoleic acid as its major constituent (which is the precursor of JA). (19).

The manganese showed positive effect and iron showed negative effect might be related with the metal ion required for the catalytic center of the lipoxygenase enzyme (20). MgSO$_4$ 7H$_2$O is also very important medium component showing main effect same as sucrose. The requirement for Mg is evident as Mg is required as a catalyst for many intracellular enzymatic reactions. Mg salts have been found most useful in other metabolite and enzyme production as well (21, 22).

By selection of medium components using Plackett-Burman design in this study, about three-fold increase in the JA production was increased compared to earlier experiment where in BSB medium, which supported the maximum JA production as well as growth of $L$. theobromae, 80 mg/l of JA was produced (7).

Present study has short-listed the few significant nutrients useful in increasing the yield of JA and has also identified the nutrients, which should be used in less concentration.

Acknowledgement

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References


Table 1: Medium components (Variables) and their respective high (+) and low (-) concentration levels used in Plackett-Burman Design

<table>
<thead>
<tr>
<th>Variable</th>
<th>Medium Components</th>
<th>+ Value (g/l)</th>
<th>- Value (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>X1</td>
<td>Malt Extract</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td>X2</td>
<td>Sucrose</td>
<td>50</td>
<td>5</td>
</tr>
<tr>
<td>X3</td>
<td>NaNO₃</td>
<td>7.5</td>
<td>0.75</td>
</tr>
<tr>
<td>X4</td>
<td>Yeast Extract</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>X5</td>
<td>FeSO₄ 7H₂O</td>
<td>0.6</td>
<td>0.06</td>
</tr>
<tr>
<td>X6</td>
<td>MnSO₄ H₂O</td>
<td>0.01</td>
<td>0.001</td>
</tr>
<tr>
<td>X7</td>
<td>MgSO₄ 7H₂O</td>
<td>6</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Table 2: Plackett-Burman design generated by fractional rotation of full factorial design where X1 to X7 are independent variables and D1 to D4 are dummy variables

<table>
<thead>
<tr>
<th>Batch</th>
<th>Component</th>
<th>JA produced (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>X1 X2 X3 X4 X5 X6 X7 D1 D2 D3 D4</td>
<td>175.3</td>
</tr>
<tr>
<td>2.</td>
<td>- + + - + + - - - + -</td>
<td>225.3</td>
</tr>
<tr>
<td>3.</td>
<td>+ - + + - + + + - - -</td>
<td>121.3</td>
</tr>
<tr>
<td>4.</td>
<td>- - + - + + - + + - -</td>
<td>145.7</td>
</tr>
<tr>
<td>5.</td>
<td>- - + - + + - + + - -</td>
<td>177.3</td>
</tr>
<tr>
<td>6.</td>
<td>- - - + - + - + + + +</td>
<td>74.6</td>
</tr>
<tr>
<td>7.</td>
<td>+ - - - + - - + + - +</td>
<td>187.2</td>
</tr>
<tr>
<td>8.</td>
<td>+ + - - - + - + - + -</td>
<td>151.1</td>
</tr>
<tr>
<td>9.</td>
<td>+ + - - - + - + - + -</td>
<td>162.2</td>
</tr>
<tr>
<td>10.</td>
<td>- + + + - - - + - - +</td>
<td>56.2</td>
</tr>
<tr>
<td>11.</td>
<td>+ - + + + - - - + - -</td>
<td>129.03</td>
</tr>
<tr>
<td>12.</td>
<td>- - - - - - - - - - -</td>
<td>59.75</td>
</tr>
</tbody>
</table>
Table 3: $t$-value, probability, confidence level and ranking of variables for JA production

<table>
<thead>
<tr>
<th>Component</th>
<th>$\overline{x}_i$ absolute value</th>
<th>Standard Error</th>
<th>$t_{(xi)}$</th>
<th>$p$</th>
<th>Confidence (%)</th>
<th>Ranking</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malt Extract</td>
<td>31.2250</td>
<td>4.4462</td>
<td>7.0228</td>
<td>0.0021</td>
<td>99.89</td>
<td>3</td>
</tr>
<tr>
<td>Sucrose</td>
<td>27.7716</td>
<td>4.4462</td>
<td>6.2461</td>
<td>0.0033</td>
<td>99.66</td>
<td>6</td>
</tr>
<tr>
<td>NaNO$_3$</td>
<td>12.9316</td>
<td>4.4462</td>
<td>2.9084</td>
<td>0.0437</td>
<td>95.73</td>
<td>7</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>43.4650</td>
<td>4.4462</td>
<td>-9.7757</td>
<td>0.0006</td>
<td>99.94</td>
<td>1</td>
</tr>
<tr>
<td>FeSO$_4$ 7H$_2$O</td>
<td>36.3083</td>
<td>4.4462</td>
<td>-8.1661</td>
<td>0.0012</td>
<td>99.88</td>
<td>2</td>
</tr>
<tr>
<td>MnSO$_4$ H$_2$O</td>
<td>30.7816</td>
<td>4.4462</td>
<td>6.9231</td>
<td>0.0022</td>
<td>99.78</td>
<td>4</td>
</tr>
<tr>
<td>MgSO$_4$ 7H$_2$O</td>
<td>27.9583</td>
<td>4.4462</td>
<td>6.2881</td>
<td>0.0032</td>
<td>99.68</td>
<td>5</td>
</tr>
</tbody>
</table>

Figure. 1: Effect of media components on JA production in batch culture