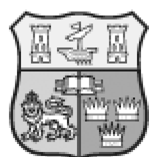
View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Cork Open Research Archive

Title	Selection of best conditions of inoculum preparation for optimum performance of the pigment production process by Talaromyces spp. using the Taguchi method
Author(s)	Morales-Oyervides, Lourdes; Oliveira, Jorge C.; Sousa-Gallagher, Maria J.; Méndez-Zavala, Alejandro; Montañez, Julio C.
Publication date	2017-04-03
Original citation	Morales-Oyervides, L., Oliveira, J. C., Sousa-Gallagher, M. J., Méndez-Zavala, A. and Montañez, J. C. 'Selection of best conditions of inoculum preparation for optimum performance of the pigment production process by Talaromyces spp. using the Taguchi method', Biotechnology Progress, doi:10.1002/btpr.2470. In Press
Type of publication	Article (peer-reviewed)
Link to publisher's version	http://dx.doi.org/10.1002/btpr.2470 Access to the full text of the published version may require a subscription.
Rights	**© 2017 American Institute of Chemical Engineers. This is the peer reviewed version of the following article: 'Selection of best conditions of inoculum preparation for optimum performance of the pigment production process by Talaromyces spp. using the Taguchi method', Biotechnol Progress, which has been published in final form at http://dx.doi.org/10.1002/btpr.2470. This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Self-Archiving.**
Embargo information	Access to this article is restricted until 12 months after publication at the request of the publisher
Embargo lift date	2018-04-03
Item downloaded from	http://hdl.handle.net/10468/4049

Downloaded on 2018-08-23T20:12:15Z



DOI 10.1002/btpr.2470

Selection of best conditions of inoculum preparation for optimum performance of the pigment production process by *Talaromyces* spp. using the Taguchi method

L. Morales-Oyervides[1,2], J.C. Oliveira [1*], M.J. Sousa-Gallagher [1], A. Méndez-Zavala [2] & J.C. Montañez [2]

[1] Process & Chemical Engineering, School of Engineering, University College Cork, Cork, Ireland.

[2] Department of Chemical Engineering, Universidad Autónoma de Coahuila. Saltillo, Coahuila, México.

* Author to whom correspondence should be addressed:

Dr. Jorge Oliveira

School of Engineering (Process & Chemical), University College Cork, Ireland

Email: j.oliveira@ucc.ie

Running title:

Pigment production by *Talaromyces* spp.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/btpr.2470
© 2017 American Institute of Chemical Engineers Biotechnol Prog
Received: Apr 18, 2016; Revised: Mar 10, 2017; Accepted: Mar 12, 2017

This article is protected by copyright. All rights reserved.

Accepted Article

Abstract

Process optimisation techniques increasingly need to be used early on in Research and Development of processes for new ingredients. There are different approaches and this paper illustrates the main issues at stake with a method that is an industry best practice, the Taguchi method, suggesting a procedure to assess the potential impact of its drawbacks. The Taguchi method has been widely used in various industrial sectors because it minimises the experimental requirements to define an optimum region of operation, which is particularly relevant when minimising variability is a target. However, it also has drawbacks, especially the intricate confoundings generated by the experimental designs used.

This work reports a process optimisation of the synthesis of red pigments by a fungal strain, *Talaromyces* spp. using the Taguchi methodology and proposes an approach to assess from validation trials whether the conclusions can be accepted with confidence.

The work focused on optimising the inoculum characteristics, the studied factors were spore age and concentration, agitation speed and incubation time. It was concluded that spore age was the most important factor for both responses, with optimum results at 5 days old, with the best other conditions being spores concentration, 100,000 (spores/mL); agitation 200 rpm, and incubation time 84 h. The interactive effects can be considered negligible and therefore this is an example where a simple experimental design approach was successful in speedily indicating conditions able to increase pigment production by 63 % compared to an average choice of settings.

Keywords: confounding, design of experiments, process performance, spore age

This article is protected by copyright. All rights reserved.

Accepted Article

Accepted Article

1. Introduction

1.1. Bioproduction of natural pigments

Research on new sources of natural pigments has increased due to the toxicity of synthetic colorants used in foods, pharmaceuticals and cosmetic preparations [1–3]. Fungi provide a readily available alternative source of natural pigments. Dufossé et al. (2014) [4] emphasised the crucial role that filamentous fungi are currently playing and are likely to continue to play in the future as microbial cell factories for the production of food grade pigments due to the versatility in their pigment colour and chemical profile, amenability for easy large scale cultivation, and a long-term history of well-studied production strains. Bioprocessing routes for producing natural red pigments have been established using *Monascus* spp., but this strain also produces a mycotoxin called citrinin [5]. Recently, *Talaromyces* spp. (formerly *Penicillium* spp.) has been proposed as a potential pigment producing strain because capable to reach high productivity levels [6].

Development of new bioprocess to obtain value-added compounds usually follows a conventional approach where the produced compounds need to be characterised (physical and chemical), approved and then its industrial production is optimised. This approach can be a time consuming and expensive process. Therefore, the efficiency of the product development cycle now requires that the process potential and economics be assessed early on, so that process conditions that do not meet stringent productivity requirements are eliminated. This brings forward process optimisation targets.

Fermentation processes depend on a large number of operating variables (medium composition, pH, inoculum, temperature, aeration rate, etc.) that may interact strongly between themselves and with the specific characteristics and properties of both growth medium and microorganisms, creating a unique environment that results in a particular final yield of the process. For this

This article is protected by copyright. All rights reserved.

Accepted Article

reason it would be economically unfeasible to find the optimum conditions that lead to the best production yields with a comprehensive experimental plan for every bioprocess.

The production of pigments by *Talaromyces* spp. (Previously cited as *Penicillium purpurogenum* GH2) has been previously optimised in terms of process conditions such as pH and temperature [7] and culture media [8]. Moreover, the effect of the inoculum type on the production of pigments was also reported [9]. However, despite the fact that process, nutrient and microbial conditions were optimised, a high variability of the production was obtained batch to batch. Therefore, it is necessary to analyse the inoculum factors comprehensively prior to scale up of the process. It is noted that in modern Quality by Design approaches, consistency of performance is a critical aspect of quality. That is, an optimum performance has to include the metric of a consistent performance. It is known that seed culture development (inoculum) is an important factor in the successful performance of a fungal process for the production of any metabolite in both laboratory and industrial scale [10,11].

Thus, the objective of the present study was to optimise the inoculum preparation conditions of spore age, seed culture incubation, inoculum level and agitation to enhance pigment production by *Talaromyces* spp. It was further desired to establish the optimum potential in a rapid fashion, illustrating the advantages (and addressing the drawbacks) of the Taguchi method, which has been proposed as an effective way of establishing best conditions.

1.2. Process optimisation tools

The Taguchi method applies statistical designs that have maximum efficiency with minimum experimental requirements and it does not require fitting mathematical models to experimental data, which would add lack of fit of the model to the error of conclusions and predictions. This

This article is protected by copyright. All rights reserved.

Accepted Article

efficiency in minimising experimental requirements without relying on model fitting comes at the cost of using designs that might lead to results where the effect of factors and of interactions between factors are confounded, ending in erroneous predictions and sub-optimal regions when interactive effects have a significant impact.

A major outcome of this method that is particularly important in many industrial problems is the ability to integratedly search for an optimum that considers not only the average result of the process but also its consistency, minimising variability and thus providing a result that minimises the waste associated with product and process variability [12]. Thus, it is not surprising that this method and the overall concept of Quality by Design that it enables (and that Taguchi placed at the centre of his philosophy) has become a cornerstone of manufacturing excellence, and a very important tool in modern industrial processing strategies (such as lean 6-sigma).

Given the need to bring upstream process optimisation techniques to be more efficient in evaluating process potential early on, this strategy has been employed in various bioprocessing lab studies, such as in the optimisation of the production of enzymes [13–17] and of process conditions to improve production yield of microbial added-value compounds [18–20]. However, the reported studies tend to stress the advantages of the method while ignoring the drawbacks and limitations that have been pointed out in statistical literature [21]. It is very important to be clear about what the Taguchi method does and does not do, and why there is in fact no perfect method, as others that mitigate Taguchi's drawbacks bring their own disadvantages too.

The most important end result therefore must be, as suggested by Taguchi, a validation of the predictions of optimum processing conditions. Very often that validation will provide evidence for a region of optimum to have been found, even if not the absolute optimum, but when this is not the case, it is important to know what to do to disentangle the confoundings between factors

This article is protected by copyright. All rights reserved.

Accepted Article

and interactions. This fits exactly the modern thinking of manufacturing excellence, in particular as designated by DMAIC (Design, Measure, Analyse, Improve and Control), as well as continuous process improvement (also known by its Japanese term *Kaizen*).

Therefore, the objective of finding optimum conditions in terms of pigment specific production and extracellular pigment production of natural red pigments was served by a two-stage approach: first, applying an orthogonal array design, which will reveal which process factors are more influential and what settings of those lead to best results with maximum consistency (minimum variability), provided that interactions are negligible. Second, validating with further trials that the conclusions and predictions are verified within the acceptable margin of confidence.

A method is proposed to verify the underlying assumption of the first-stage Taguchi predictions (that interactive effects are negligible).

2. Materials and methods

2.1 Microorganism

Talaromyces spp. was used for production of red pigments (DIA-UAdeC). The purified strain had been previously isolated and characterised as *Penicillium purpurogenum* GH2 [22,23]. *Penicillium purpurogenum* has however been transferred to *Talaromyces* spp.[24]. According to the morphological characterisation, the strain used in this study presents the characteristics of *T. atroroseus* due to the diffusible red pigments and the dark green thick walled ellipsoidal conidia produced [24], but a definite characterisation with gene sequencing and extrolite metabolites

John Wiley & Sons

This article is protected by copyright. All rights reserved.

Accepted Article

analyses is not yet available, hence *Penicillium purpurogenum* GH2 is designated here generically as *Talaromyces* spp.

The strain was maintained on PDA (Potato dextrose agar) slants at 4°C and sub-cultured periodically.

2.2 Culture Media

A Potato Dextrose Broth (PDB, ATCC medium: 336) was prepared by boiling 0.3 kg of finely diced potatoes in 500 mL of water until thoroughly cooked; the potatoes were then filtered through cheesecloth and water was added to the filtrate to complete a volume of 1.0 L. Finally, 20.0 g of glucose were added before sterilisation. Potato Dextrose Agar was prepared equally to PDB medium but with the addition of agar (15 g/L). The Czapek-dox modified medium reported by Mendez-Zavala (2011)[8] for the production of pigments consisted in (g/L): D-xylose 15.0, $NaNO_3$ 3.0, $MgSO_4 \cdot 7H_2O$ 0.5, $FeSO_4 \cdot 7H_2O$ 0.1, K_2HPO_4 1.0, KCl 1.0 and ethanol 20.0.

2.3 Experimental design

The experimental designs recommended by Taguchi are a particular case of fractional factorial designs defined by orthogonal arrays. The method will only make predictions for combinations of the settings defined by these arrays, avoiding interpolations which would require models, and hence, be prone to model bias and lack of fit. Therefore, if one were to use a full factorial design, the Taguchi method would offer no benefit at all in terms of experimental efficiency, its benefits in minimising experimental requirements are particularly strong when using many factors, and/or many levels, where by testing only some combinations one can infer the best of all. The Taguchi method, just like its alternatives (e.g. response surface analysis with experimental designs

This article is protected by copyright. All rights reserved.

without confoundings) are black-box methods and thus the results will always be specific of the range of values of the factor used in the experiments, and apply only to the actual equipment used. These are not first-principle approaches enabling scale-up; the conclusions only apply to the scale and range of variables covered in the experiments. Thus, the first step of the process (the D in DMAIC) is really critical. The smallest advisable experimental design to use for optimisation is the L-9 array, which permits analysing the effect of up to 4 factors (operating variables), and where each is tested at 3 different levels (values of the operating variables).

Process steps needed for the production of pigments are shown in Figure 1: a) pre-culture step (Inoculum), b) production step and c) pigment recovery.

Optimising a process performance implies maximising its consistency and therefore experimental plans must be repeated a minimum numbers of times which implies that experimental designs that can minimise requirements are important. This may require preliminary studies to assess relevant factors and ranges of values of interest to be sure of covering the solution space well. Focusing on the inoculum preparation parameters that significantly affect a fermentation process [25], preliminary tests were made (data not shown) to assess the un-controlled factors previously used for preparation of inoculum [8], this means that, using Taguchi's nomenclature, the focus is now on what were previously noise factors, responsible for a too high variability, in order to further minimise it. The preliminary data allowed to select only 4 factors for a more comprehensive study (namely, a 2-level design indicated that pH was not a relevant factor influencing the variability of the process in the range studied, contrasted against spore concentration and agitation), and hence a L-9 design could be used (control factors: spores age, spores concentration, agitation speed and incubation time).

John Wiley & Sons

This article is protected by copyright. All rights reserved.

Accepted Article

Accepted Article

Due to the natural variability of fermentations, it would not make sense to study the process without replicates, as it is critical to define best conditions overall, that is, those that provide the best results with consistency (and not something which can be excellent some times and very poor in others). Regardless of how tightly one controls the processing factors of importance, there are always uncontrolled factors (termed noise factors), which are responsible for process and product variability, which also include the errors of the methods of measurement.

2.4 Preparation of inoculum

The variables, their levels, and the combinations tested with the experimental design are given in Table 1.

To control spores age, the strain was inoculated on PDA medium in petri dishes before the medium was solidified and the plate was gently agitated to ensure that the microorganism grew homogeneously all over the plate. The plates were incubated at 30 ^{o}C and after the desired spores age level (Table 1) was reached, 10 mL of Tween 80 solution (0.01 % v/v) was poured onto the cultured agar plate, the spores were gently removed using a sterile glass rod and transferred to a sterile slant tube. Spores were homogenised and their concentration was estimated by using a haemocytometer (Neubauer cell). Dilutions were prepared to achieve the desired concentration level (Table 1).

Erlenmeyer flasks (125 mL of capacity) containing 25 mL of PDB medium were sterilised and inoculated with the spore suspension previously prepared to achieve the desired initial concentration of spores presented in Table 1.

This article is protected by copyright. All rights reserved.

Inoculum was incubated at 30 °C and conditions were varied according to the L-9 combination of levels given in Table 1.

2.4 Cultivation conditions

The initial pH of the Czapek-dox modified medium was adjusted to 5 before sterilising by using 0.22 m sterile membranes (Millipore, USA). A mycelial suspension was inoculated at 10% (v/v) in 125 mL Erlenmeyer flasks containing 25 mL of medium. The inoculated flasks were incubated at 30 °C on an orbital shaker (Inova 94, New Brunswick Scientific, USA) at 200 rpm for 8 days.

2.5 Analytical methods

The pigment recovery was performed according to the methodology reported by Méndez-Zavala et al. (2011) [26].

Briefly, each sample was centrifuged at 10000 x g for 20 min at 4°C (Sorball, Primo R Biofuge Centrifugation Thermo, USA). The supernatant was then filtered through a 0.45 μm cellulose filter (Millipore, USA).

Concentration of red pigments produced by *Talaromyces* spp. (formerly *Penicillium* spp.) has been expressed by various authors as optical density units [27–31] measured at 490-500 nm (a wavelength that represent the maximum adsorption for red colourants), and therefore pigment concentration was quantified indirectly by simply measuring the optical density at 500 nm using a spectrophotometer (Cary 50, UV-Visible Varian, USA).

John Wiley & Sons

This article is protected by copyright. All rights reserved.

Accepted Article

Accepted Article

The biomass concentration was determined using the gravimetric method. All analyses were replicated.

Pigment specific production (P_s, OD_{500nm}/gL^{-1}) was defined as the extracellular pigment per biomass [32]:

$$P_s = Y/B \quad (1)$$

where Y is extracellular pigment (OD_{500nm}) and B is the biomass (g/L).

3. Theory/Calculation

3.1 Optimisation targets

It is desired to achieve both a high extracellular pigment concentration and a high pigment specific production (P_s). Pigments produced by this fungus are synthesized under certain stress conditions, and therefore the production is not related necessarily to cellular growth. The microorganism could rapidly adapt to the stress conditions and use the available substrate for its growth by synthesis inhibition of other metabolites. A high extracellular pigment concentration could be obtained with low P_s but high biomass growth, but this would increase downstream processing costs due to removal of excess of biomass. On the other hand, high P_s levels could also be obtained with low extracellular pigment concentration due to minor formation of biomass.

Therefore, this is an example of a multiple response problem where more than one performance indicator needs to be considered to reach an integrated improvement of the process: to maximise the total pigment production but also the pigment per cell obtained.

Most studies in the bioprocessing area where the Taguchi method was applied consider single response processes [33,34], although in fermentation it is common to have to consider multiple

This article is protected by copyright. All rights reserved.

responses. A desirability function can be used to integrate the responses into a single objective [35], and a simple un-weighted combination of the normalised responses was used. Overall extracellular pigment and pigment specific production are both responses where the larger the better, so the normalised functions ($d_{(Y,P_s)}$) are:

$$d_{(Y,P_s)} = \begin{cases} 0, & X_{i(Y,P_s)} \leq min\left[X_{(Y,P_s)}\right] \\ \frac{X_{i(Y,P_s)} - min\left[X_{(Y,P_s)}\right]}{max\left[X_{(Y,P_s)}\right] - min\left[X_{(Y,P_s)}\right]}, & min\left[X_{(Y,P_s)}\right] \leq X_{i(Y,P_s)} \leq max\left[X_{(Y,P_s)}\right] \\ 1, & X_{i(Y,P_s)} \geq max\left[X_{(Y,P_s)}\right] \end{cases} \quad (2)$$

where $X_{i(Y,P_s)}$ is the value obtained in each experiment, $max\left[X_{(Y,P_s)}\right]$ represents the maximum value in the whole set of data and $min\left[X_{(Y,P_s)}\right]$ the minimum value in the whole set of data. The subscript Y denotes extracellular pigment and P_s the pigment specific production. The global desirability function (D_G) giving equal weight to both responses is defined as a geometric mean of the individual desirabilities [35]:

$$D_G = \left(d_Y * d_{P_s}\right)^{(1/2)} \quad (3)$$

where d_Y and d_{P_s} are the normalised response for extracellular pigment production and pigment specific production, respectively.

If both responses achieve their ideal values (max) each normalized function is equal to one, thus the global desirability function is also one. If any fails to reach its ideal value, the global desirability is less than one, and if a compromise is the best overall result, the global desirability gives equal weight to the two performance indicators.

John Wiley & Sons

This article is protected by copyright. All rights reserved.

Accepted Article

Accepted Article

3.2 Data analysis

The main drawback of the experimental designs used by Taguchi, the orthogonal arrays, is that the effect of each variable is confounded with interactive effects. Alas, the literature cited in the introduction regarding the application of the Taguchi method to optimise fermentations has not been clear about this, and largely ignored the potential problems. As the main benefit of the Taguchi method is expediency (inferring many conclusions with confidence out of a limited amount of data), there is a tendency for saturated designs (such as the one in this work with 4 factors used in the L-9 array). Azmi et al. (2013) [18], optimising yeast fermentation, used a simple L-8 with 7 factors at 2 levels, whereas Hwang et al. (2012) [19] optimising a bacterial fermentation and Chung et al. (2007) [20] optimising a fungal one used a modified L-16 (also known as M-16), testing 5 factors at 4 levels each. All these designs are saturated, that is, they use all columns of the array and thus all interactions are confounded with a factor. Furthermore, interactions between factors with 3 or 4 levels are very intricate because different components of the interaction are confounded in different columns of the design (hence, each factor is confounded with parts of many interactions, and each interaction is present in several confoundings [21]).

Confounding between a factor and an interaction implies that if an interaction is relevant, then the effect attributed to the factor is overestimated, while the impact of a factor involved in the interaction may be wrongly assessed from considering only its average effect. This could lead to very erroneous predictions for any combination of settings of the factors that is not part of the fractional design.

Hence, it is critical to analyse the data, predict the outcome of combinations not used in the design (specially the one considered to be the best, if it is not one of those used) and the

This article is protected by copyright. All rights reserved.

confidence interval of the prediction, and then run a validation test with those conditions. If the experimental outcome of the validation trial is within the confidence interval of the prediction, the assumption of neglecting interactive effects is accepted, within the margin of error of the confidence interval.

The data is analysed first with a two-way Analysis of Variance (ANoVA), and by attributing all effect of one design column to the factor assigned to it, the relative importance and statistical significance of each can be assessed. It is also possible, if desired, to break down this average effect of each factor in a linear and quadratic effect with a 3-level design (as used in this work). The response for any combination of settings of the factors is estimated with simple marginal means addition.

If all interactions are negligible, then the best setting for each factor can be chosen independently, and it will be the one with the subset of data giving the best average response (maximum pigment production, maximum pigment specific production, or maximum desirability).

An estimate using marginal means addition is given by:

$$y_{est,me} = \bar{y} + \sum_{j=1}^{n_f} \left(\bar{y}_{ic,j} - \bar{y} \right) \quad (4)$$

where $y_{est,me}$ is the estimated value of the response (considering only main effects and neglecting all interactions), $\bar{y}_{ic,j}$ is the average of the data points obtained with factor j at the chosen setting ic, and n_f is the number of factors. It is noted that eq. 4 is mathematically equal to a polynomial model with linear and quadratic terms of each factor and no interactive terms.

The confidence interval of the predictions for 90% confidence level (c.int) is given by:

$$c.int = \sqrt{\frac{V_{error} \times F_{0.1,2,18}}{n_{eff}}} \quad (5)$$

John Wiley & Sons

This article is protected by copyright. All rights reserved.

Accepted Article

Accepted Article

where V_{error} is the variance attributed to the error from the ANoVA, $F_{0.1,2,18}$ is the F-distribution value for 90% confidence level and 2 (number of levels minus 1) by 18 (number of degrees of freedom of the error) and n_{eff}, the so-called number of effective data points, is equal to:

$$n_{eff} = \frac{n}{1+\sum_{j=1}^{n_f}(n_{l,j}-1)} \quad (6)$$

In this case, with 3 replicates giving 27 points for 4 factors with 3 levels each, $n_{eff} = 3$. It is noted that the confidence level used for the F distribution was 90%, because this is a two-sided distribution, and 0.95 x 0.95 ≈ 0.9, so this corresponds roughly to a 95% confidence in one-sided distributions.

3.3 Consequences of confounding

As previously discussed, if the validation trial is successful, then a region of optimum performance was found. Further fine tuning may lead to better results still, but just like the law of diminishing returns in economics, further improvements are likely to require much more effort for lesser marginal benefits. At the stage of screening potentially promising bioprocesses, this suffices.

However, if validation is not confirmed, then the most likely reason is that one or more interactive effects are significant and confoundings must be disentangled. Taguchi method literature suggests utilising severity indexes to assess which interactions are potentially more relevant [36]. However, the authors experience in analysing many systems with easy to assess interactions (2-level designs) suggests that severity indexes tend to be high for interactions that are confounded with factors that are important (and *vice versa*) just because of the confounding itself, and thus their use is not recommended. At this stage it would be more useful to follow the advice of critics of the Taguchi method and apply a design that does not generate confoundings

This article is protected by copyright. All rights reserved.

[21]. The most commonly used ones are Box-Benkhen and Central Composite Designs. Indeed, Hwang et al. (2012) [19] applies a good practice to the optimisation of bacterial fermentation: in a first step orthogonal arrays were used with a modified L-16 revealing the 3 most important factors out of 5 and the potential region of optimum for all 5, then in a second step a Box-Benkhen design of the most relevant 3 factors was used, with the data analysed with a quadratic surface to pinpoint optimum conditions. It is noted that when the region of the solution space of the potential optimum is identified with a first step, it is likelier that the range of variability of the factors in the second step will be sufficiently small that a simple quadratic surface might provide a suitable model.

While Box-Benkhen and Central Composite Designs obviate the problem of confounding between factors and interactions, they also have drawbacks compared to the Taguchi method, namely that the predictions rely on a fit between a model and the data. If the fit is good (as in the case of Hwang et al. 2012 [19]), this is fine, otherwise there are two problems: the lack of fit of the model increases the error of conclusions and predictions, and more worryingly, quadratic surfaces have a particular shape (parabolic), which is symmetrical, and that tends to predict points of minimum or maximum that do not really exist. In many cases curvatures are due to a region of lower influence and another of higher influence in the range of the dependent variable (with a gradual evolution to a constant, like in an exponential variation), and in such cases a fit to a parabola with only 3 points will usually predict a maximum or minimum that actually does not exist (it is a mathematical artefact due to the shape of a parabola). Another limitation of quadratic surfaces is that the interactive effects are reduced to their linear by linear component, so even though interactions are considered, they are not quantified fully.

This article is protected by copyright. All rights reserved.

Accepted Article

Accepted Article

3.4 Suggested procedure to analyse validation trials

One of the reasons for the popularity of the Taguchi method and its widespread use in many industrial sectors is that industrial practice has often found that the application of the method provides significant gains in process or product targets [37]. It follows from the previous discussions that this must mean that not many interactive effects influence results significantly to negate benefits in practical applications. However, even when benefits are obtained, it is possible that one is being misled to suboptimal regions by the erroneous predictions obtained by neglecting interactions that are relevant. Thus, while Taguchi's method does provide follow-up strategies to handle interactions, it may become too cumbersome to continue using confounded designs. Thus, the procedure applied by Hwang et al. (2012) [19] is a good approach: using the Taguchi method first and a response surface analysis with an un-confounded design later (although it would have been better to analyse all data in the end integratedly; Hwang et al. (2012) [19], accept the prediction of the quadratic response surface as optimum, but this is not necessarily the case, as discussed previously, due to the bias induced by the model caused by the shape of parabolic surfaces drawn out of only 3 levels.

However, if practice shows that often interactive effects, compared to main effects, are negligible, why doing more work than needed? The main target is expediency, being able to speedily identify best process performances to assess economic potential, and therefore if in many cases the outcome of the Taguchi method with a saturated design is valid, there is no need to continue.

Taguchi's method recommends a final validation trial using the factor settings that were found to be the best. However, this does not necessarily prove that the predictions are correct, because if the best combination happens to be one of those tested there is no real validation (for instance, in

This article is protected by copyright. All rights reserved.

this case the probability of that occurring is 11%). Thus, while a validation trial with the recommended best settings should obviously be run, if necessary, it should be supplemented by another run that challenges the predictive ability of the marginal means addition estimates. There should be at least one run in the validation phase where the predictions would be erroneous if interactive effects are not negligible.

It is suggested to determine the potential impact of interactive effects just with some further analysis of the saturated design data by defining scenarios of extremes: the original estimates with marginal means addition are one extreme scenario, where all interactions are assumed negligible and all the results are assigned to main effects. Another extreme would be that only two factors are significant, the other two are not, and hence the effect attributed to them in the initial main effects ANoVA would actually be due to the components of the interactions between the others that are confounded in that column. Thus, it is possible to do several estimates, depending on what are the extreme interpretations of the causes of the results. These estimates are very easy to do, for every pair of parameters, they come from analysing the data as if it was the result of a full factorial design to obtain the full surface model, which is then used for the estimate of the conditions in the validation trial, that is:

$$y_{est,m,n} = a_{0,m,n} + a_{1,m,n}ic_m + a_{2,m,n}ic_n + a_{3,m,n}{ic_m}^2 + a_{4,m,n}{ic_n}^2 + a_{5,m,n}ic_m ic_n + a_{6,m,n}{ic_m}^2 ic_n + a_{7,m,n}ic_m {ic_n}^2 + a_{8,m,n}{ic_m}^2 {ic_n}^2 + error \quad (7)$$

where $y_{est,m,n}$ is the estimate obtained by assigning the results exclusively to the main and interactive effects of factors m and n (assuming the other factors to be negligible), ic_m and ic_m are the settings used for factors *m* and n, respectively, in the chosen combination of the validation trial and the coefficients a are obtained from the analysis of the L-9 data as a full factorial design of factors *m* and *n*. The error terms incorporates the actual effect of the other 2 factors.

John Wiley & Sons

This article is protected by copyright. All rights reserved.

Accepted Article

Accepted Article

It is noted that, just like with the marginal means addition (eq. 4), all these models fit 9 different setting combinations with 9 model parameters and therefore there is no lack of fit for the set of 27 data points; the error is totally due to white noise.

Analysing the estimate obtained with the marginal means addition ($y_{est,me}$) and those of all pairs $y_{est,m,n}$ reveals how challenging the validation trial actually is to ensure that interactive effects are indeed negligible. In case the prediction of the performance (pigment specific production, extracellular pigment, desirability function) at the optimum conditions suggested by the main effects analysis (eq. 4) does not differ significantly from other extreme justifications of the data (eqs. 7 for all pairs), then more runs are needed with at least one ensuring that the validation trial(s) is significantly challenging.

4. Results and discussion

4.1. L-9 results and main effects ANoVA

Table 2 shows the experimental design and the average results of the 3 replicates for each response. It can be seen that the range of values chosen for the factors was sufficient to cause a variation in extracellular pigment from 4 to almost 10 (as measured by colour intensity with the OD at 500 nm) and the pigment specific production from 1.6 to 4.8 (OD/g.L). The extracellular pigment and pigment specific production at the average values of the inoculum conditions were 8.2 and 4.1, respectively, showing a good potential for optimisation by finding best conditions: even just from these 9 trials, choosing the best of the 9 would mean an improvement of 22% and 17%, respectively, on the responses compared to an average choice. There is however only an 11% probability that this would indeed be the best combination of all 81 possible.

This article is protected by copyright. All rights reserved.

Accepted Article

It can be seen that runs 2, 3 and 4 gave maximum extracellular pigment with no significant difference between them, that is, it would appear that similar titers are being obtained even though different conditions are used. On the other hand, the biomass production was lower in comparison with runs 3 and 4. Therefore, the highest specific production was obtained in run 2, which is nearly 18% higher than those obtained in runs 3 and 4.

This indicates that the factors studied have a different effect on production, biomass growth and the pigment per biomass obtained and the full Analysis of Variance must be considered; it is not possible to take simple conclusions just from a subset of the data.

The appropriate method of analysis is to determine the relative importance of each factor, the best choice of setting for each, and then infer the best of the 81.

Table 3 shows the results of the main effects ANoVA, which attributes all system variability to the independent effects of the 4 variables and the error (due to noise factors, experimental variability, etc.). It can be seen that the error explains just a very small portion of the system variability (2% or less of the total sum of squares), that is, the 4 control factors dominate the system performance. If all interactive effects are negligible, then Table 3 shows that spores age is by far the most important effect on extracellular pigment and pigment specific production, accounting for more than half and more than two thirds of the sum of squares, respectively.

However, the other 3 factors have clear statistical significance ($p<0.05$), but with a different relative importance: while spores concentration is the least important of the other 3 for the extracellular pigment, it is the most relevant of the 3 for pigment specific production, and the reverse occurs for incubation time.

Figure 2 shows the means plots (averages of all data obtained with each level of each factor), from where the identification of the optimum conditions is straightforward: in each case, the best

This article is protected by copyright. All rights reserved.

Accepted Article

level is the one with highest mean. Thus, the lowest spores age (5 days) is the best for extracellular pigment and pigment specific production, as well as the lowest spore concentration (10^6 spores/mL) and the intermediate agitation speed of 200 rpm. However, the best setting for the incubation time would be the maximum (84 hrs) for the extracellular pigment, but the intermediate (72 hrs) for the pigment specific production. As Figure 2 shows that incubation time is more important for the extracellular pigment than it is for the pigment specific production, this would suggest that the highest incubation time would be the best overall. The more objective way of deciding is to use the global desirability function, giving equal weight to the importance of optimising the two responses. As Fig. 1 shows, the maximum desirability is indeed obtained with the maximum incubation time.

4.2. Phenomenological interpretation of the results

It was concluded that spores age was the most dominant effect on pigment production and pigment specific production, which were significantly higher with the lower spore age level used (5 days). Many studies have stated that spores age (slant age) has a remarkable effect on the production of any metabolites in bioprocesses [11,38,39]. Spores age might influence the mycelial morphology in submerged fermentations affecting the final yield of the process [40].

Regarding the effect of spore's age on biomass, this factor showed relatively low influence (~10% of the total sum of squares explained by this factor) on the growth presented by the microorganism.

It was also found that both responses (Y and P_s) decreased with increasing spores concentration. Many studies have related spores concentration to final morphology in fungal fermentations [40–42]. It was observed that pellets were formed at the lower level ($1x10^5$ spores/mL). Fungal morphology can determine the final pigment specific production of a fermentation process. It has

This article is protected by copyright. All rights reserved.

been stated that pellets are formed at inoculum levels below 10^{11} spores/m^3 in fungal fermentation [40].

Formation of mycelial pellets is known to be an important factor for production of certain metabolites [41,42], however, in this study spores concentration showed a very small influence on pigment production (explaining less than 7 % of the total sum of squares).

The agitation speed showed an optimum effect at the intermediate level (200 rpm), especially for the extracellular pigment, suggesting that lower or higher agitations are detrimental. Many reports have stated that the morphological changes occurring during fermentation are due to agitation or mechanical forces [25,43].

Agitation is an important control parameter in aerobic fermentations due to it is correlation with the oxygen transfer rate. The strain showed its maximum growth at an intermediate setting. This may be a consequence of two effects with reverse consequences: mechanical stress suffered by the cells at higher levels of agitation and poor oxygen supply at the lowest level, giving a maximum at an intermediate setting.

The optimal levels of inoculum age (incubation time) were different for extracellular pigment and pigment specific production, while higher incubation times provided bigger yields, the pigment specific production shows a maximum, decreasing beyond a too high incubation time. The pigment is a secondary metabolite, and therefore its pigment specific production is not a linear relation of growth (the microorganism could grow significantly and yet produce little pigment).

Higher biomass levels were obtained using the highest level of incubation time, this result indicates that the microorganism was in an active phase and thus it kept growing, which led to higher biomass accumulation and thus obtaining less pigment production per cell.

John Wiley & Sons

This article is protected by copyright. All rights reserved.

Accepted Article

Accepted Article

4.3. Validation trial

The best conditions according to the Taguchi method and the L-9 results (Fig. 3) are lowest age and concentration of spores, intermediate agitation speed and higher incubation time (coded values -1, -1, 0 and 1). This is not one of the 9 combinations tried, but in order to assess if a validation trial with this combination alone is sufficiently challenging to assess whether the results could be explained by significant interactive effects (which would mean that the conclusions taken earlier could be wrong), the L-9 data (27 points) were fitted to full factorial surfaces (eq. 7) for every pair of factors, resulting in models that could be used to predict what the extracellular pigment and pigment specific production should be with the chosen combination of settings. The model fit results and the respective predictions, compared to the Taguchi method estimate using marginal means addition (eq. 4), are shown in Table 4.

Considering first whether such a trial is sufficiently challenging, it is noted that the extracellular pigment predicted with marginal means addition for the optimum settings is significantly different from all other predictions, whereas in the case of pigment specific production there is no statistically significant difference between the prediction with marginal means or with a full factorial polynomial (eq. 7) considering that the results are due just to the effect of spores age and agitation speed and its full interaction (eq. 7 with factors A and C only). Thus, the optimum combination of settings will not be sufficiently challenging regarding pigment specific production. Of the 81 possible combinations, only 19 generated predictions of the marginal means addition that are statistically different from all models with the 6 pairs of factors (eq. 7) for both extracellular pigment and pigment specific production. One of those was the same settings for concentration, agitation and incubation time, but maximum spores age (coded values

This article is protected by copyright. All rights reserved.

1,-1,0,1). Thus, one more validation run was needed to ensure that the validation trials were sufficiently challenging. The predictions of values of this additional run are also given in Table 4. It can be seen that the marginal means estimates are actually the only that predicted the experimental outcome of the validation trial for the optimum combination of settings successfully. Thus, the conclusions of the main effects ANoVA could be accepted just with this trial and there is no evidence that interactive effects could be sufficiently relevant to affect the final result. However, that could not have been known prior to the outcome, so the other experiment with settings 1, -1, 0, 1 needs to be considered. In this case, marginal means addition was again the only model that provided an estimate that agrees with the experimental results for extracellular pigment and the two-factor model prediction that was statistically similar for the optimum conditions is now completely different and too far from the experimental results. The average pigment specific production of the 4 experimental runs under these conditions was however outside the interval of the marginal means addition plus or minus its error. However, given that the validation trial had 4 repeats, a t-test with the standard deviations given in Table 4 would give a 94.8% probability of the null hypothesis which is just marginally outside the usual 95% confidence level. The model with eq. 7 for factors A and C only would give very poor estimates for this combination of settings.

For the purpose of establishing the potential of this process, this result suffices, as further improvements are unlikely to be so dramatic. The grand average of the extracellular pigments was 6.96 and that of the pigment specific production was 3.21, these are the values obtained at the centre of the solution space, and thus have the highest probability of being selected by simple random choice of values for the 4 factors. The best conditions gave an extracellular pigment

John Wiley & Sons

This article is protected by copyright. All rights reserved.

Accepted Article

production that was 63% higher and a pigment specific production 44% higher by moving to a more favourable region of the solution space.

Several batches have been carried out with these conditions, which prove that selected settings also provide the best robustness. Figure 3 shows a distribution of the quality criterion (Pigment and Pigment specific production) of the performance of the studied system for a large number of batches (54 batches) before and after the improvement of the inoculum preparation conditions. It can be seen that before the inoculum optimisation, there is an increased spread of unacceptable batches. After the optimisation, the average performance has been improved and also the performance distribution is narrower for both responses.

This illustrates well the significant benefits that can be speedily obtained with the Taguchi method when interactions are negligible. The process optimisation outcome of a Taguchi analysis of a set of data like this is which of the 81 possible combinations of 3 values in each of 4 variables provides the best result with the maximum consistency.

While not promising to be an absolute optimum (fine tuning within the range may reveal better specific values), it suffices to provide a fair assessment of optimum productivities potentially achievable, and with just 3 repeats of 9 trials, the scanning efficiency is evident.

4.4. Further fermentation experiments on the effect of spores age

As the age of spores is the most significant factor affecting the inoculum for the production of pigments, and there was no evidence of significant interactive effects, this factor was further studied over a wider range of values. This is important in the case of an optimum setting being an extreme (as in this case, where it was the lowest), because it is conceivable that a better

John Wiley & Sons

This article is protected by copyright. All rights reserved.

Accepted Article

setting could be found outside of the range originally considered. Fermentation experiments were carried out by setting factors B, C and D at the optimum levels determined (10^5 spores/mL spores concentration, 200 rpm and 84 hrs of incubation time) and the age of spores was studied from 3 to 11 days (3 days being the lowest time where spores are formed, 11 days being the highest level previously used [8]).

Four replicates were performed in each case (the value for 5 days of age being the validation trial). Results of extracellular pigment are shown in Figure 4. It can be seen that production of pigments reaches a maximum precisely with 5-day-old spores (11.45±0.17 OD_{500nm}).

These results are higher than those previously reported for the production of pigments by *Talaromyces purpurogenus* (formerly *Penicillium purpurogenum*)[29,30], however, mycelial disks and spores were used as inoculum on those studies.

In order to identify which is the growth phase of the microorganism at 5 days old (where maximum production can be obtained) and acquire a better understanding of the decrease in pigment production by using older spores, the biomass growth kinetics of the spores used was studied. The microorganism was grown in PDA plates and the biomass dry weight was analysed from 0 to 11 days every 24 h. Results are shown in Figure 5, typical microorganism growth phases (lag, exponential, stationary and death) are highlighted in the figure.

When the microorganism is 3-4 days old it is still in an active growing phase and it uses the substrate primarily to grow and not to produce pigments. The maximum pigment production occurs when using spores that are at the beginning of the stationary phase, when they are no longer growing significantly. On the other hand, with older spores (6-11 days) there is a decrease, which could be attributed to microorganisms entering their death phase.

This article is protected by copyright. All rights reserved.

Accepted Article

Similar findings have been reported in a different bioprocess production process, determining the effect of spore's age on the production of citric acid by *Aspergillus niger* [44].

5. Conclusions

The inoculum preparation conditions have a significant effect on the pigments production process by *Talaromyces* spp. (previously cited as *Penicillium purpurogenum* GH2). Spores age was the most important effect, with an optimum at 5 days old. The Taguchi method including a validation trial in conditions deemed challenging to test the relevance of interactive effects allowed to establish overall optimum conditions with confidence as all interactions indeed proved to be negligible, and these were: spores age (days), 5; spores concentration (spores/mL), 10^5, agitation (rpm), 200 and incubation time (h), 84. Compared to the extracellular pigment and pigment specific production that would be obtained with average settings of these factors, the optimum settings thus identified with only 9 combinations of the 4 factors represented at increase of 63% in extracellular pigment and 44% in pigment specific production.

Acknowledgements

Author L. Morales-Oyervides acknowledges CONACyT-México for the financial support provided for conducting her PhD studies (215490-2011). The authors acknowledge to FINNOVA-CONACyT (Project No. 175230) for the financial support of this project.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

This article is protected by copyright. All rights reserved.

Accepted Article

Accepted Article

References

1. El-Wahab HMFA, Moram GSE-D. Toxic effects of some synthetic food colorants and/or flavor additives on male rats. *Toxicol Ind Health*. 2013;29(2):224-232.

2. Gomes KMS, Oliveira MVGA de, Carvalho FR de S, Menezes CC, Peron AP. Citotoxicity of food dyes sunset yellow (E-110), bordeaux red (E-123), and tatrazine yellow (E-102) on *Allium cepa* L. root meristematic cells. *Food Sci Technol*. 2013;33(1):218-223.

3. Kapadia GJ, Tokuda H, Sridhar R, et al. Cancer chemopreventive activity of synthetic colorants used in foods, pharmaceuticals and cosmetic preparations. *Cancer Lett*. 1998;129(1):87-95.

4. Dufossé L, Fouillaud M, Caro Y, Mapari SAS, Sutthiwong N. Filamentous fungi are large-scale producers of pigments and colorants for the food industry. *Curr Opin Biotechnol*. 2014;26:56-61.

5. Liao C-D, Chen Y-C, Lin H-Y, Chiueh L-C, Shih DY-C. Incidence of citrinin in red yeast rice and various commercial *Monascus* products in Taiwan from 2009 to 2012. *Food Control*. 2014;38:178-183.

6. Mapari SAS, Hansen ME, Meyer AS, Thrane U. Computerized screening for novel producers of *Monascus*-like food pigments in *Penicillium* species. *J Agric Food Chem*. 2008;56(21):9981-9989.

7. Méndez-Zavala A, Pérez C, Montañez JC, Martínez G, Aguilar CN. Red pigment production by *Penicillium purpurogenum* GH2 is influenced by pH and temperature. *J Zhejiang Univ Sci B*. 2011;12(12):961-968.

8. Méndez-Zavala A. Identificación de factores que afectan la producción de pigmentos por *Penicillium purpurogenum* GH2 y obtención de perfiles cromatográficos. 2011.

9. Morales-Oyervides L, Oliveira JC, Sousa-Gallagher MJ, Méndez-Zavala A, Montañez JC. Quantitative assessment of the impact of the type of inoculum on the kinetics of cell growth, substrate consumption and pigment productivity by *Penicillium purpurogenum* GH2 in liquid culture with an integrated stochastic approach. *Food Bioprod Process*. 2015;96:221-231.

10. Gutarra MLE, Godoy MG, Castilho LR, Freire DMG. Inoculum strategies for *Penicillium simplicissimum* lipase production by solid-state fermentation using a residue from the babassu oil industry. *J Chem Technol Biotechnol*. 2007;82(3):313-318.

11. Dasu VV, Panda T. Optimization of microbiological parameters for enhanced griseofulvin production using response surface methodology. *Bioprocess Eng*. 2000;22(1):45-49.

12. Taguchi G, Clausing D. Robust quality. *Harv Bus Rev*. 1990;68(1):65-75.

John Wiley & Sons

This article is protected by copyright. All rights reserved.

13. Canli O, Tasar GE, Taskin M. Inulinase production by *Geotrichum candidum* OC-7 using migratory locusts as a new substrate and optimization process with Taguchi DOE. *Toxicol Ind Health*. 2013;29(8):704-710.

14. Uysal E, Akcan N, Baysal Z, Uyar F. Optimization of α-amylase production by *Bacillus subtilis* RSKK96: using the Taguchi experimental design approach. *Prep Biochem Biotechnol*. 2011;41(1):84-93.

15. Lakshmi GS, Rao CS, Rao RS, Hobbs PJ, Prakasham RS. Enhanced production of xylanase by a newly isolated *Aspergillus terreus* under solid state fermentation using palm industrial waste: A statistical optimization. *Biochem Eng J*. 2009;48(1):51-57.

16. Das Mohapatra PK, Maity C, Rao RS, Pati BR, Mondal KC. Tannase production by *Bacillus licheniformis* KBR6: Optimization of submerged culture conditions by Taguchi DOE methodology. *Food Res Int*. 2009;42(4):430-435.

17. Teng Y, Xu Y. Culture condition improvement for whole-cell lipase production in submerged fermentation by *Rhizopus chinensis* using statistical method. *Bioresour Technol*. 2008;99(9):3900-3907.

18. Azmi A, Ngoh C, Mel M. Prediction of significant factors in the production of ethanol by ragi tapai co-culture using Taguchi methodology. *African J Biotechnol*. 2013;10(81):18833-18841.

19. Hwang C-F, Chang J-H, Houng J-Y, Tsai C-C, Lin C-K, Tsen H-Y. Optimization of medium composition for improving biomass production of *Lactobacillus plantarum* Pi06 using the Taguchi array design and the Box-Behnken method. *Biotechnol Bioprocess Eng*. 2012;17(4):827-834.

20. Chung CC, Chen HH, Hsieh PC. Aplication of the Taguchi method to optimize *Monascus* spp. culture. *J Food Process Eng*. 2007;30(2):241-254.

21. Montgomery DC. *Design and Analysis of Experiments*. John Wiley & Sons; 2008.

22. Cruz-Hernández M, Rodríguez R. Aislamiento y caracterización morfológica de cepas microbianas degradadotas de taninos. In: *XXII Encuentro Nacional de La AMIDIQ. Mexico.* ; 2001.

23. Espinoza-Hernández T. Caracterización morfológica, fisiológica y molecular de tres cepas fúngicas productoras de pigmentos. 2004.

24. Frisvad JC, Yilmaz N, Thrane U, et al. *Talaromyces atroroseus*, a New Species Efficiently Producing Industrially Relevant Red Pigments. Baker SE, ed. *PLoS One*. 2013;8(12):e84102.

25. Krull R, Wucherpfennig T, Esfandabadi ME, et al. Characterization and control of fungal morphology for improved production performance in biotechnology. *J Biotechnol*. 2013;163(2):112-123.

26. Méndez A, Pérez C, Montañéz JC, Martínez G, Aguilar CN. Red pigment production by Penicillium purpurogenum GH2 is influenced by pH and temperature. *J Zhejiang Univ Sci B*. 2011;12(12):961-968.

This article is protected by copyright. All rights reserved.

Accepted Article

Accepted Article

27. Santos-Ebinuma VC, Teixeira MFS, Pessoa A. Submerged culture conditions for the production of alternative natural colorants by a new isolated *Penicillium purpurogenum* DPUA 1275. *J Microbiol Biotechnol*. 2013;23(6):802-810.

28. Celestino J dos R, Carvalho LE de, Lima M da P, Lima AM, Ogusku MM, Souza JVB de. Bioprospecting of Amazon soil fungi with the potential for pigment production. *Process Biochem*. 2014;49(4):569-575.

29. Santos-Ebinuma VC, Roberto IC, Teixeira MFS, Pessoa Jr. A. Improvement of submerged culture conditions to produce colorants by *Penicillium purpurogenum*. *Brazilian J Microbiol*. 2014;45(2):731-742.

30. Patil S, Sivanandhan G, Thakare D. Effect of physical and chemical parameters on the production of Red exopigment from *Penicillium purpurogenum* isolated from spoilt onion and study of its antimicrobial activity. *Int J Curr Microbiol Appl Sci*. 2015;4(7):599-609.

31. Morales-Oyervides L, Oliveira JC, Sousa-Gallagher MJ, Méndez-Zavala A, Montañez JC. Effect of heat exposure on the colour intensity of red pigments produced by *Penicillium purpurogenum* GH2. *J Food Eng*. 2015;164:21-29.

32. Velmurugan P. Studies on the production and dyeing properties of water soluble pigments from filamentous fungi. 2008.

33. Das SP, Das D, Goyal A. Statistical optimization of fermentation process parameters by Taguchi orthogonal array design for improved bioethanol production. *J Fuels*. 2014;2014:1-11.

34. El Aty AAA, Wehaidy HR, Mostafa FA. Optimization of inulinase production from low cost substrates using Plackett-Burman and Taguchi methods. *Carbohydr Polym*. 2014;102:261-268.

35. Chang M-Y, Tsai G-J, Houng J-Y. Optimization of the medium composition for the submerged culture of *Ganoderma lucidum* by Taguchi array design and steepest ascent method. *Enzyme Microb Technol*. 2006;38(3-4):407-414.

36. RanjitK R. Design Of Experiment Using the Taguchi approach. In: *Taguchi's Quality Engineering Handbook.* ; 2001.

37. Taguchi G, Chowdhury S, Wu Y. Taguchi's Quality Engineering Handbook. 2005.

38. Chen H-C, Liu T-M. Inoculum effects on the production of γ-linolenic acid by the shake culture of *Cunninghamella echinulata* CCRC 31840. *Enzyme Microb Technol*. 1997;21(2):137-142.

39. Muralidhar R, Gummadi SN, Dasu VV, Panda T. Statistical analysis on some critical parameters affecting the formation of protoplasts from the mycelium of *Penicillium griseofulvum*. *Biochem Eng J*. 2003;16(3):229-235.

40. Papagianni M. Fungal morphology and metabolite production in submerged mycelial processes. *Biotechnol Adv*. 2004;22(3):189-259.

41. Driouch H, Hänsch R, Wucherpfennig T, Krull R, Wittmann C. Improved enzyme production by bio-pellets of *Aspergillus niger*: targeted morphology engineering using

This article is protected by copyright. All rights reserved.

titanate microparticles. *Biotechnol Bioeng*. 2012;109(2):462-471.

42. Zhou Z, Du G, Hua Z, Zhou J, Chen J. Optimization of fumaric acid production by *Rhizopus delemar* based on the morphology formation. *Bioresour Technol*. 2011;102(20):9345-9349.

43. Posch AE, Herwig C, Spadiut O. Science-based bioprocess design for filamentous fungi. *Trends Biotechnol*. 2013;31(1):37-44.

44. Alam MZ, Bari MN, Muyibi SA, Jamal P. Development of culture inoculum for scale-up production of citric acid from oil palm empty fruit bunches by *Aspergillus niger*. *Procedia Environ Sci*. 2011;8:396-402.

Accepted Article

This article is protected by copyright. All rights reserved.

List of Figures.

Figure 1. Process steps needed for the production of pigments produced by *Talaromyces* spp.: a) pre-culture step (Inoculum), b) production step and c) pigment recovery.

Figure 2. Means plots of extracellular pigment, pigment specific production and global desirability.

The points indicate the averages of all data obtained with the respective setting of the factor, the error bars the standard deviation caused by the 3 other factors and the error, and the horizontal dotted line is the global average of all data

Figure 3. Distribution of a) Pigment and b) Pigment specific production of 54 batches produced before (pattern bars) and after (full bars) inoculum improvement.

Figure 4. Extracellular pigment obtained with different age of spores (3-11 days).

Figure 5. Spores biomass kinetics and growth phases.

Accepted Article

John Wiley & Sons

This article is protected by copyright. All rights reserved.

Accepted Article

Table 1. Inoculum preparation conditions and levels for the L-9 experimental design.

Factor	Process Variable	Low level (-1 coded)	Intermediate level (0 coded)	High level (1 coded)
A	Spores age (days)	5	7	9
B	Spores concentration (spores/mL)	10^5	10^6	10^7
C	Agitation (rpm)	150	200	250
D	Incubation time (hrs)	60	72	84

This article is protected by copyright. All rights reserved.

Table 2. Experimental Design and average results (3 replicates) of extracellular pigment and pigment specific production by *Talaromyces* spp.

Run	Coded values				Actual				Experimental results		
	A	B	C	D	Spores Age (days)	Spores Concentration (spores/mL)	Agitation Speed (rpm)	Incubation Time (h)	Biomass (g/L)	Extracellular pigment (OD_{500nm})	Pigment specific production (OD_{500nm}/g.L^{-1})
1	-1	-1	-1	-1	5	10^5	150	60	1.96 ± 0.20	7.20 ± 0.73	3.67 ± 0.06
2	-1	0	0	0	5	10^6	200	72	2.08 ± 0.10	9.92 ± 0.30	4.76 ± 0.08
3	-1	1	1	1	5	10^7	250	84	2.43 ± 0.13	9.50 ± 0.68	3.90 ± 0.08
4	0	-1	0	1	7	10^5	200	84	2.53 ± 0.04	9.93 ± 0.20	3.92 ± 0.04
5	0	0	1	-1	7	10^6	250	60	1.68 ± 0.03	6.07 ± 0.37	3.61 ± 0.28
6	0	1	-1	0	7	10^7	150	72	2.09 ± 0.24	5.63 ± 0.91	2.69 ± 0.27
7	1	-1	1	0	9	10^5	250	72	2.19 ± 0.22	6.07 ± 0.37	2.78 ± 0.16
8	1	0	-1	1	9	10^6	150	84	2.25 ± 0.34	4.32 ± 0.52	1.92 ± 0.12
9	1	1	0	-1	9	10^7	200	60	2.52 ± 0.08	4.00 ± 0.30	1.59 ± 0.15

John Wiley & Sons

This article is protected by copyright. All rights reserved.

Accepted Article

Accepted Article

Table 3. Analysis of variance with main effects (all interactions deemed negligible). The p-value gives the probability of the null hypothesis (factors with values below 5% are statistically significant).

Cause of variability	Degrees of freedom	Raw Sum of Squares		Relative importance*		Variance explained		p-value	
		Y	P_s	Y	P_s	Y	P_s	Y	P_s
Spores age	2	78.225	18.317	56.8%	71.5%	39.113	9.159	0.0000%	0.0000%
Spores concentration	2	6.786	3.388	4.9%	13.2%	3.393	1.694	0.0015%	0.0000%
Agitation	2	18.944	2.547	13.8%	9.9%	9.472	1.274	0.0000%	0.0000%
Incubation time	2	24.937	0.904	18.1%	3.5%	12.469	0.452	0.0000%	0.0067%
Error	18	2.797	0.473	2.0%	1.8%	0.155	0.026		
Total	26	137.691	25.632						

**The relative importance of each term is the percentage of the total sum of squares explained by the raw sum of squares attributed to the factor*

This article is protected by copyright. All rights reserved.

Accepted Article

Table 4. Experimental results (average of 4 replicates) for different age of spores (A, minimum 5 and maximum 9 days) with factors B, C and D at the optimum levels (10^5 spores/mL, with 82 hrs incubation and 200 rpm) and predictions with marginal means additions and with full factorial combinations with 2 factors only (eqs. 7)

Responses	Confidence intervals	A	Experimental	Marginal means	A and B only	A and C only	A and D only	B and C only	B and D only	C and D only
Extracellular pigment (OD_{500nm})	0.54	-1	11.45±0.17	11.59	7.20	9.92	9.50	9.93	9.93	9.93
		1	7.12±0.50	7.52	6.07	4.00	4.32	9.93	9.93	9.93
Pigment Specific Production (OD_{500nm}/gL^{-1})	0.16	-1	4.51±0.42	4.63	3.67	4.76	3.90	3.92	3.92	3.92
		1	3.01±0.20	2.61	2.78	1.59	1.92	3.92	3.92	3.92

This article is protected by copyright. All rights reserved.

Accepted A

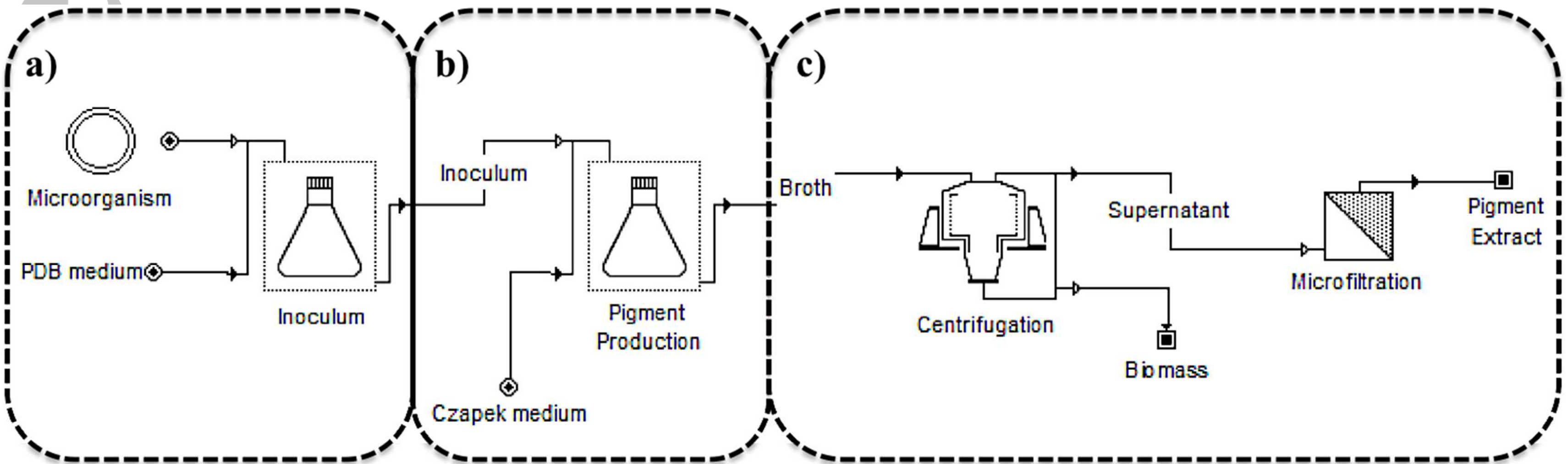


Figure 1

John Wiley & Sons

This article is protected by copyright. All rights reserved.

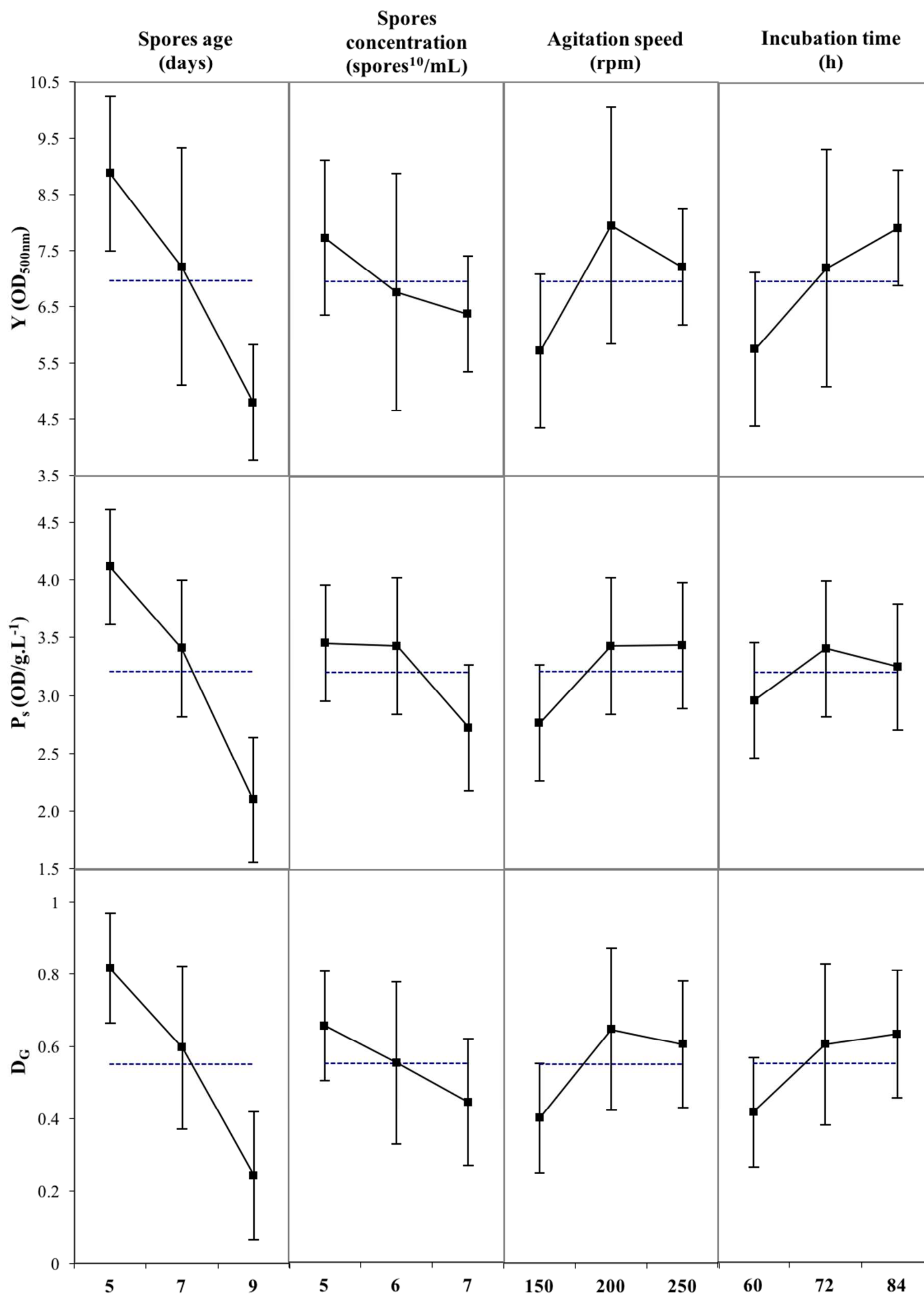


Figure 2

John Wiley & Sons

This article is protected by copyright. All rights reserved.

Accepted Article

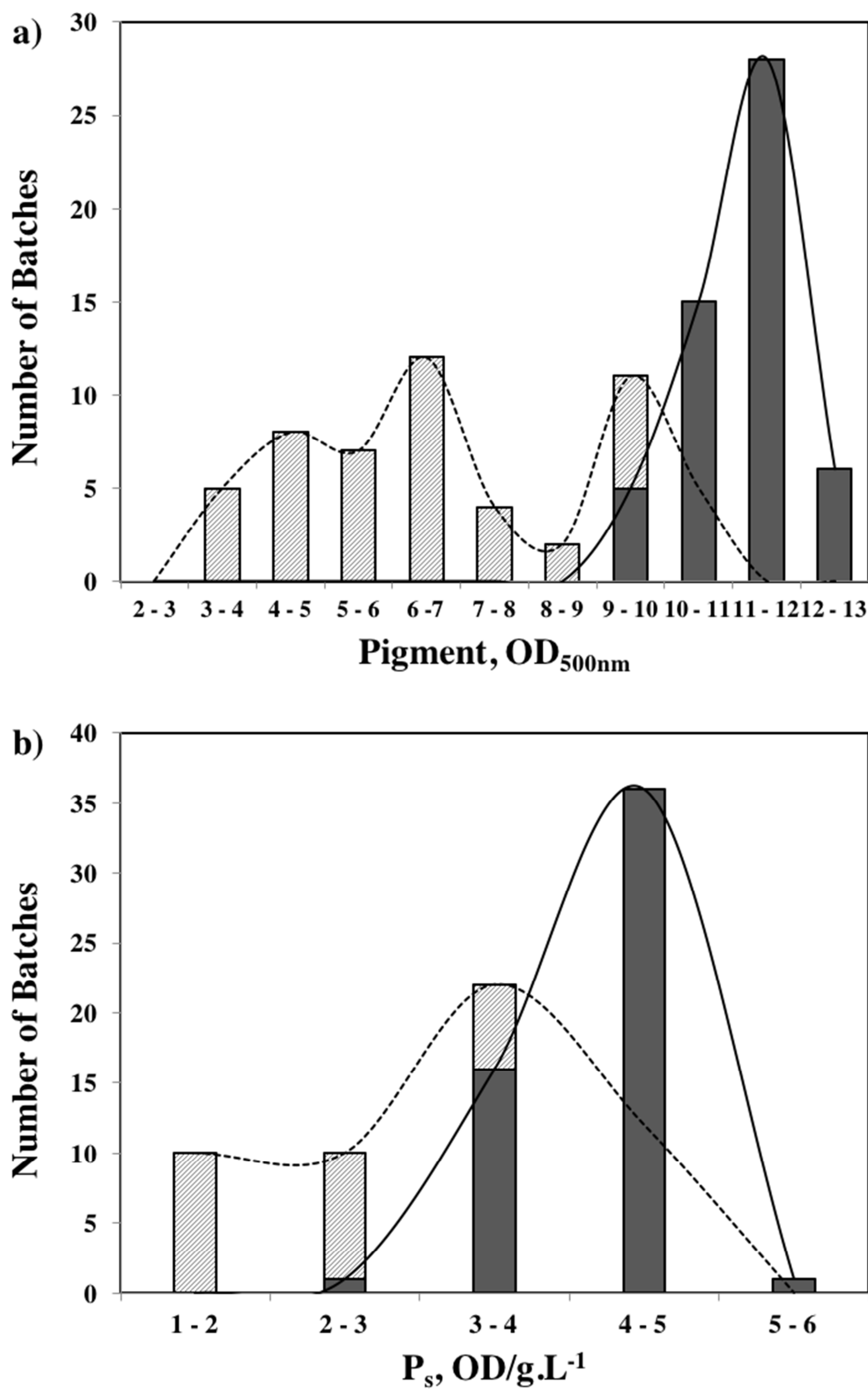


Figure 3

This article is protected by copyright. All rights reserved.

Accepted Article

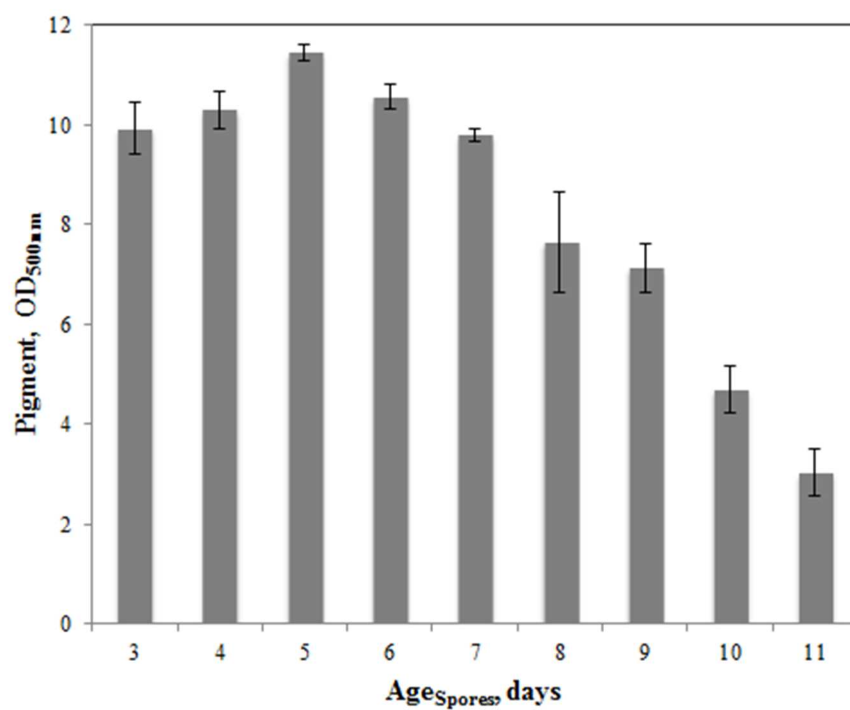


Figure 4

John Wiley & Sons

This article is protected by copyright. All rights reserved.

Accepted Article

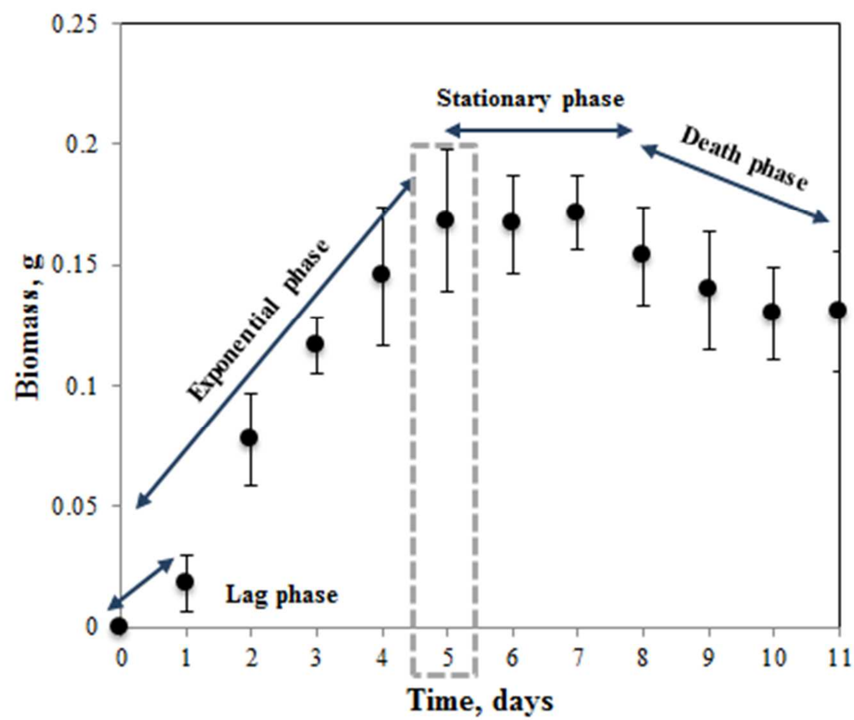


Figure 5

This article is protected by copyright. All rights reserved.