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

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2 **transformation using optimization algorithms**

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1 **Abstract:** The insect pathogenic fungus, *Aschersonia placenta* is a highly effective
2 pathogen of whiteflies and scale insects. Few genetic tools, however, are currently
3 available for this organism. Here we report on the conditions for the production of
4 transformable *A. placenta* protoplasts using an optimized protocol based on the
5 response surface method (RSM). Critical parameters for protoplast production were
6 modelled by using a Box-Behnken design (BBD) involving three levels of three
7 variables, and subsequently experimentally verified in its predictability of protoplast
8 production ($R^2 = 0.9465$). The optimized conditions resulted in the highest yield of
9 protoplasts ($4.41 \pm 0.02 \times 10^7$ cells/ml of culture, mean $\pm$ SE) when fungal cells were
10 treated with 26.1 mg/ml of lywallzyme for 4 h of digestion, and subsequently allowed
11 to recover for 64.6 h in 0.7 M NaCl-Tris buffer. The latter used as an osmotic
12 stabilizer. The yield of protoplasts was approximately 10-fold higher than that of the
13 non-optimized conditions. Generated protoplasts were transformed with vector
14 PbarGPE containing *bar* gene as the selection marker. Transformation efficiency was
15 300 colonies/ μ g DNA/ 10^7 protoplasts, and integration of the vector DNA was
16 confirmed by PCR. The results show that rational design strategies (RSM and BBD
17 methods) are useful to increase the production of fungal protoplasts for a variety of
18 downstream applications.

19

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

22

1 **Introduction**

2 Biologically based insecticides are of wide-spread interest in biological control
3 approaches due to their more environmentally friendly effects when compared to
4 chemical agents, and are considered more renewable and sustainable (Glare et al.
5 2012). *Aschersonia*, a fungal pathogen of insects, is specifically used to control
6 whiteflies (*Aleyrodidae*) and scale insects (*Coccidae*) (Meekes et al. 2002; Qiu et al.
7 2010, 2013; Wang et al. 2013) and can cause significant epizootic infections in these
8 insect populations in the tropics and subtropics (Evans and Hywel-Jones 1990).
9 Natural *Aschersonia* infections have been shown to impact the population dynamics
10 of the woolly whitefly, *Aleurothrixus floccus*, on sweet orange (Umeh et al. 2011), and
11 new species of this insect pathogen have been continuously isolated (Qiu et al. 2010,
12 2012). These fungi are also rich in the production of secondary metabolites of
13 biotechnological interest including cytotoxic xanthenes, cyclodepsipeptide toxins, and
14 antimicrobial compounds (Isaka et al. 2005, 2012; Kornsakulkarn et al. 2013).

15 For biological control applications, many wild-type strains are not ideal since
16 obstacles exist due to low survival/persistence in natural environments, difficulties in
17 (large-scale) propagation as well as storage of virulent spores (Rodriguez-Iglesias and
18 Schmoll 2015). Genetic engineering, however, offers a possible route towards
19 improving the efficacy of fungal strains, as well as a means for natural products
20 discovery of bioactive compounds. To the best of our knowledge, to date there are no
21 reports of a transformation system for *Aschersonia*. *Agrobacterium tumefaciens*
22 mediated transformation has been successfully used for other entomopathogenic fungi,

1 including *Metarhizium anisopliae* and *Beauveria bassiana* (Fang et al. 2004, 2006),
2 however preliminary attempts to use such a system for *Aschersonia* were unsuccessful
3 (Qiu unpublished observation). Production of cellular protoplasts via enzymatic
4 treatment of the fungal cell wall is a well-known method for production of
5 “competent” fungal cells capable of taking up exogenous DNA (Rodriguez-Iglesias
6 and Schmoll 2015). These methods require the efficient isolation and regeneration of
7 protoplasts, a process yet to be developed for *Aschersonia*.

8 The objective of this study was to develop a system to promote protoplast
9 production of *Aschersonia*. We employed a rational experimental design strategy
10 incorporating response surface methodology (RSM) and Box–Behnken design (BBD),
11 a process more time and cost effective than traditional variation of one parameter at a
12 time (Ghosh and Hallenbeck 2010). RSM combines mathematical and statistical
13 techniques to design experimental trajectories including model building, identification
14 of effective factors, examining potential interactions between parameters, and
15 searching for optimal conditions, which thereby eliminates the limitations of
16 single-factor optimization (Bandaru et al. 2006; Tabandeh et al. 2008; Tan et al. 2010).
17 RSM was applied to find appropriate enzymatic hydrolysis conditions for *A. placenta*
18 protoplast formation and cultivation conditions that would enhance protoplast
19 production. We also used Box–Behnken design (BBD) to examine important
20 conditions and to identify optimal production parameters. BBD has been previously
21 used in biological control applications and optimization of the medium composition
22 resulted in a defined biphasic production system for mycelium growth and spore

1 production of *Aschersonia placenta* (Feng et al. 1994; Shih et al. 2007; Dong et al.
2 2009; Qiu et al. 2013). With respect to the optimized conditions, there was a 10-fold
3 increase in protoplast yield. Moreover, protoplasts displayed high transformation
4 efficiencies reaching up to 300 colonies/ μg DNA/ 10^7 protoplasts.

5

6 **Materials and methods**

7 **Culture strain**

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

14

15 **Induction of protoplasts**

16 A set of initial experiments were performed to determine the optimal osmotic
17 stress stabilizer for *A. placenta* conidia, which was determined to be 0.7 M NaCl in 20
18 mM Tris buffer, pH = 6.0 (NaCl-Tris buffer). Lywallzyme (Sigma Chemical Co,
19 Beiruo, China) was dissolved in this buffer with different concentrations as detailed
20 below. Conidia produced on PDA (20 d) were scraped and suspended in sterile water
21 containing 0.05% (v/v) Tween 80 (Sigma Chemical Co, Beiruo, China). Conidial
22 concentration was measured using a haemocytometer and diluted to desired

1 concentration (typically 1×10^8 conidia/ml) for further use (Qiu et al. 2013). One ml of
2 conidial suspension was inoculated onto 100 ml potato dextrose broth (PDB) liquid
3 media and cultured for 2-3 d at 26°C before harvesting for protoplast production.
4 Fungal biomass was collected on a sterilized filter (Whatman filter papers, pore size
5 15-18 μm ; Sigma Chemical Co, Beiruo, China) and the hyphae were washed using
6 NaCl-Tris buffer until the wash fluid was achromatic. The washed hyphae were
7 ground in a mortar and pestle with a small amount of buffer on ice. The ground
8 hyphae were placed into a 50 ml centrifuge tube with 20 ml potato dextrose broth
9 (PDB) and incubated at 28°C for 16 h with aeration (150 rpm). After incubation, the
10 hyphae were collected by filtration and resuspended in the indicated solution
11 containing lywallzyme. Treatment variables included temperature and time, after
12 which the cells were harvested by centrifugation (4000 rpm for 10 min), and the cell
13 pellet was resuspended in NaCl-Tris buffer with various pH values. Experimental
14 factor parameters, i.e. cell culture time, pH, lywallzyme concentration, digestion
15 temperature and regeneration time are shown in Tables 1 and 2.

16

17 **Experimental design and statistical analyses**

18 *Preliminary experiments: The effects of enzyme concentration, digestion time, and* 19 *other parameters on A. placenta protoplast formation*

20 To investigate the influence of enzyme concentration and digestion time on
21 production of *A. placenta* protoplasts, different concentrations of lywallzyme (1, 5, 10,
22 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 Prabhakar 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

1 coefficient R^2 (Qiu et al. 2013). Response surface contour plots were formed to
2 demonstrate the possibility of enhancing yield of protoplasts.

3

4 ***Treatment of data***

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

9

10 **PEG-mediated transformation of protoplasts**

11 Regenerated protoplasts (200 μ l per tube, $\sim 5 \times 10^7$ protoplasts/ml) of *Aschersonia*
12 *placenta* were gently mixed with 15 μ l of 100 μ g/ml plasmid PbarGPE, containing the
13 *bar* selection marker for resistance to the herbicide phosphinothricin (PPT) and used
14 for PEG-mediated transformation as described elsewhere (Datta et al. 1992; Fan et al.
15 2011). Briefly, cells were incubated on ice with plasmid DNA for 45 min and then
16 subjected to heat shock at 42°C for 20 min. The cells were harvested by centrifugation
17 (6000g, 3 min) at 4°C. The transformation mixture was plated on Czapek-Dox (CZA)
18 agar containing 0.12 mg/ml PPT. Transformants were single spore purified and
19 subcultured on CZA-PPT plates as indicated.

20

21 **Characterization of transformants by PCR**

22 Purified transformants were grown in CZA and their genomic DNA were extracted

1 as described (Fang et al. 2004). Polymerase chain reaction (PCR) analysis was
2 performed in 25 µl reaction mixture containing 1 µl each of primers (10 µM) designed
3 to the *bar* gene, F1 (5'- GCAGGAACCGCAGGAGTGGA -3') and R1
4 (5'-ATCTCGGTGACGGGCAGGAC-3'), 0.5 U of *Taq* polymerase, 5 µl buffer, and
5 genomic DNA, using the following protocol: 5 min 94°C, followed by 35 cycles of
6 denaturation (60 s at 94°C), annealing (45 s at 60°C), and extension (60 s at 72°C).
7 PCR products were analyzed by agarose gel electrophoresis.

8

9 **Results**

10 The influence of different enzyme concentrations on the production of protoplasts
11 was initially examined. Production of protoplasts was highest at a concentration of 25
12 mg/ml, yielding $1.11 \pm 0.13 \times 10^7$ cells/ml. With respect to digestion time, production
13 of protoplasts was highest when 4 h were chosen as the tested period, yielding $1.02 \pm$
14 0.11×10^7 cells/ml. The series of experiments demonstrated that the major factors
15 affecting the protoplast yields were 25 mg/ml lywallzyme, digestion time with also
16 including digestion temperature, pH and regeneration time (data not shown). Thus,
17 these conditions were tested for further optimization of the variables.

18

19 **Plackett–Burman design**

20 Plackett-Burman design (PBD) was used to assess the relative contributions of
21 different variables in impacting *A. placenta* protoplast yield. Preliminary experiments
22 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 ($1-5\Delta$). Protoplast yield peaked ($3.22 \pm 0.03 \times 10^7$ cells/ml) in the second step ($X + 2\Delta$), 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

1 displayed in Table 5, with the statistical analyses (ANOVA) of the results given in
2 Table 6. The results show a high confidence that the model equation sufficiently
3 depicts the response surface. The polynomial models were mathematically expressed
4 as follows:

$$\begin{aligned} \text{YP} = & 3.93 + 0.2125X_1 + 0.03375X_2 + 0.18875X_3 - 0.94625X_1^2 - 0.64375X_2^2 - \\ & 0.58875X_3^2 + 0.315X_1X_2 - 0.02X_1X_3 - 0.0425X_2X_3 \end{aligned}$$

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

18

19 Transformation of the protoplasts

20 Protoplasts (1×10^7) were prepared using the optimized condition as described
21 above and transformed with the plasmid PbarGPE containing the *bar* selection gene
22 marker. A high transformation efficiency of 300 colonies/ μg DNA/ 10^7 protoplasts was

1 obtained in the test. However, untreated fungal cells gave no transformants. The
2 stability of gene transformation was proved after subculturing transformants for four
3 generations on CZA plates in the presence of phosphinothricin. Genomic DNA of four
4 selected transformants obtained from each generation was extracted and the existence
5 of the *bar* gene selection marker was examined using PCR. A PCR amplified
6 fragment of 500 bp corresponding to the expected size was detected in all of the
7 transformants (Fig. 4).

8

9 Discussion

10 Entomopathogenic fungi are promising biological control agents capable of
11 targeting a wide range of agricultural pests (Lomer et al. 2001). The ability to
12 genetically manipulate these organisms can be a powerful tool in increasing the
13 efficacy for diverse applications. Moreover, genetic approaches allow fundamental
14 research aimed towards understanding the molecular aspect of the host-pathogen
15 interaction. It is of significant importance to study *A. placenta* because of its host
16 specificity. Towards developing genetic tools for *A. placenta*, preliminary
17 experiments using *A. tumefaciens*, an approach successful with many other fungi
18 (include several insect pathogens) were not successful due to the low transformation
19 efficiency (Keyhani, unpublished data). Protoplasts, cells in which the outer cell wall
20 of the fungus has been partially degraded, are well known to be “competent” in their
21 ability to uptake DNA as compared to their untreated counterparts (Rodriguez-Iglesias
22 and Schmoll 2015). We therefore sought to optimize the production of protoplasts

1 useful for various downstream applications including transformation.

2 Optimized algorithm combining sequential experiments based upon

3 Plackett-Burman and Box-Behnken designs, and response surface outputs were used

4 to optimize *A. placenta* protoplast yield. Validation at various stages was examined

5 using the R^2 coefficient of determination. The R^2 value indicates the degree of

6 variability on the basis of the experimental parameters and their interactions (Qiu et al.

7 2013). The ability of models to forecast response values increases as R^2 values

8 approach 1.0 (Acikel et al. 2010). In the results presented, the observed high R^2 values

9 (> 0.946) support the idea that the model equations can adequately predict the

10 responses. The 3-D response surfaces and 2-D contour plots generated based on the

11 model equations were used to identify the optimal parameter conditions (Figs. 1–3).

12 Their shapes show the nature and extent of the interactions of experimental variables

13 (Prakash et al. 2008; Qiu et al. 2013). The peaks in the 3-D response surface and 2-D

14 contour plots indicate the optimal points (i.e. the best predicted experimental

15 conditions) within the design limits. Each figure examines the effects of two factors

16 on protoplast yield when the third factor is maintained at the peak level. The 3-D plot

17 also shows that the interactions among the three individual factors significantly

18 influenced protoplast yield. By coupling modeling to select experimental verification,

19 our results indicate a significantly streamlined process in which experimental

20 conditions were optimized, reducing both effort and cost. As noted earlier, the

21 optimum values for the three variables affecting protoplast yield were 26.1 mg/ml for

22 enzyme concentration, 4 h for digestion time and 64.6 h for regeneration time and the

1 predicted yield was $4.38 \pm 0.07 \times 10^7$ cells/ml whereas the actual yield was $4.41 \pm$
2 0.02×10^7 cells/ml. However, other biological factors related to the increase of
3 protoplast yield were not investigated.

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

10

11 Conclusion

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

20

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4

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Figure legends

Fig. 1: Interactive effects of enzyme concentration (X_1) and digestion time (X_2) on protoplast yield (Y1) 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 (Y1) 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 (Y1) 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).

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Table 1. Plackett-Burman design matrix and results of screening of factors affecting protoplast yield by *A. placenta*.

RUN	X ₁	X ₂	X ₃	X ₄	X ₅	X ₆	X ₇	X ₈	Protoplast yield (×10 ⁷ cells/ml)
1	1	-1	1	-1	-1	-1	1	1	2.39 (0.11)
2	1	1	-1	1	-1	-1	-1	1	2.71 (0.34)
3	-1	1	1	-1	1	-1	-1	-1	2.16 (0.21)
4	1	-1	1	1	-1	1	-1	-1	2.12 (0.04)
5	1	1	-1	1	1	-1	1	-1	2.73 (0.15)
6	1	1	1	-1	1	1	-1	1	2.98 (0.06)
7	-1	1	1	1	-1	1	1	-1	2.00 (0.17)
8	-1	-1	1	1	1	-1	1	1	2.35 (0.05)
9	-1	-1	-1	1	1	1	-1	1	2.65 (0.06)
10	1	-1	-1	-1	1	1	1	-1	2.84 (0.25)
11	-1	1	-1	-1	-1	1	1	1	2.33 (0.26)
12	-1	-1	-1	-1	-1	-1	-1	-1	1.62 (0.32)

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.

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Table 2. *A. placenta* protoplast yield as affected by levels of preparation conditions and as predicted by the Plackett–Burman design.

code	Parameter name	Levels		Statistics for observed data	
		Low (-1)	High(+1)	<i>t</i>	Pr> <i>t</i>
X ₁	Enzyme concentration (mg/ml)	20	30	50.73327	0.0057**
X ₂	Dummy	-1	1	6.335564	0.0864
X ₃	pH	5.8	6.2	5.552581	0.0997
X ₄	Dummy	-1	1	0.413002	0.5662
X ₅	Digestion time (h)	1.0	5.0	46.25908	0.0065**
X ₆	Dummy	-1	1	6.608031	0.0824
X ₇	Digestion temperature (°C)	28	32	1.147228	0.3626
X ₈	Regeneration time (h)	36	72	26.98566	0.0139*

Note: *Statistical significance for comparison of Low vs. High level at the 95% probability level.

** Statistical significance for comparison of Low vs. High level at the 99% probability level.

Table 3. Design and results of the steepest ascent experiment for protoplast yield by *A. placenta*.

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

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.

Code	Condition for protoplast production	Level		
		-1	0	1
X ₁	Enzyme concentration (mg/ml)	25.5	26.0	26.5
X ₂	Digestion time (h)	3.5	4.0	4.5
X ₃	Regeneration time (h)	59	64	69

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*.

Run	X ₁	X ₂	X ₃	Protoplast yield (×10 ⁷ cells/ml)
1	-1	-1	0	2.44 (0.19)
2	-1	1	0	1.82 (0.03)
3	1	-1	0	2.23 (0.12)
4	1	1	0	2.87 (0.11)
5	0	-1	-1	2.28 (0.06)
6	0	-1	1	2.99 (0.13)
7	0	1	-1	2.32 (0.13)
8	0	1	1	3.20 (0.09)
9	-1	0	-1	2.18 (0.05)
10	1	0	-1	2.65 (0.05)
11	-1	0	1	2.18 (0.17)
12	1	0	1	2.57 (0.14)
13	0	0	0	3.91 (0.06)
14	0	0	0	4.00 (0.07)
15	0	0	0	3.88 (0.09)

Note: See Table 4 for explanation of X₁, X₂, X₃, 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.

Source	DF	SS	MS	F	Pr > F
Model	9	6.420965	0.713441	9.824975	0.0108
Error	5	0.363075	0.072615		
(Lack of fit)	3	0.355275	0.118425	30.36538	0.0321
(Pure error)	2	0.0078	0.0039		
Total	14	6.78404			
R^2	0.9465	Adjusted R^2	0.8501		

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