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REVIEW

Statistical experimental designs for the production of secondary metabolites in plant cell suspension cultures

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Abstract Statistical experimental designs, also known as the "design of experiments" (DoE) approach, are widely used to improve not only technical processes but also to answer questions in the agricultural, medical and social sciences. Although many articles have been published about the application of DoE in these fields, few studies have addressed the use of DoE in the plant sciences, particularly in the context of plant cell suspension cultures (PCSCs). Compounds derived from PCSCs can be developed as pharmaceuticals, chemical feedstocks and cosmetic ingredients, and statistical experimental designs can be used to improve the productivity of the cells and the yield and/or quality of the target compounds in a cost efficient manner. In this article, we summarize recent findings concerning the application of statistical approaches to improve the performance of PCSCs and discuss the potential future applications of this approach.

Keywords Design of experiments (DoE) · Factorial design · One-factor-at-a-time (OFAT) · Plant cell cultivation · Process optimization

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Introduction

Plants provide a wide range of industrially relevant secondary metabolites with applications in the food, pharmaceutical, chemical and cosmetic industries (Gaosheng and Jingming 2012). In nature, plants synthesize secondary metabolites mainly for defense against herbivores and pathogens, and for communication with the environment (Kennedy and Wightman 2011). The pathways are distinct from those used in primary metabolism. Well known secondary pathways include the mevalonate and 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathways which yield isoprenoids, both comprising multiple steps catalyzed by different enzymes (Liao et al. 2016). Plants produce nearly four times as many secondary compounds as microbes (Rao and Ravishankar 2002) and the products can be broadly categorized according to their biochemical origin, e.g. terpenoids derived from

terpene, alkaloids derived from amino acids or polyamines, and phenylpropanoids derived from phenylalanine (Bourgaud et al. 2001). The diverse biochemical properties of secondary metabolites make them suitable for a large number of commercial applications, particularly in the context of food, feed, chemicals, pharmaceuticals and cosmetics. The market has grown from ~US$ 3 billion at the turn of the last century to more than ~US$ 4 billion today, and is expected to exceed ~US$ 4.5 billion by 2020 (Glaser 1999; Markets and Markets 2015). This reflects the expansion of multiple market segments in addition to pharmaceuticals, including plant-derived natural colors, flavors and sweeteners (Murthy et al. 2014).

Secondary metabolites are mainly extracted from the tissues of whole plants collected from nature but are also isolated from plants grown in greenhouses or cultivated fields (Souza et al. 2015). However, the use of whole plants increases the risk of contamination with agrochemicals and fertilizers, the plants are susceptible to pests and diseases, and performance depends on cultivation conditions which may vary due to local differences in soil quality and climate (Hellwig et al. 2004; Bhatia et al. 2015). This has driven the development of contained production systems that can be maintained under constant conditions, particularly hairy root cultures and plant cell suspension cultures (PCSCs). The latter comprise cells originating from de-differentiated plant donor material that have subsequently been adapted to controlled conditions and are cultivated with continuous shaking in liquid media. Several PCSCs have been established for the production of commercially valuable secondary metabolites such as catechin, gallic acid, aloin, betalains, dopamine, and acacetin (Ali et al. 2013; Raei et al. 2014; Tariq et al. 2014; Guadarrama-Flores et al. 2015; Manivannan et al. 2016). However, only minute amounts of secondary metabolites accumulate within the plant cells (Hussain et al. 2012). A break-even point of US$ 1500 kg^{-1} has been proposed for the commercial production of secondary metabolites in PCSCs (Dörnenburg and Knorr 1995). The commercialization of valuable secondary metabolites therefore involves two key objectives: (1) drive secondary metabolism towards maximum product accumulation to increase intrinsic productivity, and (2) boost PCSC biomass production to increase volumetric productivity (Holmberg et al. 2014). This increases the amount of product while limiting the culture volume, fermentation time and downstream processing costs (Rasche et al. 2016).

PCSCs can be optimized by manipulating cultivation parameters such as light, temperature and medium constituents, and their influence can affect both product accumulation and biomass production (Smetanska 2008). However, there are multiple interactions between different cultivation parameters so the overall effect on productivity can be unpredictable. For example, the O_2 intake of PCSCs is not dependent solely on the O_2 partial pressure, but also the flask filling volume and shaking frequency (Vasilev et al. 2014b). In standard optimization approaches, such interactions are not taken into considerations because only one parameter is varied while the others are held constant, which is known as the one-factor-at-a-time (OFAT) strategy. More recently, statistical experimental designs, also known as the "design of experiments" (DoE) approach, have been more widely adopted as a tool for process optimization (see Montgomery 2013 for review). Statistical analysis can be used to simultaneously examine the effects of multiple input parameters on a given output, allowing cultivation processes to be optimized in terms of product accumulation and biomass production by considering how input parameters interact (Buyel and Fischer 2014; Vasilev et al. 2013). Although there have been few reports thus far describing the optimization of PCSCs using statistical analysis, the advantages of this approach make it likely to be adopted more widely in the future, especially in a commercial context. Here we discuss the advantages and disadvantages of PCSCs for the production of secondary metabolites and highlight systematic DoE strategies that can be used to enhance the production of valuable compounds in plant cells.

Trial-and-error experiments compared to statistical experimental designs

PCSCs are often used as model production systems because they have a number of advantages over whole plants: (1) they take up less space; (2) cultivation is not dependent on the climate, season, weather or geopolitical factors, and is not affected by pests and diseases; (3) the manipulation of secondary metabolism is more straightforward in uniform cultures than whole plants; (4) no agrochemicals are required; (5) the containment and controlled conditions make PCSCs suitable for

good manufacturing practice (GMP) production (Gaosheng and Jingming 2012); and (6) scale up from shake flasks to large fermenters is straightforward (Raven et al. 2015). These benefits mean that PCSCs are the most appropriate plant system for small-scale laboratory experiments before process optimization and scaled-up production (Vasilev et al. 2014a). However, several obstacles remain to be addressed when cultivating plant cells. The growth rate of PCSCs is slower than microbes because there is a longer gap between each round of cell division, and the accumulation of secondary metabolites is lower than whole plants because undifferentiated plant cells lack the biochemical and morphological characteristics of differentiated cells that promote product accumulation, e.g. desirable metabolic pathways can be switched off during dedifferentiation (Yue et al. 2016), and undifferentiated cells lack features such as extracellular storage compartments (Smetanska 2008). PCSCs derived from different plant tissues may also favor different physical cultivation parameters (light, temperature, shaking frequency and humidity) and chemical environments (medium composition and the presence of specific plant growth regulators).

The impact of physical and chemical cultivation parameters is poorly understood in most cases, and PCSC optimization studies tend to be based on the trial-and-error testing of individual parameters using the OFAT approach which yields suboptimal results. However, because OFAT is quick and convenient, researchers are inclined to accept the consequences of losing significant information and drawing hasty conclusions, especially when such experiments do yield significant improvements. In most reports, only one cultivation parameter is investigated intensively, e.g. one medium component at different concentrations (Raei et al. 2014; Fazal et al. 2016; Manivannan et al. 2016). The synergistic effects of two or more cultivation parameters are thereby concealed and their potential impact on the process is neglected. In contrast, statistical experimental designs consider the interactions between multiple parameters to gain deeper insight into the mechanistic basis of secondary metabolism. There are several specialized designs for mixtures (mixture designs), screening factors (factorial designs) and the quantitation of factor impacts on responses (response surface methods) available for process optimization studies. The advantages of DoE in comparison to the OFAT approach can easily be illustrated with an example for a factorial design. Assuming that the effect of two factors A_0 and B_0 on the desired output are investigated, information could be gained by varying (index "1") the factors one at a time. For the OFAT approach this results in two factor level scenarios: A_1 B_0 and A_0 B_1. If the OFAT design showed that A_1 B_0 and A_0 B_1 gave better results than A_0 B_0 the conclusion would be that A_1 B_1 improve the results even further. However, if there is an interaction or interdependency between both factors this conclusion may be false. An interaction of A and B moreover means that the effect of A on the results depends on the level of B and vice versa. In a factorial design an additional combination where both factors are varied simultaneously is added (A_1 B_1). By this approach it is possible to obtain data just as concise as those from the OFAT approach and additionally decipher potential interactions of the investigated factors. In Fig. 1, a factorial design (a) and the OFAT approach (b) are displayed side by side (Montgomery 2013) demonstrating that the factorial design investigates a larger experimental design space than the OFAT approach.

OFAT and DoE strategies to optimize the production of secondary metabolites

The composition of cultivation media containing macronutrients, micronutrients and additional elicitors has a strong impact on the activity of inducible secondary pathways induced by external influences. Furthermore, physical cultivation parameters such as light, temperature, humidity and shaking velocity also influence the behavior of PCSPs in terms of biomass production and the accumulation of secondary metabolites (Rao and Ravishankar 2002). The physiological mechanisms underlying these influential factors have been investigated in detail, but the diversity of PCSCs and the complexity of metabolic pathways make it challenging to draw general principles. Therefore, researchers must investigate and optimize each PCSC biomass/product combination on a case-by-case basis to maximize productivity.

Illumination

Light influences plant growth, development, and the biosynthesis of specific secondary metabolites (Zhong

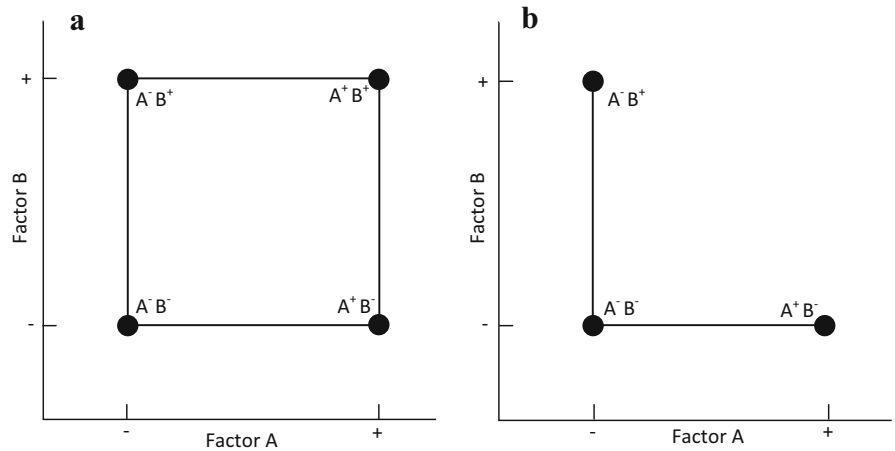

Fig. 1 Comparison of a factorial design experiment (**a**) and the OFAT approach (**b**). **a** The factorial experiments allows the investigation of all possible combinations of two factors (A, B) on two levels (− low level; + high level) thus considering potential interactions (A+, B+) between the factors. **b** In the OFAT approach, apart from the original factor levels (A−, B−) two factor combinations are investigated neglecting factor interactions that leads to the loss of meaningful information

2001). Recent studies have shown that light-emitting diodes (LEDs) with different spectral ranges and intensities can affect callogenic responses, biomass production and secondary metabolite accumulation in callus cultures of *Prunella vulgaris* (Tariq et al. 2014; Fazal et al. 2016). Similarly, Kuo et al. (2015) showed that secondary pathways are modulated by red and far-red light in agarwood (*Aquilaria* spp.). However, these studies focused on the effect of light alone and did not consider interactions with other physical parameters such as temperature and humidity, nor did they consider interactions with components of the medium. DoE approaches could address this because different LED spectra could be combined with variations in other physical factors and medium components simultaneously, resulting in the identification of optimum illumination settings that stimulate the accumulation of secondary metabolites or biomass production more effectively.

Illumination has a major impact on the large-scale cultivation of PCSCs (Rorrer and Mullikin 1999). During scale-up from incubation shakers to stirred tank reactors or wave bioreactors, it is necessary to ensure that the cells have constant access to light (Seydel et al. 2009). This has been achieved using various designs of photobioreactors, including tubular, horizontal and Christmas tree variants. However, these systems are expensive and it is more practical to redesign existing fermenters using the increasing diversity of available LEDs. However, we are unaware of any reports in which PCSCs have been cultivated in stirred tank bioreactors equipped with post-fitted supplementary light sources, which would allow the DoE-based analysis of light on biomass and/or product accumulation.

Optimization of medium composition and physical parameters

Medium optimization often begins with a commercial formula such as Murashige & Skoog (MS) medium or Gamborg's B5, followed by the OFAT approach using each macronutrient or micronutrient as a variable (Wu et al. 2007). As discussed above, this approach tends to mask interdependencies and interactions among the ingredients, and the DoE approach is therefore recommended because multiple factors are varied simultaneously. Although advantageous, statistical medium optimization is challenging because most commercial media have a large number of components, and a full factorial design would need to test each component at different concentrations in order to cover the design space. This requires the laborious preparation of many individual nutrient stock solutions that are mixed in many different combinations. Any pipetting errors or undetected contamination would weaken or disrupt the resulting model and yield false optima.

One way to avoid the drawbacks of full factorial designs is to develop simpler DoE models with

fewer combinations, but still enough to cover 'design space'. For example, one simplification involves the preparation of blended commercial media at different ratios but with a constant working volume. The most suitable ratio can be determined by the statistical evaluation of the model and this also provides data concerning the way individual ingredients interact, which can be investigated in more detail using full factorial designs if necessary.

The optimization of physical cultivation parameters by DoE is restricted by the number of flasks and shakers/incubators that can be used in a single experiment. For example, full factorial designs testing different modes of illumination against variations in shaking velocity and humidity will quickly expand the number of incubators required and the experiments soon become impractical. For this reason, there are few reports describing the use of DoE to optimize multiple physical parameters. Rasche et al. (2016) investigated and optimized the combinatorial effect of temperature, incubation time, inoculum density and illumination on biomass production in pear cell suspension cultures, and a similar experimental setup has been used in tobacco cells to study the essential physical parameters affecting biomass accumulation and the production of geraniol, an intermediate in the secoiridoid pathway (Vasilev et al. 2014b). An orthogonal array design was used to investigate the effects of light, shaking frequency, inoculum size, filling volume, osmolality, solubilizers, and the addition of conditioned medium. Osmolality and light showed a potent interdependent effect on the accumulation of geraniol indicating that a unique influence could be revealed by DoE that would remain unrecognized with an OFAT approach (Vasilev et al. 2014b).

If technical limitations restrict the deployment of powerful DoE models, a single physical factor such as the cultivation temperature can be investigated beforehand using the OFAT approach. A simplified DoE can then be applied to other parameters using the optimum temperature as a constant. It is not advisable to compare smaller groups of cultivation parameters across separate DoE models because inter-experimental variations and the inability to assess the dependencies among factors considered in separate DoE runs will generate unreliable results and false optima.

Elicitation

Elicitors are organic molecules (e.g., polysaccharides, glycoproteins, organic acids) or abiotic factors (ultraviolet irradiation, heavy metal salts, temperature shifts, osmotic stress) that can induce the biosynthesis of secondary metabolites (Ramirez-Estrada et al. 2016). Certain synthetic elicitors can mimic natural effectors, such as pathogens, that induce biochemical responses in plants. Several recent studies have focused on the successful optimization of product accumulation using elicitors (Komaraiah et al. 2003; Raei et al. 2014; Gadzovska Simic et al. 2015; Saiman et al. 2015; Manivannan et al. 2016). However, different elicitors have diverse effects on plant physiology and the mechanism of action is often poorly understood, so the impact of synthetic elicitors on particular metabolic pathways can only be deduced empirically for each plant system. Optimization studies therefore usually test the effect of several elicitors at different concentrations, either in parallel or at different times during cultivation. The potential synergistic interactions among multiple elicitors and other medium components are often overlooked in such studies. Elicitation provides too many degrees of freedom for sophisticated optimization using the OFAT approach, but DoE models accommodate such interdependent effects. Several elicitors can be tested at different concentrations and/or different application times in one statistical experimental design, revealing the optimal combinations and concentrations of elicitors throughout the fermentation run. However, to our knowledge, dedicated DoE studies concerning elicitation of PCSCs were not yet reported by literature.

Precursor feeding

Precursor feeding is another approach that can be used to enhance the production of specific products in plant cells (Koca and Karaman 2015; Kumar et al. 2015; Verma et al. 2015; Chen et al. 2016). The cultivation medium is supplemented with intermediates from the biosynthesis pathway leading to the desired product, thus overcoming any bottlenecks that restrict product accumulation by relieving the pressure at rate-limiting steps. However, the nature and dose of the precursor must be chosen carefully to avoid the potential for feedback inhibition or the suppression of growth and cell division by precursor-related toxicity. Optimal

feeding protocols are established by testing PCSCs with different precursors at different concentrations to determine the effect on the target product concentration. As discussed above for other parameters, this OFAT approach cannot determine optimal concentrations for different precursors in the same medium, or potential synergistic interactions between precursors and other medium components. This is a significant drawback particularly in the context of commercial processes because precursors are secondary metabolites in their own right and are therefore expensive to manufacture. Even if marginal improvements can be achieved with a lower quantity of precursor then the economic benefits are likely to be substantial in a large-scale process. DoE models provide the means to optimize precursor feeding strategies precisely, e.g. by assessing the effect of several intermediates at different concentrations, or by revealing interactions between precursors and other ingredients that can be used to reduce costs. Statistical experimental designs can also be used to identify critical concentrations of precursors that avoid feedback inhibition. Studies where DoE was applied to assess optimal precursor feeding conditions are not reported by literature yet.

Conclusions and future perspectives

The cultivation of PCSCs and their use for the production of secondary metabolites is based on complex biochemical and physiological mechanisms that are not fully understood. Several different approaches can be used to enhance the accumulation of target compounds, the simplest of which is the OFAT strategy in which one factor is varied while all others remain constant, but this can only test a restricted linear range of the design space and truly optimal parameters cannot be determined. The alternative approach of statistical experiments designs improves on the OFAT approach by sampling different combinations of parameter values throughout the design space, helping to identify optimal combinations based on factor interactions which can then be investigated in more detail. This DoE approach has not yet been adopted widely, but it offers a powerful mathematical solution to process optimization that achieves four major advantages: (1) multiple cultivation parameters can be investigated simultaneously in a single experiment; (2) interactions between parameters are taken into consideration; (3) statistical analysis allows optimum cultivation conditions to be predicted even if those exact conditions have not been tested directly; and (4) there are immense time and costs savings compared to the OFAT approach.

A breakthrough in the application of DoE approaches in PCSCs, such as a substantial increase in productivity leading to significantly higher yields of secondary metabolites, will eventually elevate plant cells to the status of an economically feasible platform for the commercial production of valuable metabolites. Other approaches not discussed in this review include the in situ removal of metabolites, cell immobilization and metabolic engineering, each of which can also be used to improve the accumulation of metabolites. However, even with these approaches, the complex interactions between physical and chemical cultivation parameters will still remain in force, and the complex regulation of metabolic pathways will still present a challenge, so process optimization will remain necessary and statistical designs are likely to become an essential strategy to maximize productivity and product quality.

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