### Efficient production of Aschersonia placenta protoplasts for transformation using optimization algorithms

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| Keyword:     | Aschersonia placenta, Protoplast yield, Production condition, Response surface method |
Efficient production of Aschersonia placenta protoplasts for transformation using optimization algorithms

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Abstract: The insect pathogenic fungus, *Aschersonia placenta* is a highly effective pathogen of whiteflies and scale insects. Few genetic tools, however, are currently available for this organism. Here we report on the conditions for the production of transformable *A. placenta* protoplasts using an optimized protocol based on the response surface method (RSM). Critical parameters for protoplast production were modelled by using a Box-Behnken design (BBD) involving three levels of three variables, and subsequently experimentally verified in its predictability of protoplast production ($R^2 = 0.9465$). The optimized conditions resulted in the highest yield of protoplasts ($4.41 \pm 0.02 \times 10^7$ cells/ml of culture, mean ± SE) when fungal cells were treated with 26.1 mg/ml of lywallzyme for 4 h of digestion, and subsequently allowed to recover for 64.6 h in 0.7 M NaCl-Tris buffer. The latter used as an osmotic stabilizer. The yield of protoplasts was approximately 10-fold higher than that of the non-optimized conditions. Generated protoplasts were transformed with vector PbarGPE containing *bar* gene as the selection marker. Transformation efficiency was 300 colonies/µg DNA/10^7 protoplasts, and integration of the vector DNA was confirmed by PCR. The results show that rational design strategies (RSM and BBD methods) are useful to increase the production of fungal protoplasts for a variety of downstream applications.

Key words: *Aschersonia placenta*, Protoplast yield, Response surface method, Production condition.
Introduction

Biologically based insecticides are of widespread interest in biological control approaches due to their more environmentally friendly effects when compared to chemical agents, and are considered more renewable and sustainable (Glare et al. 2012). *Aschersonia*, a fungal pathogen of insects, is specifically used to control whiteflies (*Aleyrodidae*) and scale insects (*Coccidae*) (Meekes et al. 2002; Qiu et al. 2010, 2013; Wang et al. 2013) and can cause significant epizootic infections in these insect populations in the tropics and subtropics (Evans and Hywel-Jones 1990). Natural *Aschersonia* infections have been shown to impact the population dynamics of the wooly whitefly, *Aleurothrixus floccus*, on sweet orange (Umeh et al. 2011), and new species of this insect pathogen have been continuously isolated (Qiu et al. 2010, 2012). These fungi are also rich in the production of secondary metabolites of biotechnological interest including cytotoxic xanthones, cyclodepsipetide toxins, and antimicrobial compounds (Isaka et al. 2005, 2012; Kornsakulkarn et al. 2013).

For biological control applications, many wild-type strains are not ideal since obstacles exist due to low survival/persistence in natural environments, difficulties in (large-scale) propagation as well as storage of virulent spores (Rodriguez-Iglesias and Schmoll 2015). Genetic engineering, however, offers a possible route towards improving the efficacy of fungal strains, as well as a means for natural products discovery of bioactive compounds. To the best of our knowledge, to date there are no reports of a transformation system for *Aschersonia*. *Agrobacterium tumefaciens* mediated transformation has been successfully used for other entomopathogenic fungi,
including *Metarhizium anisopliae* and *Beauveria bassiana* (Fang et al. 2004, 2006), however preliminary attempts to use such a system for *Aschersonia* were unsuccessful (Qiu unpublished observation). Production of cellular protoplasts via enzymatic treatment of the fungal cell wall is a well-known method for production of “competent” fungal cells capable of taking up exogenous DNA (Rodriguez-Iglesias and Schmoll 2015). These methods require the efficient isolation and regeneration of protoplasts, a process yet to be developed for *Aschersonia*.

The objective of this study was to develop a system to promote protoplast production of *Aschersonia*. We employed a rational experimental design strategy incorporating response surface methodology (RSM) and Box–Behnken design (BBD), a process more time and cost effective than traditional variation of one parameter at a time (Ghosh and Hallenbeck 2010). RSM combines mathematical and statistical techniques to design experimental trajectories including model building, identification of effective factors, examining potential interactions between parameters, and searching for optimal conditions, which thereby eliminates the limitations of single-factor optimization (Bandaru et al. 2006; Tabandeh et al. 2008; Tan et al. 2010).

RSM was applied to find appropriate enzymatic hydrolysis conditions for *A. placenta* protoplast formation and cultivation conditions that would enhance protoplast production. We also used Box-Behnken design (BBD) to examine important conditions and to identify optimal production parameters. BBD has been previously used in biological control applications and optimization of the medium composition resulted in a defined biphasic production system for mycelium growth and spore
production of *Aschersonia placenta* (Feng et al. 1994; Shih et al. 2007; Dong et al. 2009; Qiu et al. 2013). With respect to the optimized conditions, there was a 10-fold increase in protoplast yield. Moreover, protoplasts displayed high transformation efficiencies reaching up to 300 colonies/µg DNA/10^7 protoplasts.

### Materials and methods

#### Culture strain

*Aschersonia placenta* FJSM, used in the current study, was isolated from an infected camellia whitefly (*Aleurotrachelus camelliae* Kuwana) cadaver collected in a tea (*Camellia oleifera* Abel) plantation in the town of Sanming, Fujian Province, China. Strain FJSM was subsequently stored in glycerol at -80°C and cultured on potato dextrose agar (PDA, Hangzhou Baisi Biotechnology Co, China) at 25°C for 20 d for growth and spore production as described previously (Qiu et al. 2013).

#### Induction of protoplasts

A set of initial experiments were performed to determine the optimal osmotic stress stabilizer for *A. placenta* conidia, which was determined to be 0.7 M NaCl in 20 mM Tris buffer, pH = 6.0 (NaCl-Tris buffer). Lywallzyme (Sigma Chemical Co, Beiruo, China) was dissolved in this buffer with different concentrations as detailed below. Conidia produced on PDA (20 d) were scraped and suspended in sterile water containing 0.05% (v/v) Tween 80 (Sigma Chemical Co, Beiruo, China). Conidial concentration was measured using a haemocytometer and diluted to desired
concentration (typically \(1\times10^8\) conidia/ml) for further use (Qiu et al. 2013). One ml of conidial suspension was inoculated onto 100 ml potato dextrose broth (PDB) liquid media and cultured for 2-3 d at 26°C before harvesting for protoplast production. Fungal biomass was collected on a sterilized filter (Whatman filter papers, pore size 15-18 µm; Sigma Chemical Co, Beiruo, China) and the hyphae were washed using NaCl-Tris buffer until the wash fluid was achromatic. The washed hyphae were ground in a mortar and pestle with a small amount of buffer on ice. The ground hyphae were placed into a 50 ml centrifuge tube with 20 ml potato dextrose broth (PDB) and incubated at 28°C for 16 h with aeration (150 rpm). After incubation, the hyphae were collected by filtration and resuspended in the indicated solution containing lywallzyme. Treatment variables included temperature and time, after which the cells were harvested by centrifugation (4000 rpm for 10 min), and the cell pellet was resuspended in NaCl-Tris buffer with various pH values. Experimental factor parameters, i.e. cell culture time, pH, lywallzyme concentration, digestion temperature and regeneration time are shown in Tables 1 and 2.

Experimental design and statistical analyses

**Preliminary experiments: The effects of enzyme concentration, digestion time, and other parameters on A. placenta protoplast formation**

To investigate the influence of enzyme concentration and digestion time on production of A. placenta protoplasts, different concentrations of lywallzyme (1, 5, 10, 15, 20, 25, 30, 35, 40, and 50 mg/ml) and various digestion times (15 min, 30 min, 1 h,
2 h, 4 h, 5 h, 6 h, and 8 h) were examined independently while maintaining the other protoplast preparation conditions constantly as described above. Subsequently, digestion temperatures (consisting of 22°C, 24°C, 26°C, 28°C, 30°C, 32°C, 34°C, 36°C, 38°C, and 40°C), pH values (including 4.2, 4.6, 5, 5.4, 5.8, 6.2, 6.6, 7, 7.4, 7.8, and 8.2) and regeneration time (covering 12 h, 24 h, 36 h, 48 h, 60 h, 72 h, 84 h, and 96 h) were also estimated for the suitability of protoplast production.

Optimization algorithms for protoplast production

The Plackett–Burman design (PBD) as previously used (Naveena et al. 2005; Yuan et al. 2008; Singh et al. 2011; Qiu et al. 2013) was employed in which each individual factor was designed at two levels, high (+1) and low (-1). Both levels were set on the strength of the findings of the preliminary experiments as described in Table 1 and 2, i.e. included enzyme concentration, pH, digestion time, digestion temperature and regeneration time.

The steepest ascent design was used to determine the direction toward predicted higher responses (Box et al. 1978; Chen et al. 2002) and to ensure the validity and correctness of the response surface analysis results (Lin et al. 2007). The first-order model equation obtained from the PBD test was used to confirm the increasing or decreasing direction and the length of the ascent pace, leading to an iterative experimental design (Lin et al. 2007). Experiments were performed along the steepest ascent path until no further increases in the response. Experimental design details are given in Table 3 in which the steepest ascent experiment included five steps as shown
by the numbers 1–5 following the plus sign. The paths of enzyme concentration, digestion time, and regeneration time for protoplast production begins at 25 mg/ml, 3 h, and 54 h (X), with a step (Δ) of 0.5 mg/ml, 0.5 h and 5 h, respectively (Table 3).

**Box–Behnken design**

A three variable Box–Behnken design (BBD) was applied to model the optimal pretreatment protoplasting conditions and to analyze the sensitivity of the responses to variation in the experimental variables (Box and Behnken 1960) using a process similar to what has been described for other fungal physical processes (Dong et al. 2009). The BBD parameters included three factors at three levels (involving triplicates at the center point) and was modeled based upon the outcomes of the preliminary results. The ranges and levels of the individual variables are listed in Table 4 and the experimental designs with the observed responses and predicted values in relation to protoplast yield are given in Table 5. The polynomial equation generated by this experimental design was as follows:

\[ Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 \]

where \( Y \) is the predicted response; \( b_0 \) = the mean effect; \( b_1 \) to \( b_{33} \) are regression coefficients, and \( X_1, X_2, \) and \( X_3 \) are the independent variables selected from the preliminary experiments. This design was used as previously described (Rane and Prabakar 2013). Determination of the “best fit” of the polynomial model equation was shown using the coefficient of determination \( R^2 \). The model adequacy was further confirmed based on an \( F \)-test at the 5% level of significance and the determination
coefficient $R^2$ (Qiu et al. 2013). Response surface contour plots were formed to
demonstrate the possibility of enhancing yield of proplasts.

**Treatment of data**

Each experimental treatment was performed in triplicate, i.e. independent flasks for
each condition. STATISTICA 7.0 (StatSoft Inc., Tulsa, OK, USA) software was used
for analyzing the data, describing the response surface, and producing the response
surface plots.

**PEG-mediated transformation of proplasts**

Regenerated proplasts (200 µl per tube, ~5 x 10^7 proplasts/ml) of *Aschersonia*
placenta were gently mixed with 15 µl of 100 µg/ml plasmid PbarGPE, containing the
*bar* selection marker for resistance to the herbicide phosphinothricin (PPT) and used
for PEG-mediated transformation as described elsewhere (Datta et al. 1992; Fan et al.
2011). Briefly, cells were incubated on ice with plasmid DNA for 45 min and then
subjected to heat shock at 42°C for 20 min. The cells were harvested by centrifugation
(6000g, 3 min) at 4°C. The transformation mixture was plated on Czapek-Dox (CZA)
agar containing 0.12 mg/ml PPT. Transformants were single spore purified and
subcultured on CZA-PPT plates as indicated.

**Characterization of transformants by PCR**

Purified transformants were grown in CZA and their genomic DNA were extracted
as described (Fang et al. 2004). Polymerase chain reaction (PCR) analysis was performed in 25 µl reaction mixture containing 1 µl each of primers (10 µM) designed to the *bar* gene, F1 (5’-GCAGGAACCGCAGGAGTGGA-3’) and R1 (5’-ATCTCGGTGACGGGCAGGAC-3’), 0.5 U of Taq polymerase, 5 µl buffer, and genomic DNA, using the following protocol: 5 min 94°C, followed by 35 cycles of denaturation (60 s at 94°C), annealing (45 s at 60°C), and extension (60 s at 72°C). PCR products were analyzed by agarose gel electrophoresis.

**Results**

The influence of different enzyme concentrations on the production of protoplasts was initially examined. Production of protoplasts was highest at a concentration of 25 mg/ml, yielding 1.11 ± 0.13 × 10⁷ cells/ml. With respect to digestion time, production of protoplasts was highest when 4 h were chosen as the tested period, yielding 1.02 ± 0.11 × 10⁷ cells/ml. The series of experiments demonstrated that the major factors affecting the protoplast yields were 25 mg/ml lywallzyme, digestion time with also including digestion temperature, pH and regeneration time (data not shown). Thus, these conditions were tested for further optimization of the variables.

**Plackett–Burman design**

Plackett-Burman design (PBD) was used to assess the relative contributions of different variables in impacting *A. placenta* protoplast yield. Preliminary experiments revealed that protoplast production from *A. placenta* hyphal cultures grown in PDB,
showed the highest dependence on enzyme concentration ($X_1$), cell treatment digestion time ($X_5$), and regeneration time ($X_8$) with respect to the initial preparation variables. The results of a two-level PBD modeling and testing of these factors are given in Tables 1 and 2. Protoplast production ranged from $1.62 \pm 0.32 \times 10^7$ to $2.98 \pm 0.06 \times 10^7$ cells/ml. Protoplast yield was highest with high levels of enzyme concentration ($X_1$), pH ($X_3$), digestion time ($X_5$) and regeneration time ($X_8$) and low levels of digestion temperature ($X_7$) (Tables 1 and 2). The model and experimental data resulted in a value for the determination coefficient, $R^2 = 0.9796$.

**Identification of the largest response area**

Experiments were performed based upon the steepest ascent region in the model (Table 3). In these experiments, the identified experimental parameters were varied in a series of five steps (1–5Δ). Protoplast yield peaked ($3.22 \pm 0.03 \times 10^7$ cells/ml) in the second step (X + 2Δ), when enzyme concentration, digestion time and regeneration time were increased (Table 3). These data indicated the convergence point where the identified experimental conditions were approaching the maximum in terms of protoplast yield. These results (Table 3) were used to further predict the range and coded level of the next levels of factors as given in Table 4 using the Box–Behnken design (BBD) as described in the Methods section.

**Optimization of protoplast yield by response surface methodology**

The results derived from the BBD experimental design algorithm (Table 4) are
displayed in Table 5, with the statistical analyses (ANOVA) of the results given in Table 6. The results show a high confidence that the model equation sufficiently depicts the response surface. The polynomial models were mathematically expressed as follows:

\[
YP = 3.93 + 0.2125X_1 + 0.03375X_2 + 0.18875X_3 - 0.94625X_1^2 - 0.64375X_2^2 - 0.58875X_3^2 + 0.315X_1X_2 - 0.02X_1X_3 - 0.0425X_2X_3
\]

YP represents the response factors (protoplast yield), and \(X_1\), \(X_2\), and \(X_3\) are the coded variables. Using this, the quadratic regression model was estimated by ANOVA. The low \(p\) value (<0.05, Table 6) indicated that the model was statistically robust. Among these results, the value of the determination coefficient \((R^2)\) was 0.9465 for protoplast yield. To validate the model, the predicted optimized variables determined for enzyme concentration, digestion time and regeneration time, specifically 26.1 mg/ml, for 4 h, and for 64.6 h, respectively, were used to generate \(A.\) placenta protoplasts (Figs 1–3). Under these conditions, protoplast yield reached \(4.41 \pm 0.02 \times 10^7\) cells/ml, which was greater than the number predicted \((4.38 \pm 0.07 \times 10^7\) cells/ml) when 0.7 M NaCl was used as an osmotic stabilizer (Figs. 1–3).

**Transformation of the protoplasts**

Protoplasts \((1 \times 10^7)\) were prepared using the optimized condition as described above and transformed with the plasmid PbarGPE containing the \textit{bar} selection gene marker. A high transformation efficiency of 300 colonies/µg DNA/\(10^7\) protoplasts was
obtained in the test. However, untreated fungal cells gave no transformants. The stability of gene transformation was proved after subculturing transformants for four generations on CZA plates in the presence of phosphinothricin. Genomic DNA of four selected transformants obtained from each generation was extracted and the existence of the \textit{bar} gene selection marker was examined using PCR. A PCR amplified fragment of 500 bp corresponding to the expected size was detected in all of the transformants (Fig. 4).

**Discussion**

Entomopathogenic fungi are promising biological control agents capable of targeting a wide range of agricultural pests (Lomer et al. 2001). The ability to genetically manipulate these organisms can be a powerful tool in increasing the efficacy for diverse applications. Moreover, genetic approaches allow fundamental research aimed towards understanding the molecular aspect of the host-pathogen interaction. It is of significant importance to study \textit{A. placenta} because of its host specificity. Towards developing genetic tools for \textit{A. placenta}, preliminary experiments using \textit{A. tumefaciens}, an approach successful with many other fungi (include several insect pathogens) were not successful due to the low transformation efficiency (Keyhani, unpublished data). Protoplasts, cells in which the outer cell wall of the fungus has been partially degraded, are well known to be “competent” in their ability to uptake DNA as compared to their untreated counterparts (Rodriguez-Iglesias and Schmoll 2015). We therefore sought to optimize the production of protoplasts
useful for various downstream applications including transformation. Optimized algorithm combining sequential experiments based upon Plackett-Burman and Box-Behnken designs, and response surface outputs were used to optimize *A. placenta* protoplast yield. Validation at various stages was examined using the $R^2$ coefficient of determination. The $R^2$ value indicates the degree of variability on the basis of the experimental parameters and their interactions (Qiu et al. 2013). The ability of models to forecast response values increases as $R^2$ values approach 1.0 (Acikel et al. 2010). In the results presented, the observed high $R^2$ values (> 0.946) support the idea that the model equations can adequately predict the responses. The 3-D response surfaces and 2-D contour plots generated based on the model equations were used to identify the optimal parameter conditions (Figs. 1–3). Their shapes show the nature and extent of the interactions of experimental variables (Prakash et al. 2008; Qiu et al. 2013). The peaks in the 3-D response surface and 2-D contour plots indicate the optimal points (i.e. the best predicted experimental conditions) within the design limits. Each figure examines the effects of two factors on protoplast yield when the third factor is maintained at the peak level. The 3-D plot also shows that the interactions among the three individual factors significantly influenced protoplast yield. By coupling modeling to select experimental verification, our results indicate a significantly streamlined process in which experimental conditions were optimized, reducing both effort and cost. As noted earlier, the optimum values for the three variables affecting protoplast yield were 26.1 mg/ml for enzyme concentration, 4 h for digestion time and 64.6 h for regeneration time and the
predicted yield was $4.38 \pm 0.07 \times 10^7$ cells/ml whereas the actual yield was $4.41 \pm 0.02 \times 10^7$ cells/ml. However, other biological factors related to the increase of protoplast yield were not investigated.

The resultant protoplasts were readily transformable yielding mitotically stable transformants. Future experiments can thus be performed to determine whether the technique can be applied for generation of target gene knockouts in *A. placenta*, a critical step forward in genetic studies of this organism. These results have determined the parameters for the simple and efficient preparation of protoplasts from *A. placenta*.

**Conclusion**

The goal of present study was to identify the conditions for efficient production of *A. placenta* protoplasts for transformation. The individual and interactive roles of the pivotal media parameters were investigated using RSM and Box–Behnken design. The value of optimization based on RSM and Box–Behnken design was affirmed by an experiment, which indicated that high yields of protoplasts were obtained when the optimized parameters were used. Further studies are required to ensure that this optimization approach is effective for the large-scale production of *A. placenta* protoplasts.

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components using response surface methodology for lipase production by Rhizopus

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for the production of elastase by Bacillus sp. EL31410 with response surface


**Figure legends**

**Fig. 1:** Interactive effects of enzyme concentration ($X_1$) and digestion time ($X_2$) on protoplast yield ($Y_1$) of *A. placenta* keeping $X_3$ at its optimum level ($X_3 = 0$, as predicted by 3-D response surface and 2-D contour plots).

**Fig. 2:** Interactive effects of enzyme concentration ($X_1$) and regeneration time ($X_3$) on protoplast yield ($Y_1$) of *A. placenta* keeping $X_2$ at its optimum level ($X_2 = 0$, as predicted by 3-D response surface and 2-D contour plots).

**Fig. 3:** Interactive effects of digestion time ($X_2$) and regeneration time ($X_3$) on protoplast yield ($Y_1$) of *A. placenta* keeping $X_1$ at its optimum level ($X_1 = 0$, as predicted by 3-D response surface and 2-D contour plots).

**Fig. 4:** PCR characterization of phosphinothricin-resistant transformants (PCR product with a size of 500 bp).
Fig. 1
Fig. 2
Fig. 3
Fig. 4

Lane 1, marker (2000 bp); lanes 2-5, positive clones; lane 6, negative control (untreated fungal cells); lane 7, positive control (PbarGPE).
Table 1. Plackett-Burman design matrix and results of screening of factors affecting protoplast yield by *A. placenta*.

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</tbody>
</table>

Note: X₂, X₄ and X₆ were dummy variables and other codes were preparation conditions of protoplasts to be optimized. Values for protoplasts are means (SE) of triplicate treatments.
Table 2. *A. placenta* protoplast yield as affected by levels of preparation conditions and as predicted by the Plackett–Burman design.

<table>
<thead>
<tr>
<th>code</th>
<th>Parameter name</th>
<th>Levels</th>
<th>Statistics for observed data</th>
<th>Note: <em>Statistical significance for comparison of Low vs. High level at the 95% probability level.</em>* Statistical significance for comparison of Low vs. High level at the 99% probability level.</th>
</tr>
</thead>
<tbody>
<tr>
<td>X₁</td>
<td>Enzyme concentration (mg/ml)</td>
<td>20 30</td>
<td>50.73327 0.0057**</td>
<td></td>
</tr>
<tr>
<td>X₂</td>
<td>Dummy</td>
<td>-1 1</td>
<td>6.33564 0.0864</td>
<td></td>
</tr>
<tr>
<td>X₃</td>
<td>pH</td>
<td>5.8 6.2</td>
<td>5.552581 0.0997</td>
<td></td>
</tr>
<tr>
<td>X₄</td>
<td>Dummy</td>
<td>-1 1</td>
<td>0.413002 0.5662</td>
<td></td>
</tr>
<tr>
<td>X₅</td>
<td>Digestion time (h)</td>
<td>1.0 5.0</td>
<td>46.25908 0.0065**</td>
<td></td>
</tr>
<tr>
<td>X₆</td>
<td>Dummy</td>
<td>-1 1</td>
<td>6.608031 0.0824</td>
<td></td>
</tr>
<tr>
<td>X₇</td>
<td>Digestion temperature (℃)</td>
<td>28 32</td>
<td>1.147228 0.3626</td>
<td></td>
</tr>
<tr>
<td>X₈</td>
<td>Regeneration time (h)</td>
<td>36 72</td>
<td>26.98566 0.0139*</td>
<td></td>
</tr>
</tbody>
</table>
Table 3. Design and results of the steepest ascent experiment for protoplast yield by *A. placenta*.

<table>
<thead>
<tr>
<th>Run</th>
<th>Enzyme concentration (mg/ml)</th>
<th>Digestion time (h)</th>
<th>Incubation time (h)</th>
<th>Protoplast yield ($\times 10^7$ cells/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>25.0</td>
<td>3.0</td>
<td>54.0</td>
<td>2.72 (0.11)</td>
</tr>
<tr>
<td>Δ</td>
<td>0.5</td>
<td>0.5</td>
<td>5.0</td>
<td>0.00</td>
</tr>
<tr>
<td>X+Δ</td>
<td>25.5</td>
<td>3.5</td>
<td>59.0</td>
<td>2.79 (0.04)</td>
</tr>
<tr>
<td>X+2Δ</td>
<td>26.0</td>
<td>4.0</td>
<td>64.0</td>
<td>3.22 (0.03)</td>
</tr>
<tr>
<td>X+3Δ</td>
<td>26.5</td>
<td>4.5</td>
<td>69.0</td>
<td>2.98 (0.05)</td>
</tr>
<tr>
<td>X+4Δ</td>
<td>27.0</td>
<td>5.0</td>
<td>74.0</td>
<td>2.58 (0.05)</td>
</tr>
<tr>
<td>X+5Δ</td>
<td>27.5</td>
<td>5.5</td>
<td>79.0</td>
<td>2.47 (0.18)</td>
</tr>
</tbody>
</table>

**Note:** This steepest ascent experiment involved five steps as indicated by the numbers 1 - 5 following the plus sign. Values for protoplasts are means (SE) of triplicate treatments.
Table 4. The range and the level of conditions individually tested in BBD for production of *A. placenta* protoplasts.

<table>
<thead>
<tr>
<th>Code</th>
<th>Condition for protoplast production</th>
<th>Level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-1</td>
</tr>
<tr>
<td>X₁</td>
<td>Enzyme concentration (mg/ml)</td>
<td>25.5</td>
</tr>
<tr>
<td>X₂</td>
<td>Digestion time (h)</td>
<td>3.5</td>
</tr>
<tr>
<td>X₃</td>
<td>Regeneration time (h)</td>
<td>59</td>
</tr>
</tbody>
</table>

**Note:** The results of the experiment that used these levels are described in Table 5.
Table 5. Box-Behnken design matrix for optimization of protoplast production by *A. placenta*.

<table>
<thead>
<tr>
<th>Run</th>
<th>$X_1$</th>
<th>$X_2$</th>
<th>$X_3$</th>
<th>Protoplast yield ($\times 10^7$ cells/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-1</td>
<td>-1</td>
<td>0</td>
<td>2.44 (0.19)</td>
</tr>
<tr>
<td>2</td>
<td>-1</td>
<td>1</td>
<td>0</td>
<td>1.82 (0.03)</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>-1</td>
<td>0</td>
<td>2.23 (0.12)</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>2.87 (0.11)</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>-1</td>
<td>-1</td>
<td>2.28 (0.06)</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>-1</td>
<td>1</td>
<td>2.99 (0.13)</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>1</td>
<td>-1</td>
<td>2.32 (0.13)</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>3.20 (0.09)</td>
</tr>
<tr>
<td>9</td>
<td>-1</td>
<td>0</td>
<td>-1</td>
<td>2.18 (0.05)</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>0</td>
<td>-1</td>
<td>2.65 (0.05)</td>
</tr>
<tr>
<td>11</td>
<td>-1</td>
<td>0</td>
<td>1</td>
<td>2.18 (0.17)</td>
</tr>
<tr>
<td>12</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>2.57 (0.14)</td>
</tr>
<tr>
<td>13</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3.91 (0.06)</td>
</tr>
<tr>
<td>14</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4.00 (0.07)</td>
</tr>
<tr>
<td>15</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3.88 (0.09)</td>
</tr>
</tbody>
</table>

**Note:** See Table 4 for explanation of $X_1$, $X_2$, $X_3$, and their levels. Values for protoplasts are means (SE) of triplicate treatments.
Table 6. ANOVA for response surface quadratic model of *A. placenta* protoplast yield.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>9</td>
<td>6.420965</td>
<td>0.713441</td>
<td>9.824975</td>
<td>0.0108</td>
</tr>
<tr>
<td>Error (Lack of fit)</td>
<td>3</td>
<td>0.355275</td>
<td>0.118425</td>
<td>30.36538</td>
<td>0.0321</td>
</tr>
<tr>
<td>(Pure error)</td>
<td>2</td>
<td>0.0078</td>
<td>0.0039</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
<td>6.78404</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$R^2$</td>
<td></td>
<td>0.9465</td>
<td>Adjusted $R^2$</td>
<td>0.8501</td>
<td></td>
</tr>
</tbody>
</table>