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Fermentation optimization for the production of lovastatin by *Aspergillus terreus*: use of response surface methodology

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Abstract: A Box–Behnken experimental design was used to investigate the effects of five factors—ie oxygen content in the gas phase; concentrations of C, N and P; and fermentation time—on the concentrations of biomass and lovastatin produced in batch cultures of *Aspergillus terreus*. The values of the various factors in the experiment ranged widely, as follows: 20–80% (v/v) oxygen in the aeration gas; 8–48 g dm^{-3} C-concentration; 0.2–0.6 g dm^{-3} N-concentration; 0.5–2.5 g dm^{-3} phosphate-concentration; and 7–11 days fermentation time. No previous work has used statistical analysis in documenting the interactions between oxygen supply and nutrient concentrations in lovastatin production. The Box–Behnken design identified the oxygen content in the gas phase as the principal factor influencing the production of lovastatin. Both a limitation and excess of oxygen reduced lovastatin titers. A medium containing 48 g dm^{-3} C supplied as lactose, 0.46 g dm^{-3} N supplied as soybean meal, and 0.79 g dm^{-3} phosphate supplied as KH_2PO_4, was shown to support high titers (~230 mg dm^{-3}) of lovastatin in a 7-day fermentation in oxygen-rich conditions (80% v/v oxygen in the aeration gas). Under these conditions, the culture medium had excess carbon but limiting amounts of nitrogen. The optimized fermentation conditions raised the lovastatin titer by four-fold compared with the worst-case scenario within the range of factors investigated.

Keywords: lovastatin; *Aspergillus terreus*; fermentation optimization; response surface methodology

1 INTRODUCTION

Lovastatin ($C_{24}H_{36}O_5$) is a potent drug for lowering blood cholesterol. Lovastatin acts by competitively inhibiting the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA)[1,2] which catalyzes the rate limiting step of cholesterol biosynthesis. Lovastatin also inhibits tumor growth through the inhibition of nonsterol isoprenoid synthesis.[3,4] Furthermore, fermentation-derived lovastatin is a precursor for simvastatin, a powerful semi-synthetic statin commercially available as Zocor™. Simvastatin is obtained via a selective enzymatic deacylation of lovastatin.[5]

Lovastatin is produced as a secondary metabolite by a variety of filamentous fungi including *Penicillium* sp,[6] *Monascus ruber*[7,8] and *Aspergillus terreus*.[2] Commercial production of lovastatin is based on *A terreus* batch fermentation and most of the literature deals with this species.[9–14] *Aspergillus terreus* fermentations are typically carried out at ~28 °C and pH 5.8–6.3.[13] The dissolved oxygen level is controlled at ≥40% of air saturation.[13] A batch fermentation generally runs for less than 10 days. At least in some cases, pelleted growth of *A terreus* has yielded higher titers of lovastatin than obtained with filamentous growth.[13] Uncontrolled filamentous growth occurs when using rapidly metabolized substrates. The rapid increase in viscosity accompanied by filamentous growth greatly impedes oxygen transfer and this is said to explain the low titers of lovastatin.[13]

The composition of a fermentation medium influences the supply of nutrients and metabolism of cells in a bioreactor and, therefore, the productivity of a fermentation process also depends on the culture medium used. Of the major culture nutrients, carbon and nitrogen sources generally play a dominant role in fermentation productivity because these nutrients are directly linked with the formation of biomass and metabolites. Also, the nature and concentration of the carbon source can regulate secondary metabolism through phenomena such as catabolic repression. Biosynthesis of lovastatin has been found to depend on the carbon and nitrogen sources.[11,12,14]

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Because of the large number of quantitative and qualitative variables involved in a bioprocess, methods of statistical experimental design are used in many studies for optimizing fermentation media.[15] To reduce the experimental workload, simple batch cultures in a parallel approach are carried out in shake flasks.[16] In general, the procedure applied can be subdivided into four steps: identification of the most important media components, identification of the variable range, search for the optimum value of the variables and experimental verification of the optimum.[16]

Other studies have used response surface methodology (RSM) in identifying the impact of the medium composition on lovastatin production. With a high-producing mutant of *A terreus*, Lai *et al*[17] evaluated the effects of concentrations of several carbon and nitrogen sources and observed a significant interactive effect of the medium constituents on lovastatin titers. Similarly, the RSM was used to optimize the culture medium for producing lovastatin from *M ruber*.[18] No report exists on any interactive effects of dissolved oxygen and the other nutrients on the production of lovastatin.

In the present work, the fungus *Aspergillus terreus* ATCC 20542 was used to produce lovastatin by batch fermentation. Media components screening and variable range setting were carried out in a previous work.[19] The response surface method, a powerful technique for testing multiple process variables, was selected for identifying the optimum process conditions. RSM can help identify and quantify the various interactions among variables. The effects of fermentation time, oxygen availability, carbon, nitrogen and phosphate concentrations on the yield of lovastatin are reported.

A Box–Behnken statistical design of experiments was used. This design comprised five factors at three levels of variation to permit an unconfounded estimation of the regression coefficients. The factors and the levels of variation were: 20%, 50% and 80% oxygen in air; 8, 28 and 48 g dm^{-3} carbon concentration; 0.2, 0.4 and 0.6 g dm^{-3} nitrogen concentration; 0.5, 1.5 and 2.5 g dm^{-3} phosphate concentration; and 7, 9 and 11 days of culture. The responses were the biomass concentration and lovastatin concentration.

2 MATERIALS AND METHODS

2.1 Microorganism and inoculation

The fungus used was obtained from the American Type Culture Collection, as *Aspergillus terreus* ATCC 20 542. The culture was maintained on Petri dishes of PDA (potato dextrose agar). After inoculation from the original slant, the dishes were incubated at 28 °C for 5 days and subsequently stored at 5 °C. A suspension of spores was obtained by washing the Petri dish cultures with a sterile aqueous solution of 2% Tween® 20. The resulting suspension was centrifuged (~2800 *g*, 5 min) and the solids were resuspended in sterile distilled water. The spore concentration was determined spectrophotometrically at 360 nm. A standard curve was used to correlate the optical density to direct spore counts carried out with a flow cytometer (Coulter Epics XL-MCL).

2.2 Culture conditions

All fermentations were carried out at 28 °C in 100 cm^3 shake flasks filled with 50 cm^3 of the medium held on a rotary platform shaker (150 rpm, stroke 2.6 cm). Flasks without cotton closures were located in a closed air-controlled growth chamber. Gas with a given oxygen content entered the chamber through a 0.2 μm sterile Millipore air filter at a flow rate high enough to provide a constant gas composition within the chamber. Lactose was the carbon source and nitrogen was provided as soybean meal. The flasks were inoculated with 900 mm^3 of a spore suspension which had been standardized to contain 9×10^7 spores cm^{-3}. The culture lasted up to 11 days. The soybean meal used contained 8.5% w/w nitrogen and 43.3% w/w carbon. The carbon source (lactose) did not contain any nitrogen. The carbon content (% by dry weight) of lactose was 39.96. Phosphate was provided as KH_2PO_4.

In addition to the components in Table 1, the media contained the following (per dm^3): 0.52 g $MgSO_4 \cdot 7H_2O$, 0.40 g NaCl, 2 mg $Fe(NO_3)_3 \cdot 9H_2O$, 1 mg $ZnSO_4 \cdot H_2O$, 0.04 mg biotin and 1 cm^3 of a trace element solution. The trace element solution contained (for 1 dm^3 of solution): $Na_2B_4O_7 \cdot 10H_2O$, 100 mg; $MnCl_2 \cdot 4H_2O$, 50 mg; $Na_2MoO_4 \cdot 2H_2O$, 50 mg and $CuSO_4 \cdot 5H_2O$, 250 mg. The pH of the medium was adjusted to 6.5 with NaOH (0.1 N) before sterilization.

2.3 Analytical methods

2.3.1 Biomass

The biomass dry weight was determined by filtering a known volume of the broth through a 0.45 μm Millipore cellulose filter, washing with sterile distilled water and freeze-drying the solids.

2.3.2 Lovastatin

Lovastatin was determined as its beta hydroxyacid, by high performance liquid chromatography (HPLC) of the biomass-free filtered broth.[20] Because the fungus secretes lovastatin in the beta hydroxyacid form, the assay eliminated the conversion step to the active lactone form of the drug. Using the beta hydroxyacid permitted rapid analysis because this form elutes earlier from a chromatography column than does the lactone form of lovastatin. Also, the beta hydroxyacid is quite stable in solution. The filtered broth containing the beta hydroxyacid form of lovastatin was diluted 10-fold with acetonitrile–water (1:1 by vol) prior to analysis.[21]

Pharmaceutical-grade lovastatin (lactone form) tablets (Nergadan® tablets; J Uriach & Cía, SA)

Table 1. Factor values and the responses of Box–Behnken design runs (figures in bold represent central point replicates)

Run	Oxygen (% v/v)	Carbon ($g\,dm^{-3}$)	Nitrogen ($g\,dm^{-3}$)	Phosphate ($g\,dm^{-3}$)	Time (*d*)	Lovastatin ($mg\,dm^{-3}$)	Biomass ($g\,dm^{-3}$)
1	20	28	0.4	0.5	9	85.8	7.91
2	50	28	0.6	1.5	11	72.6	13.41
3	20	8	0.4	1.5	9	66.1	6.10
4	80	28	0.6	1.5	9	200.8	12.59
5	50	8	0.4	2.5	9	131.6	6.09
6	80	28	0.4	1.5	11	162.6	8.45
7	50	28	0.2	0.5	9	130.3	4.98
8	50	8	0.4	1.5	11	98.7	4.80
9	20	48	0.4	1.5	9	63.4	9.25
10	50	8	0.6	1.5	9	97.5	5.04
11	20	28	0.4	1.5	7	93.9	8.80
12	20	28	0.4	1.5	11	123.4	8.36
13	50	48	0.2	1.5	9	84.1	3.40
14	50	28	0.4	0.5	7	145.9	8.92
15	80	28	0.4	0.5	9	149.1	7.98
16	20	28	0.6	1.5	9	47.2	13.51
17	80	28	0.4	2.5	9	179.1	7.95
18	50	28	0.6	0.5	9	61.9	13.24
19	50	8	0.2	1.5	9	73.5	3.93
20	**50**	**28**	**0.4**	**1.5**	**9**	**126.1**	**9.46**
21	**50**	**28**	**0.4**	**1.5**	**9**	**131.1**	**9.66**
22	50	48	0.4	2.5	9	166.4	8.16
23	50	8	0.4	1.5	7	127.2	6.61
24	50	28	0.2	1.5	11	104.6	4.07
25	**50**	**28**	**0.4**	**1.5**	**9**	**115.0**	**5.95**
26	20	28	0.4	2.5	9	121.1	8.03
27	50	28	0.2	2.5	9	99.6	4.07
28	50	48	0.4	1.5	7	188.6	8.61
29	50	28	0.2	1.5	7	107.5	4.37
30	80	8	0.4	1.5	9	104.8	4.61
31	20	28	0.2	1.5	9	110.7	4.17
32	50	8	0.4	0.5	9	89.6	5.34
33	50	48	0.4	0.5	9	144.9	8.05
34	50	28	0.6	1.5	7	78.3	13.87
35	50	28	0.4	2.5	11	166.3	8.21
36	50	28	0.4	0.5	11	117.4	8.11
37	80	28	0.2	1.5	9	72.6	2.97
38	50	48	0.4	1.5	11	167.9	8.61
39	50	48	0.6	1.5	9	94.5	11.39
40	**50**	**28**	**0.4**	**1.5**	**9**	**148.1**	**8.24**
41	80	48	0.4	1.5	9	175.1	7.58
42	**50**	**28**	**0.4**	**1.5**	**9**	**136.3**	**8.59**
43	**50**	**28**	**0.4**	**1.5**	**9**	**149.5**	**8.15**
44	50	28	0.6	2.5	9	117.8	10.65
45	80	28	0.4	1.5	7	202.8	8.49
46	50	28	0.4	2.5	7	183.0	9.06

containing 40 mg lovastatin per tablet were used to prepare the standards for the HPLC analyses. Prior to use, the lactone form of lovastatin was converted to the beta hydroxyacid form by dissolving the tablets in a mixture of 0.1 N NaOH and ethanol (1:1 by vol), heating at 50 °C for 20 min, and neutralizing with HCl. The resulting standard stock solution contained 400 mg lovastatin (beta hydroxyacid) per dm^3. The solution was held at 5 °C until needed.

HPLC was performed on a Beckman Ultrasphere ODS (250 × 4.6 mm id, 5 μm) column. The column was mounted in a Shimadzu model Lc10 liquid chromatograph equipped with a Shimadzu MX-10Av diode array detector. The eluent was a mixture of acetonitrile and 0.1% phosphoric acid (60:40 by vol). The eluent flow rate was 1.5 $cm^3\ min^{-1}$. The detection wavelength was 238 nm. The sample injection volume was 20 mm^3.

2.4 Experimental design

Response surface methodology (RSM) using a Box–Behnken design was applied to batch cultures of *A terreus*, for identifying the effects of process variables (fermentation time; oxygen concentration in the gas

phase; the concentrations of C, N, and P), on the final biomass concentration and the titer of lovastatin. RSM and analysis of variance (ANOVA) were performed using the Statgraphics™ software package (Manugistics Inc, Rockville, Maryland, USA).

Box–Behnken design uses a selection of face and central points to span an experimental space with fewer points than a complete factorial design.[22] Box–Behnken design is similar in intent to a central composite design, but differs in that no corner or extreme points are used. Box–Behnken designs are rotatable and use only three-level factors. In the present work, the design consisted of 46 runs with six replicates of the central point to determine the experimental error. Independent variables (oxygen in the gas phase; fermentation time; the concentrations of C, N, and P) and levels together with experimental results are given in Table 1 for the various runs.

3 RESULTS

The experimental runs and results for the Box–Behnken design are shown in Table 1. The 46 runs in a single block were used to study the effects of five factors on two responses. Biomass concentration ranged from 2.97 g dm^{-3} to 13.87 g dm^{-3} and lovastatin concentration varied from 47.2 mg dm^{-3} to 202.8 mg dm^{-3} for the complete experimental range. The ANOVA tables (Tables 2 and 3) give the statistical significance of the effects for biomass (Table 2) and lovastatin (Table 3) concentration responses.

With regard to biomass concentration, four effects had *P*-values of less than 0.05 (Table 2), indicating that they were significantly different from zero at the 95% confidence level. These effects were the nitrogen concentration, carbon concentration, the quadratic effect of carbon concentration, and the interaction between carbon and nitrogen concentrations. Considering the *F*-ratio statistic (Table 2), it was concluded that a change in nitrogen concentration caused the major variation in biomass concentration. This was because nitrogen was the limiting nutrient. The effect of fermentation time was not statistically significant as biomass was harvested at the stationary growth phase. The R^2 statistic (Table 2) indicated that the model as fitted explained 90.6% of the variability in biomass concentration. The adjusted R^2 statistic, which is more suitable for comparing models with different numbers of independent variables, was 83.0% (Table 2). The standard error of the estimate showed the standard deviation of the residuals to be 1.15. The mean absolute error, or the average value of the residuals was 0.641. The Durbin–Watson (DW) statistic tested the residuals to determine if there was any significant correlation based on the order in which they occurred in the data file. Because the DW value exceeded 1.4 (Table 2), there was probably no significant autocorrelation in the residuals.

In the case of lovastatin titer, six effects had *P*-values of less than 0.05 (Table 3). Oxygen content in the gas phase was the main source of variation in lovastatin biosynthesis. The effects of carbon and phosphate concentration were also statistically significant and nitrogen appeared as an important factor through its quadratic effect and the interactions with oxygen

Table 2. Analysis of variance for biomass (R^2 = 90.6%, R^2 (adjusted for df) = 83.0%, standard error of estimate = 1.154, mean absolute error = 0.641, Durbin–Watson statistic = 1.52)

Source	Sum of squares	df	Mean square	*F*-ratio	*P*-value
A: oxygen	1.89	1	1.89	1.42	0.245
B: carbon	31.77	1	31.77	23.87	0.000
C: nitrogen	238.22	1	238.22	179.01	0.000
D: phosphate	0.34	1	0.34	0.26	0.618
E: time	1.39	1	1.39	1.05	0.316
AA	0.03	1	0.03	0.02	0.876
AB	0.01	1	0.01	0.01	0.940
AC	0.02	1	0.02	0.02	0.900
AD	0.01	1	0.01	0.00	0.950
AE	0.04	1	0.04	0.03	0.862
BB	24.06	1	24.06	18.08	0.000
BC	11.82	1	11.82	8.88	0.006
BD	0.10	1	0.10	0.08	0.786
BE	0.82	1	0.82	0.61	0.441
CC	0.20	1	0.20	0.15	0.700
CD	0.71	1	0.71	0.53	0.472
CE	0.01	1	0.01	0.00	0.945
DD	0.03	1	0.03	0.02	0.878
DE	0.00	1	0.00	0.00	0.985
EE	1.68	1	1.68	1.27	0.271
Total error	33.27	25	1.33		
Total (corr)	352.69	45			

Table 3. Analysis of variance for lovastatin (R^2 = 84.9%, R^2 (adjusted for df) = 72.9%, standard error of estimate = 20.724, mean absolute error = 11.574, Durbin–Watson statistic = 2.45)

Source	Sum of squares	df	Mean square	*F*-ratio	*P*-value
A: oxygen	17 893.70	1	17 893.70	41.66	0.000
B: carbon	5474.52	1	5474.52	12.75	0.002
C: nitrogen	9.39	1	9.39	0.02	0.884
D: phosphate	3592.80	1	3592.80	8.37	0.008
E: time	808.41	1	808.41	1.88	0.182
AA	149.01	1	149.01	0.35	0.561
AB	1333.35	1	1333.35	3.10	0.090
AC	9189.14	1	9189.14	21.4	0.000
AD	6.66	1	6.66	0.02	0.902
AE	1211.74	1	1211.74	2.82	0.106
BB	1092.71	1	1092.71	2.54	0.123
BC	45.77	1	45.77	0.11	0.747
BD	104.86	1	104.86	0.24	0.626
BE	15.37	1	15.37	0.04	0.852
CC	12 531.30	1	12 531.30	29.18	0.000
CD	1877.92	1	1877.92	4.37	0.047
CE	2.02	1	2.02	0.00	0.946
DD	401.62	1	401.62	0.94	0.343
DE	34.99	1	34.99	0.08	0.778
EE	1062.97	1	1062.97	2.48	0.128
Total error	10 737.10	25	429.48		
Total (corr)	71 328.50	45			

and phosphate concentrations. Fermentation time had no effect in comparison with the other sources of variation. The R^2 statistic (Table 3) indicated that the model as fitted explained 84.9% of the variability in lovastatin concentration.

4 DISCUSSION

4.1 Biomass concentration

From the analysis of the data in Table 1 by the least-squares method, the following second-order model was fitted:

$$\text{Biomass (g dm}^{-3}) = 0.23 + 0.13B + 7.26C - 4.21 \times 10^{-3}B^2 + 0.43BC \quad (1)$$

where B and C refer to concentration of carbon and nitrogen, respectively. The values of the variables in eqn (1) are in their original units (Table 1).

The positive effect of nitrogen concentration on biomass produced was quantified through its regression coefficient, 13.8. A pictorial representation of the effect is shown in Fig 1 where the biomass concentration is plotted for various values of the five factors (see Table 1). The interactive effect of carbon and nitrogen concentrations on biomass is clearly revealed in Fig 2. At the lowest nitrogen concentration, $0.2\,\text{g dm}^{-3}$, an increase in carbon supply had little effect on biomass production as cultures were nitrogen-limited (C/N ranged from 40 to 240). In contrast, at $0.6\,\text{g dm}^{-3}$ of nitrogen, the biomass concentration increased with increasing carbon in the culture medium because now carbon limited growth (C/N ranged from 13 to 80).

The intensive variable 'biomass dry weight per gram of nitrogen supplied' depended on the C/N ratio, as shown in Fig 3. The data in Fig 3 span the full set of experimental runs. At low values of C/N mass ratio, an increase in carbon concentration gave rise to a rapid increase in the biomass produced per gram of nitrogen because the carbon source was limiting. At high C/N values, nitrogen became the limiting nutrient and the amount of biomass obtained per gram of nitrogen supplied, no longer depended on C/N. The best-fit curve in Fig 3 obeyed the following hyperbolic saturation relationship:

$$\frac{\text{g biomass}}{\text{g N}} = \frac{24.1(\text{C:N})}{11.8 + (\text{C:N})} \qquad r^2 = 0.77; \quad p < 0.0001 \quad (2)$$

The maximum amount of biomass obtained per gram of nitrogen supplied was 24.1 g. The saturation constant (ie $11.8\,\text{g g}^{-1}$) in eqn (2) represented the C/N mass ratio value at which the biomass generated per gram of nitrogen was half the maximum. The saturation effect occurred above a C/N value of ~70. Thus, at C/N value of 13, the biomass concentration was limited by the availability of carbon. In contrast, for $48\,\text{g dm}^{-3}$ of carbon and $0.6\,\text{g dm}^{-3}$ of nitrogen, the C/N mass ratio was 80 where the nitrogen source was the limiting nutrient.

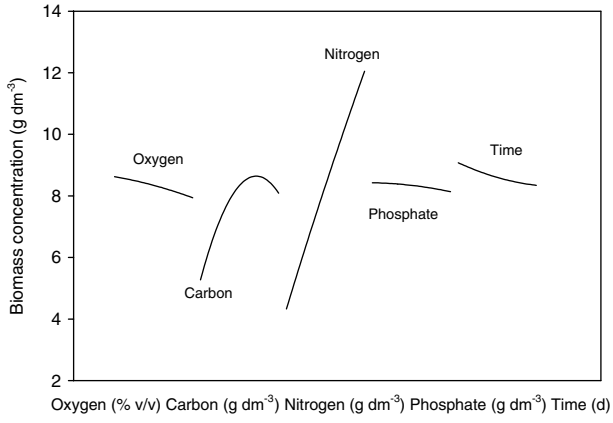

Figure 1. Main effects plot for biomass concentration. Range of variables: 20–80% (v/v) oxygen in the aeration gas; 8–48 g dm^{-3} C-concentration; 0.2–0.6 g dm^{-3} N-concentration; 0.5–2.5 g dm^{-3} P-concentration; and 7–11 days fermentation time.

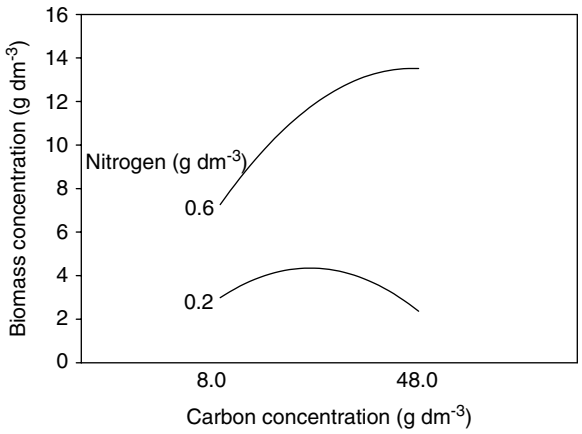

Figure 2. Biomass generation as a function of carbon concentration at two levels of nitrogen.

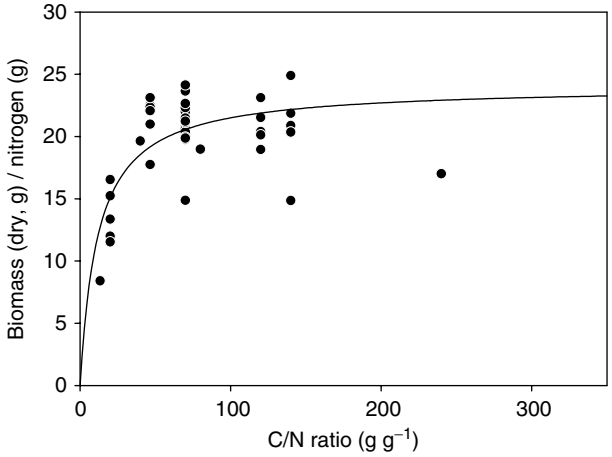

Figure 3. Influence of C/N mass ratio on the biomass generated per gram of nitrogen.

4.2 Lovastatin concentration

The main goal was to optimize lovastatin concentration which responds differently to culture conditions

than does the biomass concentration. A second-order polynomial model fitted from the data in Table 1 was as follows:

$$\text{Lovastatin (mg dm}^{-3}) = 102.95 - 2.08A + 0.92B + 203.43C - 28.35D + 7.99AC - 956.69C^2 + 108.34CD \quad (3)$$

In order to discuss the observed variations, the influence of each factor is presented in Fig 4, even though the analysis of variance suggested no statistically significant effect of fermentation time and nitrogen concentration on the concentration of lovastatin. The principal factor that influenced lovastatin biosynthesis was the concentration of oxygen in the gas phase. The lovastatin concentration increased with increasing amounts of oxygen. Molecular oxygen is required in the biosynthesis of lovastatin molecules via the polyketide pathway[23,24] and an augmentation in the oxygen tension probably affected some equilibrium step in the biosynthetic route, enhancing the final lovastatin concentration. The results clearly suggest that oxygen availability has an important role in lovastatin production and that the dissolved oxygen in the fermentation broth should be controlled for attaining a high productivity of lovastatin.

In a 500 dm^3 culture of *A terreus*, Gbewonyo *et al*[25] reported a peak oxygen consumption rate of 20 mmol dm^{-3} h^{-1} at the end of the growth phase when lovastatin biosynthesis predominantly occurs. Similar values of oxygen uptake rates were claimed by Hajjaj *et al*[14] in a 4 dm^3 fermenter with a biomass concentration of 24 g dm^{-3}. Using these data,[14] a specific oxygen consumption rate for the biomass can be estimated at 0.83 mmol g^{-1} h^{-1}. In our shake flasks, the measured value of the overall volumetric mass transfer coefficient was 17.5 h^{-1}, which is consistent with values reported by others.[26] For our maximum biomass concentration of ~14 g dm^{-3} and using the measured volumetric oxygen mass transfer coefficient an estimation of the specific oxygen uptake rate in the shake flasks under the worst case scenario was 0.31, 0.73 and 1.17 mmol g^{-1} h^{-1} for 20, 50 and 80% oxygen in the gas phase. In view of these values, no oxygen limitation occurred when there was 80% oxygen in the gas phase. However, the cultures were likely oxygen-limited when the concentration of oxygen in the gas phase was 20%.

Carbon concentration also had a positive effect on lovastatin production. A high C/N mass ratio ensured nitrogen-limitation of growth. Nitrogen-limitation has been found to be necessary for lovastatin biosynthesis. If we consider the interaction of oxygen and carbon concentrations, the influence of nitrogen-limited growth on secondary metabolism appears again. At a carbon concentration of 8 g dm^{-3}, the C/N mass ratio ranged from 13.3 to 40, or below the saturation value identified in Fig 3. Under these conditions, the oxygen availability had a minor effect as the lovastatin synthesis had not been strongly activated. At 48 g dm^{-3} carbon concentration, the C/N mass ratio ranged from 80 to 240, where nitrogen was growth limiting, carbon was in excess, and lovastatin synthesis was taking place. This concurs with an earlier study,[27] that claimed a positive effect of increased dissolved oxygen on lovastatin production by a mutant of *A terreus*. According to Lai *et al*, carbon feeding while increasing the level of dissolved oxygen can enhance the synthesis of lovastatin.[27] This agreed with our observation about the positive effect of increasing oxygen tension on lovastatin production when the carbon source was supplied in excess. The complete response surface for these two principal factors (ie oxygen concentration in the gas phase and carbon source concentration) is shown in Fig 5.

With regard to the influence of nitrogen concentration, the first-order term of the model (eqn (3)) was not statistically significant while the second-order term was one of the main sources of variation. Once again the interaction between growth, that is, primary metabolism, and secondary metabolism became relevant. At a nitrogen concentration of 0.2 g dm^{-3}, growth was nitrogen-limited and the lovastatin synthesis per unit biomass concentration was high. At

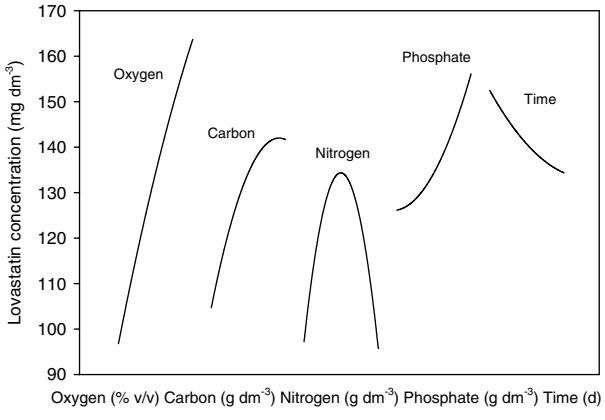

Figure 4. Main effects plot for lovastatin titer. Range of variables: 20–80% (v/v) oxygen in the aeration gas; 8–48 g dm^{-3} C-concentration; 0.2–0.6 g dm^{-3} N-concentration; 0.5–2.5 g dm^{-3} P-concentration; and 7–11 days fermentation time.

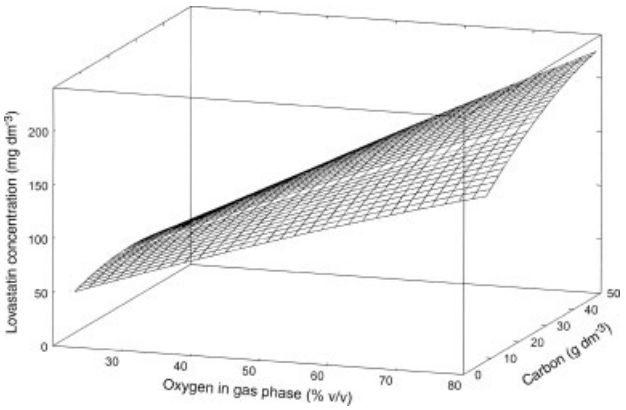

Figure 5. Estimated response surface for lovastatin concentration as a function of oxygen in the gas phase and carbon concentration (0.5 g dm^{-3} N, 0.8 g dm^{-3} P and 7 days of culture).

increasing nitrogen levels, more biomass was generated but the specific production of lovastatin was less.

From the model equation (eqn (3)), the calculated maximum (or optimum) value of lovastatin concentration was 229.8 ± 33.4 (95% confidence interval) mg dm^{-3} for the following culture conditions: 80% oxygen in the gas phase and culture medium containing 48 g dm^{-3} C, 0.46 g dm^{-3} N and 0.79 g dm^{-3} phosphate. Culture time was not a significant influence in the range investigated. The maximum calculated value of lovastatin concentration (eqn (3)) was close to the experimental maximum obtained in run 45 (Table 1). The optimal response factor levels for lovastatin differed from those for the biomass concentration, as is generally the case for secondary metabolites.

These results suggested that attaining a high titer of lovastatin required a high value of the dissolved oxygen tension. Considering this, a second set of experiments was carried out under a pure oxygen atmosphere. The concentrations of carbon, nitrogen and phosphate sources were varied in a narrow interval around the Box–Behnken optimum in a 3^3 factorial experimental design. The lovastatin titers were sharply reduced in comparison with the Box–Behnken optimal titers. Also, the use of pure oxygen altered the morphology of the fungal pellet. The pellets were smaller in comparison with the size in the Box–Behnken runs. When pure oxygen was used, the pellets were dark and dense. The effect of gas phase composition on morphology, growth and product formation by fungi has been reported for many species.[28]

In view of these results, as no increase in lovastatin titer was obtained in the new experimental range of variables, the conditions found for the maximum titers in the Box–Behnken experiment were optimal. Thus, a culture medium for maximizing lovastatin production should contain 48 g dm^{-3} C as lactose, 0.46 g dm^{-3} N as soybean meal and 0.79 g dm^{-3} phosphate. A high transfer rate of oxygen ($\sim$1.17 mmol g^{-1} h^{-1}) should be maintained in the bioreactor for the 7 days of culture.

An independent experiment was used to verify the optimum conditions identified in the Box–Behnken experiment. The verification consisted of triplicate runs comparing the control fermentation (ie original nonoptimal medium) and the optimized growth medium with 80% oxygen in the gas phase. After 7 days of culture the lovastatin concentrations obtained were 44.7, 46.0 and 45.2 mg dm^{-3} with the initial medium and 232.7, 229.3 and 230.8 mg dm^{-3} in the optimized conditions. The biomass concentrations obtained under the optimal conditions for the lovastatin production were 10.4, 10.2 and 10.1 g dm^{-3} or quite close to 9.8 g dm^{-3} estimated from the model (eqn (1)).

5 CONCLUSIONS

Based on a statistically designed search, an optimal medium for maximizing the production of lovastatin in batch cultures of *A terreus* should contain 48 g dm^{-3} C as lactose, 0.46 g dm^{-3} N as soybean meal, and 0.79 g dm^{-3} phosphate. This composition can yield a lovastatin titer of $\sim$230 mg dm^{-3} within 7 days of culture in an oxygen-rich environment. The identified optimal medium is rich in carbon but provides a limiting level of nitrogen. Maintaining a high concentration of dissolved oxygen is essential for attaining a high titer of lovastatin. Maximum values of the biomass yield on nitrogen are obtained when the C/N mass ratio in the medium is $\sim$70 g g^{-1}, or greater.

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