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ORIGINAL ARTICLE

Statistical optimization of simple culture conditions to produce biomass of an ochratoxigenic mould biocontrol yeast strain

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Abstract

Aim: To maximize biomass production of an ochratoxigenic mould–controlling strain of *Lachancea thermotolerans* employing response surface methodology (RSM).

Methods and Results: Using Plackett–Burman screening designs (PBSD) and central composite designs (CCD), an optimized culture medium containing (g l^{-1}): fermentable sugars (FS), 139·2, provided by sugar cane molasses (CMz), $(NH_4)_2HPO_4$ (DAP), 9·0, and yeast extract (YE), 2·5, was formulated. Maximal cell concentration obtained after 24 h at 28°C was 24·2 g l^{-1}cell dry weight (CDW). The mathematical model obtained was validated in experiments performed in shaken-flask cultures and also in aerated bioreactors. Maximum yield and productivity values achieved were, respectively, of 0·23 g CDW/g FS in a medium containing (g l^{-1}): FS, 87·0; DAP, 7·0; YE, 1·0; and of 0·96 g CDW l^{-1} h^{-1} in a medium containing (g l^{-1}): FS, 150·8 plus DAP, 6·9.

Conclusions: Optimized culture conditions for maximizing yeast biomass production determined in flask cultures were applicable at a larger scale. The highest yield values were attained in media containing relatively low-CMz concentrations supplemented with DAP and YE. Yeast extract would not be necessary if higher productivity is the aim.

Significance and Impact of the Study: Cells of *L. thermotolerans* produced aerobically could be sustainably produced in a medium just containing cheap carbon, nitrogen and phosphorus sources. Response surface methodology allowed the fine-tuning of cultural conditions.

Introduction

The chlorinated isocoumarin compound ochratoxin A (OTA), a potent nephrotoxic and carcinogenic toxin produced by several *Aspergillus* species within *Aspergillus* Section *Nigri*, has been reported in wine and grape juice (Visconti *et al.* 1999; Bleve *et al.* 2006). Although widespread use of chemical fungicides has decreased the incidence of fungal diseases, a growing number of consumers prefer food products free of chemical residues considering they may persist at the time of the consumption (Castoria *et al.* 2001; Masih *et al.* 2001). By this reason, biocontrol by means of microbial antagonists can be considered as a suitable alternative. Several species of yeasts are recommended to inhibit fungal growth in fruits because they can grow and colonize efficiently their surfaces (Taqarort *et al.* 2008).

In previous studies, nine yeast strains isolated from grapes in a vineyard showed antagonistic actions against *Aspergillus* section *Nigri*. These strains were evaluated in '*in vitro*' screening assays, by determining the reduction in growth rate and OTA production of the ochratoxigenic

fungi. Some of the isolates were identified as *Lachancea thermotolerans*, and one strain, RCKT4, was selected, because it exhibited a marked antagonist activity assayed at different pH, a_w and temperatures conditions (Ponsone 2009).

To test in the field the antagonistic activity of the yeast strain chosen, cell production needs to be produced at a larger scale. So, optimization of biomass production and costs reduction is included in the strategic analysis of the viability of the biotechnological process. One of the major contributors to the overall cost is the price of the carbon source. Molasses are one of the most suitable and economically available sugar-bearing raw materials to serve as feed stocks for biotechnological purposes (Spencer and Spencer 1997; Smith 2000; Enfors 2001) and also contain nitrogen-rich compounds, growth factors and microelements necessaries for yeast's growth.

As the biomass production of any organism is a multivariable process, experimental design techniques give a balanced approach to culture conditions improvement (Thiry and Cingolani 2002). Methods for experimental design can be used for the exploration of a potential large number of input variables to discover those which are statistically significant and estimate their magnitude (Strobel and Sullivan 1999). Then, optimization designs and RSM analysis are applied to a reduced number of variables to find out the values that maximize the response (Box and Wilson 1951). Finally, after model building and optimization, the predicted optimum must be verified.

In this study, the main culture conditions of a simple and economical aerobic batch process for maximal biomass production of a strain of *L. thermotolerans*, aiming an industrial scale, were optimized for the first time using experimental designs.

Materials and Methods

Chemicals, microorganism and culture conditions

Except for CMz (58% w/w FS), culture media components were of the highest purity available. The yeast strain used was isolated from grapes in the Province of Mendoza (Argentine) and identified using morphological markers and by restriction fragment length polymorphisms according to Guillamón *et al.* (1998) and Esteve-Zarzoso *et al.* (1999) as described in Ponsone (*op. cit.*) and was identified as *Lachancea thermotolerans* and named RCKT4. The strain was maintained at 4°C on slants of YPD agar. For Erlenmeyer flasks inoculation, a loopful of the slants cultures was transferred to 20-ml YPD broth contained in Erlenmeyer flasks and incubated for 18–24 h with agitation (200 rev min^{-1}) provided by an orbital shaker, keeping the ratio 'volume flask/volume medium' in 5 : 1 (pre-inoculum). Before inoculation, pre-inoculum cells were washed three times with sterilized distilled water by centrifugation (9000 ***g*** for 5 min) and suspension. Filter-sterilized (0·22-μm-pore membranes of cellulose acetate) YE solutions were aseptically added to the other media components previously sterilized at 121°C for 15 min. Unless indicated, temperature for all experimental cultures was maintained at 28 ± 1°C, and pH values between 5·2 and 5·4.

For bioreactor assays, batch fermentations were carried out in a mechanically stirred 5-l New Brunswick FS300 fermentor (New Brunswick Scientific Co., Edison, NJ, USA) equipped with pH, temperature and dissolved oxygen concentration sensors. Agitation and aeration were varied to maintain dissolved oxygen concentration above 40% saturation. The pH was maintained between 5·2 and 5·4 units by addition of 18 N H_2SO_4 or 20% w/v Na_2CO_3 solutions. Foam production was controlled by the addition of antifoam (silicone Antifoam 289; Sigma, St Louis, MO, USA). The fermentation media in the bioreactor (3·5-l working volume) contained different concentrations of CMz, DAP and YE as it is described in Results. The sterilized media were inoculated with cells previously incubated at the same temperature, time and agitation conditions, as those described for Erlenmeyer flask inoculum, in a medium containing (g l^{-1}): CMz (58% w/w FS), 80·0; DAP, 3·0 and YE, 3·0), with initial pH of 5·4 units after autoclaving. Aliquots of 30-ml culture were withdrawn as indicated in Results for biomass and FS determinations.

Statistical experimental designs

Plackett–Burman screening designs were set up for five or eight factors with three coded levels (−1, 0, +1), to evaluate their linear effect on cell growth. Three centre points were run for each experiment. The results were fitted with a first-order model, estimating the coefficient (slope) for each factor and their levels of significance.

Circumscribed CCD (CCCD) (Box and Wilson 1951) with five levels for each of the three factors (CMz, DAP and YE) were run to evaluate the quadratic effects and two-way interactions among the variables.

In both PBSD and CCCD, the variable levels X_i were coded as x_i according to Eqn (1):

$$x_i = \frac{X_i - X_0}{\Delta X_i}, i = 1, 2, 3, \ldots, k \quad (1)$$

where x_i and X_i are the dimensionless (codified) value and the actual value of an independent variable, respectively, X_0 is the real value of an independent variable at centre point and ΔX_i the step change.

In the CCCD with $\alpha = 1{\cdot}682$, upper and lower limits of the variables CMz (X_1), DAP (X_2) and YE (X_3) were (in g l^{-1}), respectively, (80·0–400·0), (3·0–15·0) and (0·0–5·0); RSM was used to analyse this experimental design. The second-degree model used to fit the response to the independent variables is shown in Eqn (2):

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{j=2}^{k} \sum_{i=1}^{j-1} \beta_{ij} x_i x_j + \sum_{i=1}^{k} \beta_{ii} x_i^2 \quad (2)$$

where Y is the predicted response, $x_i x_j$ are the input variables that influence the response variable Y, β_0 is the intercept, β_i is the ith linear coefficient, β_{ii} is the ith quadratic coefficient and β_{ij} is the ijth interaction coefficient.

Statistical and numerical analyses were carried out by means of the analysis of variance (ANOVA) and multiple regressions using the software Essential Regression (ver. 2003; developed by D.D. Steppan, J. Werner and R.P. Yeater, Gibsonia, PA).

Responses measured were subjected to multiple regressions by minimal sum of squares. Statistical significance of the regression coefficient was evaluated by the Student's t-test. Adequacy of the mathematical models of regression was pondered by F test.

Experimental designs were carried out in shaken-flask cultures (vol. flask: vol. medium, 5 : 1). Culture media were prepared by dissolving the selected solutes in sterilized distilled water at the concentrations given by the designed matrices.

Analytical determinations

For routine assays, biomass production was monitored by the optical density at 640 nm (OD_{640nm}), after washing the cells three times with phosphate buffer 0·05 mol l^{-1} (pH 6·5) by centrifugation (5000 rpm, 5 min) and suspension. Biomass was also gravimetrically evaluated as CDW (g l^{-1}) after cells were washed with distilled water as described before. Growth was also evaluated as viable cell number by CFU counting by spotting 20 μl of each appropriate dilution by duplicate onto YPD-agar plates incubated for 48 h at 28°C. Viability counts were performed routinely, and no loss of viability was observed in the aliquots of the cultures tested.

The equivalences obtained among these three parameters were as follows:

CFU ml^{-1} = $OD_{640nm} \times 3{\cdot}85 \times 10^7$ CFU ml^{-1}

CDW (g l^{-1}) = $OD_{640nm} \times 0{\cdot}59$ g l^{-1}

Fermentable sugars concentrations in culture media were determined as concentrations of sucrose, D-glucose and D-fructose, with an enzymatic kit (Boehringer Mannheim/R-Biopharm, Cat. no. 10716260035). All experiments described herein were carried out at least in duplicate and repeated at least twice.

Results

Screening of essential culture conditions for biomass production

As there are no reports on statistical optimization of cultural conditions for biomass production of *L. thermotolerans*, the main variables involved with strain RCKT4 growth were screened by means of PBSDs. These variables were assayed at three coded levels (−1, 0, +1) in the following concentrations (X_1–X_5, in g l^{-1}): X_1, glucose (5·0–50·0); X_2, $(NH_4)_2SO_4$ (2·0–20·0); X_3, KH_2PO_4 (0·5–5·0); X_4, $MgSO_4{\cdot}7H_2O$ (0·25–2·5); X_5, YE, (0–4·0); X_6, trace elements solution (0·2–2·0, ml l^{-1}), (Cerrutti and Galvagno 2004); X_7, inoculum (0·01–0·3, initial OD_{640nm}); and X_8, incubation time (24–48 h).

The results obtained showed that concentrations of N as $(NH_4)_2SO_4$ and P as KH_2PO_4, $MgSO_4{\cdot}7H_2O$ and trace elements did not significantly affect cell growth in the ranges selected, but glucose and YE concentrations, incubation time and inoculum size significantly affected ($P < 0{\cdot}05$) the growth of the strain studied.

The equation obtained for cell growth was as follows:

Response measured = OD_{640nm} = 0·298 + **0·014** x_1 + 0·002 x_2 − 0·039 x_3 − 0·03 x_4 + **1·022** x_5 + 0·128 x_6 + **2·138** x_7 − **0·017** x_8

[*The P values for regression coefficients in bold characters were significant at $P < 0{\cdot}05$.] The lack of fit of the regression model was not significant, and the P value of the F test ($F_{significative} = 0{\cdot}0069 < 0{\cdot}05$) demonstrated a high significance for the regression. The F value (6·80) was higher than the critical value (3·48) obtained from tables for a 5% significance level with 5 and 9 degrees of freedom. The goodness of fit of the model was checked by the determination of the coefficient $R^2 = 0{\cdot}994$.

Subsequent PBSD assays were focused on the influence of the variables that significantly impacted biomass production; the incubation temperature was also taken into account, as it is a very important variable mainly in large-scale fermentations. The use of CMz was now proposed because it is a cheaper and readily available carbon source, and it is also a supply of biologically available source of N, P, vitamins and microelements (Enfors 2001).

The ranges chosen for the variables were as follows: CMz (X_1 = 20·0–80·0 g l^{-1}), YE (X_2 = 0·0–3·0 g l^{-1}), inoculum size (OD_{640nm}) (X_3 = 0·05–0·50), temperature (X_4 = 25–37°C) and incubation time (X_5 = 24–48 h). According to the results, the equation obtained was:

OD_{640nm} = 26·14 + **0·589** x_1 + 0·313 x_2 + 6·759 x_3 − **0·848** x_4 + 0·0727 x_5 [The P values for regression coefficients in bold characters were significant at $P < 0{\cdot}05$ (x_1) and $P < 0{\cdot}1$ (x_4)]

The value for R^2 was 0·781 meaning a high significance of the model. Furthermore, the lack of fit was not significant. The results demonstrated that cell growth was two- to three-fold higher when CMz (standardized by FS concentration) were used as carbon source instead of glucose. It is also shown that growth was negatively and significantly affected when temperature was increased from 25 to 37°C. Besides, the μ_{max} values obtained from growth curves in batch cultures performed at 28, 32 and 37°C were 0·37, 0·35 and 0·28 h^{-1}, respectively. Accordingly, incubation temperature was maintained at 28°C in the following experiments. The inoculum size chosen for the subsequent experiments, as suggested by PBSD, was 1·5–2·0 × 10^7 CFU ml^{-1}.

Finally, as the incubation time had no significant effect on the response, the following fermentations were stopped at 24-h incubation, taking into account the improvement of the bioprocess productivity. Previous experiments had showed that when CMz were used as raw material, biomass exhibited only a slight increase (<0·05 log cycles) between 24 and 48 h incubation.

Optimization of selected culture conditions for biomass production

Taking into account the results obtained, optimization of biomass production of *L. thermotolerans* RCKT4 was conducted to define the optimal levels of the chosen variables that maximize cell growth in a batch culture. Therefore, CCCD designs were carried out to optimize concentrations of CMz, DAP and YE (see Materials and methods). The maximal cell concentration obtained was 41·6 units of OD_{640nm}, corresponding to 1·6 × 10^9 CFU ml^{-1}, after 24-h fermentation at 28°C and was the average of the values corresponding to the three centre points (g l^{-1}): CMz, 240·0; DAP, 9·0; and YE, 2·5. Data were analysed by multiple regressions. Table 1 shows the significance of each coefficient determined by Student's *t*-test and *P* values. Judging by the regression coefficients and *t* values, it can be concluded that *L. thermotolerans* RCKT4 cell production is determined primarily by CMz concentration (in its linear and quadratic terms, x_1 and x_1x_1, respectively) and DAP concentration (in its linear and quadratic terms x_2 and x_2x_2, respectively). No significant interactions between any two of the three factors were found.

Table 1 Analysis of the design optimization for biomass production

Term	Regression coefficient	Standard error	*t* value	*P* value*
x_1*	**4·50**	**0·58**	**7·75**	**1·1x10^{-4}**
x_2	**51·6**	**15·5**	**3·33**	**0·013**
x_3	24·4	32·6	0·75	0·478
x_1x_1	**−0·08**	**0·01**	**−8·23**	**7·6x10^{-5}**
x_2x_2	**−28·3**	**6·93**	**−4·08**	**0·0047**
x_3x_3	−75·1	39·9	−1·88	0·102
x_1x_2	−0·49	0·31	−1·59	0·156
x_1x_3	0·20	0·74	0·27	0·792
x_2x_3	3·06	19·8	0·16	0·881
Intercept	−35·8	13·1	−2·74	0·029

*The *P* values for regression coefficients in bold characters are significant at $P < 0·05$.

The experimental results obtained were fitted and explained with a second-order polynomial function [eqn (2), see Materials and methods], which for this work corresponded to a quadratic regression model with ten terms. The correlation coefficient value $R^2 = 0·938$ indicated a high significance of the model. The lack of fit of the regression model was not significant, and Fisher's *F* test demonstrated a high significance for the regression ($P < 0·05$).

Another indicative proof of the high significance of the model obtained for yeast cell production is the plot representing predicted OD_{640nm} *vs* experimental values. The plot was very close to $y = x$ (data not shown), and it could be assumed that the experimental data prediction by the model applied was highly satisfactory.

Employing RSM, the combination of factors that maximized biomass production of the strain RCKT4 was (g l^{-1}) as follows:

(a) CMz, 262·0; DAP, 7·0; YE, 2·1; for which the model predicts an OD_{640nm} = 43·7 (=1·7 × 10^9 CFU ml^{-1}).

The combination of the variables concentrations of CMz and DAP that maximized the response in absence of YE was also analysed and corresponded to (g l^{-1}):

(b) CMz, 260·0; DAP, 6·9; YE, 0·0; for which the model predicts an OD_{640nm} = 40·4 (=1·6 × 10^9 CFU ml^{-1}).

Surface plots showing both combinations (a and b) are depicted in Fig. 1(a,b). As it is observed, there was a high similarity in the plot's shape, although the maximal biomass value obtained was higher in the presence of YE.

Validation of the model in shaken flasks and bioreactors

Different combinations of the cultural variables involved were used, and the experimental responses were compared with the predicted by the mathematical model both in shaken flasks and bioreactors. These assays were carried out at an inoculum concentration of 0·5 units of OD_{640nm} developed in the same culture medium as for the experimental fermentations. The results of validation experiments after 24-h incubation at 28°C at the concentrations of CMz, DAP and YE used are showed in Table 2. As can be seen, the results obtained widely validated the mathematical model. On the other hand, the absolute values of the

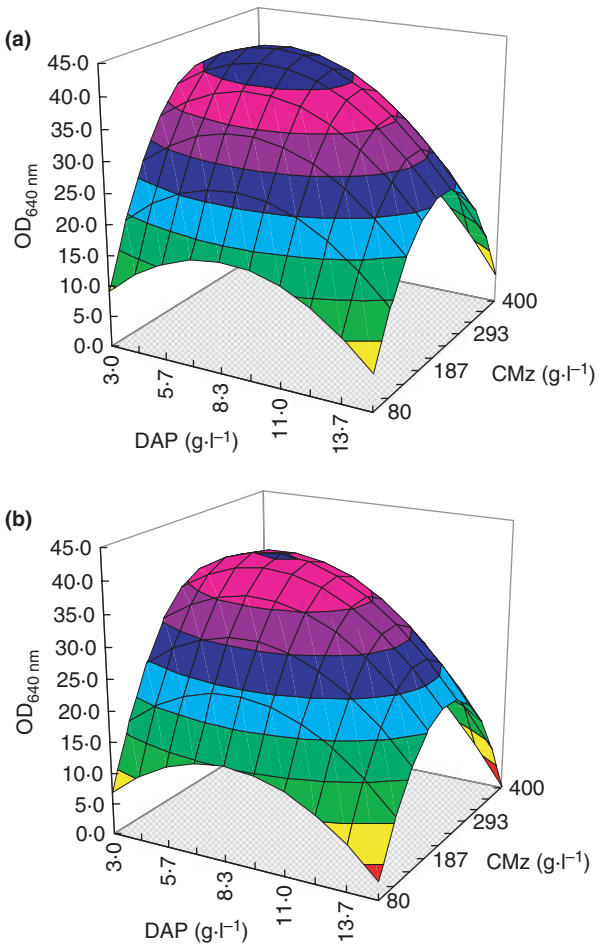

Figure 1 Response surface plots showing the effect on biomass production of cane molasses (CMz) and $(NH_4)_2HPO_4$ (DAP) concentrations at yeast extract (YE) concentrations of: a = 2·1 g l^{-1}, b = 0·0 g l^{-1}.

standardized residues were within the acceptable limits (data not shown). In bioreactor assays in which CMz concentration was reduced by 42%, in the presence of 1·0 g l^{-1} YE, volumetric productivity diminished only about 17%. Yield values obtained after 24-h fermentation were 1·5-fold higher in the 'low-concentration' CMz medium, which could indicate that in the presence of YE, FS were more efficiently used to produce yeast biomass instead of being directed to by-products formation. In bioreactor assays at 'high-concentration' CMz medium, biomass production reached a maximum value at 24-h fermentation when FS concentration was 5% of the original value. Thereafter, biomass values did not differ significantly up to the end of the fermentation.

Discussion

Till date, there is no information on statistical optimization of the culture conditions for biomass production of *Lachancea* (=*Kluyveromyces*) *thermotolerans*. In this work, experimental designs as a whole proved to be adequate for the design and optimization of biomass production intended to be used at an industrial scale, because it allowed fine-tuning of the economically most important production variables. Response surface methodology analysis indicated that the optimized concentrations for the main substrates as well as for other operating conditions for yeast production determined in shaken-flask cultures could be applied at a larger scale. The validity of the model was proved by fitting the values of the variables into a model equation and by actually carrying out the experiments at the variable setting selected, both in shaken flasks and in an aerated bioreactor. Yield values obtained (expressed as biomass standardized by sugar consumed) were as high as those expected for Crabtree-positive yeast species (Christen and Sauer 2011). High-productivity values were attained in media containing a cheap and locally available source of sugars and growth factors as CMz, supplemented only with small concentrations of an inorganic nitrogen and phosphorous source and YE. In the medium containing high-concentration CMz, some amounts might be devoted to the production of metabolites or other by-

Table 2 Validation results, yields and productivities in shaken-flask cultures and in bioreactor

Validation assay	Obtained biomass (g CDW l^{-1})	Predicted biomass (g CDW l^{-1})	Absolute standardized residual	Yield (g CDW g^{-1} FS)	Productivity (g CDW l^{-1} h^{-1})
Optimized medium with YE* (CMz, 262·0 g l^{-1}; DAP, 7·0 g l^{-1}; YE, 2·1 g l^{-1})	24·2	25·8	0·88	0·165	1·04
Optimized medium without YE† (CMz, 260·0 g l^{-1}; DAP, 6·9 g l^{-1})	22·5	23·8	0·78	0·154	0·96
Medium with low-CMz concentration† (CMz, 150·0 g l^{-1}; DAP, 7·0 g l^{-1}; YE, 1·0 g l^{-1})	18·9	19·5	0·30	0·227	0·80

CCD, cell dry weight; YE, yeast extract.
*In shaken-flask cultures.
†In bioreactor.

products different from biomass, as the remaining FS concentration at the end of the fermentation period was quite similar for both 'high' and 'low' CMz concentration medium (5·2 and 4·1 g l^{-1}, respectively).

Only a 7·6% reduction in biomass production occurred by eliminating YE, a high-cost substrate. This fact is very important in the bioprocess economy improvement. It is worth to mention that YE has to be added in the fermentation medium if biomass yield is the target, but no growth factors additions would be necessary if productivity is the aim. The production medium developed in this work may provide an industrial solution to the biocontrol of mould infections in grapes. Our results also strongly suggest that the strain RCKT4 of *L. thermotolerans*, an ochratoxigenic mould biocontrol yeast, is a good candidate for cell biomass production if fermentation at an industrial scale is intended. High-productivity values can be obtained in a batch fermentation using a cheap and simple medium. Currently, greenhouse trials of biocontrol activity on grapes sprayed with cell suspensions of *L. thermotolerans* (10^4–10^6 CFU ml^{-1}) produced in bioreactors as described here are successfully in progress.

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References

Bleve, G., Grieco, F., Cozzi, G., Logrieco, A. and Visconti, A. (2006) Isolation of epiphytic yeasts with potential for biocontrol of *Aspergillus carbonarius* and *A. niger* on grape. *Int J Food Microbiol* **108**, 204–209.

Box, G.E.P. and Wilson, K.B. (1951) On the experimental attainment of optimum conditions. *J Royal Stat Soc Series B* **13**, 1–45.

Castoria, R., De Curtis, F., Lima, G., Caputo, L., Pacifico, S. and De Cicco, V. (2001) *Aureobasidium pullulans* (LS-30) an antagonist of postharvest pathogens of fruits: study on its mode of action. *Postharvest Biol Technol* **22**, 7–17.

Cerrutti, P. and Galvagno, M.A. (2004) Aumento de la actividad panificante de levaduras comerciales por aplicación de condiciones de estrés durante su propagación. *Rev Arg Microbiol* **36**, 41–46.

Christen, S. and Sauer, U. (2011) Intracellular characterization of aerobic glucose metabolism in seven yeast species by ^{13}C flux analysis and metabolomics. *FEMS Yeast Res* **11**, 263–272.

Enfors, S. (2001) Baker's yeast. In *Basic Biotechnology* ed. Ratledge, C. and Kristiansen, B. pp. 377–389. Cambridge, UK: Cambridge University Press.

Esteve-Zarzoso, B., Belloch, C., Uruburu, F. and Querol, A. (1999) Identification of yeasts by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. *Int J Systematic Bacteriol* **49**, 329–337.

Guillamón, J., Sabaté, J., Barrio, E., Cano, J. and Querol, A. (1998) Rapid identification of wine yeast species based on RFLP analysis of the ribosomal internal transcribed spacer (ITS) region. *Arch Microbiol* **169**, 387–392.

Masih, E.I., Slezack-Deschaumes, S., Marmaras, I., Ait Barka, E., Vernet, G., Charpentier, C., Adholeya, A. and Paul, B. (2001) Characterisation of the yeast *Pichia membranifaciens* and its possible use in the biological control of *Botrytis cinerea*, causing the grey mould disease of grapevine. *FEMS Microbiol Lett* **202**, 227–232.

Ponsone, L. (2009) *Evaluación del riesgo y manejo integrado de ocratoxina A en uvas y vinos. Ecofisiología de Aspergillus subgénero Circumdati Sección Nigri.* PhD Thesis in Biological Sciences. Provincia de Córdoba, República Argentina: Universidad Nacional de Río Cuarto, Facultad de Ciencia Exactas, Físico-químicas y Naturales.

Smith, J.E. (2000) Substrates for biotechnology. In *Biotechnology*, 3rd edn pp. 19–26. Cambridge, UK: Cambridge University Press.

Spencer, F. and Spencer, D. (1997) Yeasts and the life of man: Part I: helpers and hinderers. ''Traditional'' yeast-based industries, Spoilage yeasts. In *Yeasts in Natural and Artificial Habitats* ed. Spencer, F. and Spencer, D. pp. 226–242. Berlin, New York: Springer.

Strobel, R.J. and Sullivan, G.R. (1999) Experimental design for improvement of fermentations. In *Manual of Industrial Microbiology and Biotechnology* ed. Demain, A. and Davies, J. pp. 80–93. Washington, DC, USA: ASM Press.

Taqarort, N., Echairi, A., Chaussod, R., Nouaim, R., Boubaker, H., Benaoumar, A.A. and Boudyach, E. (2008) Screening and identification of epiphytic yeasts with potential for biological control of green mold of citrus fruits. *World J Microbiol Biotechnol* **24**, 3031–3038.

Thiry, M. and Cingolani, D. (2002) Optimizing scale-up fermentation processes. *Trends Biotechnol* **20**, 103–105.

Visconti, A., Pascale, M. and Centonze, G. (1999) Determination of ochratoxin A in wine by means of immunoaffinity column clean-up and high-performance liquid chromatography. *J Chromatography A* **864**, 89–101.