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DESIGN OF EXPERIMENTS AND DESIGN SPACE APPROACHES IN THE PHARMACEUTICAL BIOPROCESS OPTIMIZATION

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Design of experiments, Quality by design, Design space, Critical process parameters, Critical quality attributes

Abstract

The optimization of pharmaceutical bioprocesses suffers from several challenges like complexity, upscaling costs, regulatory approval, leading to the risk of delivering substandard drugs to patients. Bioprocess is very complex and requires the evaluation of multiple components that need to be monitored and controlled in order to attain the desired state when the process ends. Statistical design of experiments (DoE) is a powerful tool for optimizing bioprocesses because it plays a critical role in the quality by design strategy as it is useful in exploring the experimental domain and providing statistics of interest that enable scientists to understand the impact of critical process parameters on the critical quality attributes. This review summarizes selected publications in which DoE methodology was used to optimize bioprocess. The main objective of the critical review was to clearly demonstrate potential benefits of using the DoE and design space methodologies in bioprocess optimization.

Introduction

Pharmaceutical companies are facing bioprocess optimisation challenges like upscaling costs, regulatory approval, and possibility of process failure and risk of delivering substandard drugs to patients. It is thus important to have a reliable and trustable methodology to control and monitor the large number of factors involved in optimizing bioprocesses while saving time and money [1]. A well-designed experiment would play a critical role in the identification of optimal operating conditions to maximize product outputs and quality at the lowest possible cost by minimizing process variation. For most bioprocess development, largescale production using bioreactor systems are carried out to meet up the demand for products like stem cells, antibodies among others used in drug testing. The adoption of the quality by design (QbD) approach, where quality is inbuilt within the process, and process analytical tools (PAT) for process monitoring has greatly enhanced strategies for biopharmaceutical production [2,3].

Traditionally bioprocesses are optimized by means of the quality-by- testing (QbT) approach, which is completely based on trial and error. QbT involves testing different experimental conditions ordinarily by varying one factor at a time (OFAT) meaning that all but one factor are held constant [4–7]. For bioprocess, this approach has a major setback that for a particular cell line there are many critical

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process parameters (CPPs) to control and monitor in order to achieve optimal conditions. Therefore, testing each factor at a time would require a very large number of experiments to achieve optimal conditions, and hence would consume more time and money [8]. It is worth mentioning that the OFAT approach ignores interactions among CPPs that increases the risk of missing the optimal conditions and having a poor understanding of the process dynamics itself. Estimating only main effects in complex processes that require optimization of multiple inputs may give misleading conclusions about the process thus complicating the approval process by the regulatory authorities.

As opposed to the OFAT methodology, the design of experiments (DoE) would help to reveal both main factors, the nature of their effects and how they interact to influence the process [9–12]. The DoE strategy is an asset to an improved understanding of the process and building a design space (DS) [9]. The DoE strategy decreases the time and costs of the optimization of a large number of variables involved in most bioprocessing procedures and also maximises the amount of information gained about the process with fewer experiments [13,14]. It is evident from Fig. 1 that the DoE methodology has been more and more successfully adopted to optimize bioprocess. The main reason for this exponential trend in the number of publications is the recent campaign of the regulatory authorities for pharmaceutical business to move from the traditional QbT to a QbD approach. Consequently, there has been an increase in knowledge about the DS concept.

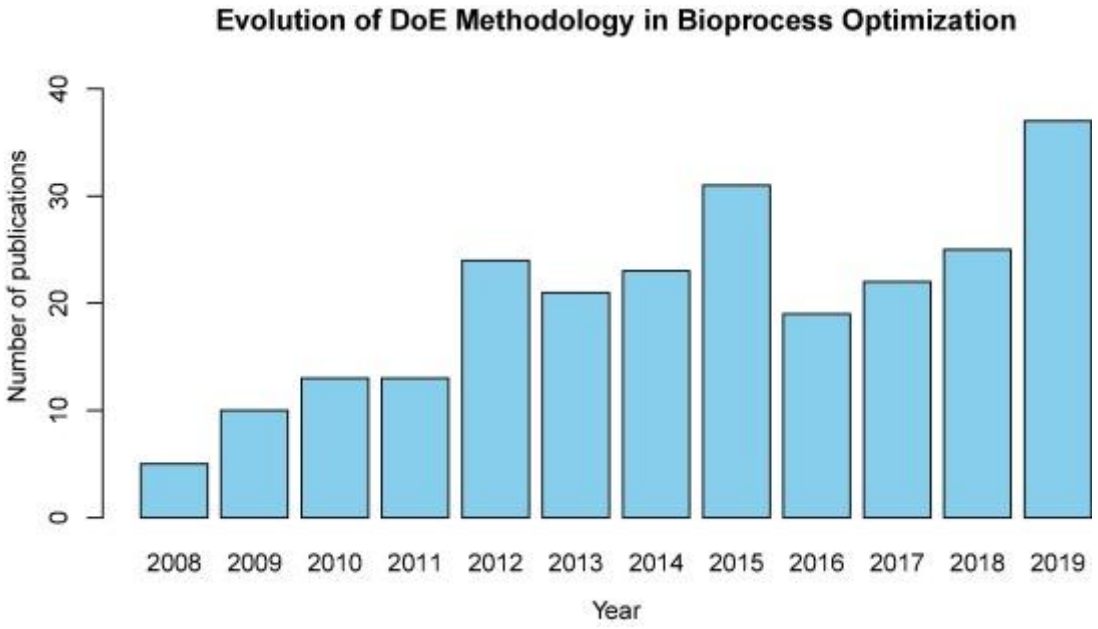

Fig. 1. Number of publications by year period of 2008 – 2019 that have applied the DoE methodology to optimize bioprocess., Scopus database (2008–2019). These trends represent the number of publications involving bioprocesses optimized with the DoE methodology between 2009 and 2019, using PubMed data base. Three keywords including design of experiments or experimental design, bioprocesses and optimization or optimization were used for the search.

The ICH Q8 (R2) defines the DS as "*the multidimensional combination and interaction of input variables (e.g., material attributes) and process parameters that have been demonstrated to provide assurance of quality*". Working within the DS is not considered as a change but movement out of the DS is taken

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as a change and would normally initiate a regulatory post approval change process. A proposal of the DS is given by the applicant and is subjected to regulatory assessment and approval [15]. A DS could be presented inform of ranges of CPPs and CQAs or alternately described through more complex mathematical relationships. The expectation is that operating within the DS will lead to a product meeting the defined quality no matter how the DS was developed. The association between DS, knowledge space and normal operating range (NOR) is best illustrated as in Fig. 2. Knowledge space is the summary of all knowledge gained from the whole process of product development this includes all information gained through screening and optimization experiments. The NOR describes a region around the target operating conditions that contain operational variability which contain common operational variability that cannot always be controlled.

Bioprocesses are very dynamic and not easily predictable, the QbT methodology would not be cost effective and the process quality would not be guaranteed. Since most bioprocesses are expensive and require regulatory approval, adopting the QbD approach where quality is built into the process instead of being confirmed through testing should be the gold standard. The International Conference on Harmonization (ICH) Q8 defines QbD as a *'systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and control, based on sound science and quality risk management'* [15]. The idea of QbD is the ability to identify, monitor and control the CPPs like pH, aeration rate, agitation rate, media composition, yeast extract and temperature among others which influence the critical quality attributes (CQAs) like cell density, cell quality, protein production yield, production of antibodies and enzyme production, among others. This results in the ability to understand and control the process, detect faults earlier in the process and achieve continuous process improvement, which finally pays off with a greater likelihood of high-quality products at the end of the process [16].

The main advantages of the QbD methodology are the ability to efficiently use available time and costs, ability to conform to regulatory authorities' expectations before product submission and reduce the time taken by the latter to approve products to the market. There has been a wide implementation of QbD in the pharmaceutical industry and today drug products with high and reproducible quality can be well predicted [17–19]. According to the FDA, optimal conditions can be realized for almost all pharmaceutical unit operations through the application of DoE-DS strategies. In the context of bioprocesses, utilizing QbD methodology, that makes use of statistical modeling, can greatly improve the quality of the final product like cell yield, antibodies among others to be used for drug testing as well as the effectiveness of the bioprocess [20–22].

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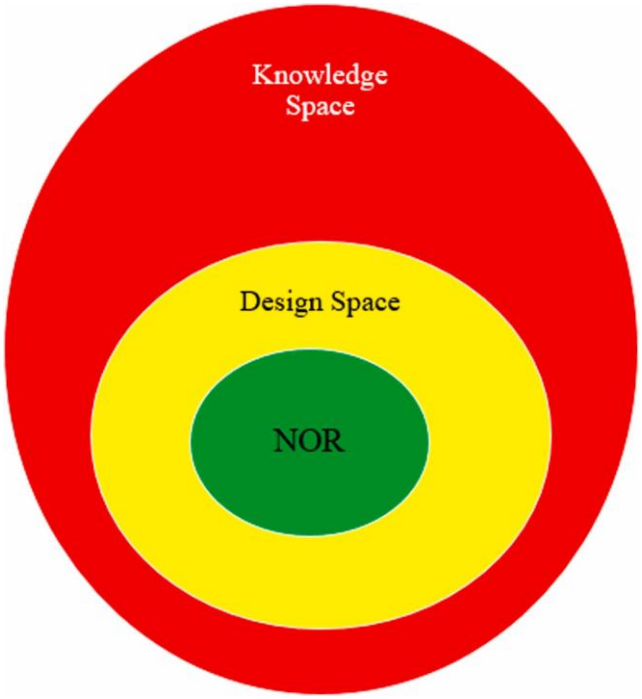

Fig. 2. Illustration of the relationship between Design space, Knowledge space and Normal operation range (NOR).

Despite the wealth of papers in bioprocess optimization, there is still insufficient information on DoE-DS applications to cell culture as most of its applications have been in the optimization of drug formulations. DoE- DS has been widely applied and accepted in the pharmaceutical framework for drug formulation. This can be explained by the fact that

pharmaceutical processes are less variable than biological processes like cell culture and cell differentiation. It could be very interesting to apply the DoE-DS strategy to such complex processes since this would guarantee the quality of drugs delivered to patients if the drug testing must be done on final products of bioprocess like antibodies and stem cells. In this paper, recent publications regarding how the DoE-DS methodology has been applied in the optimization of bioprocess have been reviewed. The main objective of the review was to demonstrate potential benefits of using the DoE-DS methodology in bioprocess optimization.

Overview of the design of experiments and design space approach

The DoE is an outstanding tool that presents the opportunity to manipulate several factors with a pre-defined design to efficiently explore the behavior of CQAs in the multidimensional space formed by these factors. Typically, the DoE-DS procedure proceeds as follows. First, the candidate CPPs are screened using the so-called screening designs so that only those that mostly influence the CQAs are selected. Second, the selected CPPs are used in an optimization phase to determine optimal ranges for each of them [10,23]. To that end, an optimization design is used, with optimally pre-defined

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experiments where the levels of the CPPs are varied simultaneously. Then an appropriate mathematical model is used to connect CPPs to the CQAs. This model is used to explore the behavior of the CQAs in the complex multidimensional space formed by the CPPs and identify an optimal subspace for the CQAs. Hence, the DoE methodology is advantageous because it optimally sets the number of experiments needed to reach these optimal conditions, while maximizing information to understand the whole process and reducing the cost of production. Moreover, when it is appropriately used, the DoE-DS approach can quantify the probability that the product quality is within specifications increasing regulator confidence of robust products [24]. It is also worth noting that DoE is part of the process validation and thus plays a central role in defining the acceptable ranges for the CPPs known as the DS. It is the expectation of the FDA that DoE be part of the National Drug Authority (NDA) submissions [15]. Fig. 3 summarizes the DoE-DS methodology for a QbD bioprocess development.

As already mentioned above, designs are generally categorized into two types depending on the objective of the study, screening designs used for identifying the most important factors influencing the process and optimization designs used to define the DS.

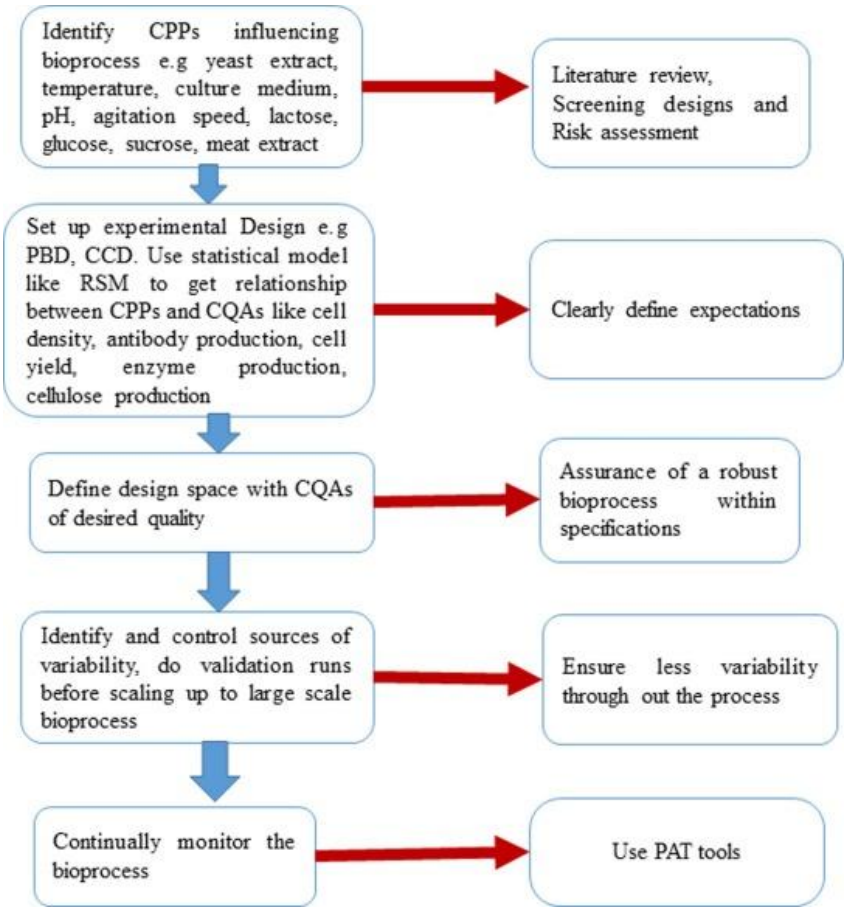

Fig. 3. Stages of a DoE-DS approach.

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SCREENING EXPERIMENTAL DESIGNS

Bioprocesses involve many CPPs believed to possibly influence the final product quality, which are determined through a review of literature, past knowledge and through risk assessment of the process. Therefore, prior to the process optimization, a screening DoE will likely be helpful in selecting the most important factors influencing the process, while at the same time maximizing as much information derived from the process as possible and planning the fewest number of experiments. The main advantage of screening designs is the reduction of the number of factors by eliminating those that are not significantly influencing the CQAs. Therefore, only the most influential CPPs are further studied, hence saving time and financial resources. The commonly used designs for screening are factorial designs, fractional factorial designs (FFD), Taguchi designs, Plackett-Burman designs (PBD) and D-optimal designs among others explained in detail elsewhere [1,25]. The design to use typically depends on the number of CPPs to screen, constraints on the CPPs, and budget considerations

OPTIMIZATION DESIGNS

After the screening phase, the most important variables influencing the process are determined and then used in the optimization phase by applying more complex designs, response surface models (RSM) being the most utilized [26–29]. RSM is a statistical method used for analyzing and modeling a process where the outcome of interest is (e.g cell yield, antibody production) influenced by several CPPs. The aim of this method is also to maximize the outcome. In RSM, higher order terms like quadratic, cubic or quartic effects of the factors on the outcome are estimated. The most commonly used designs for optimization phase include the central composite design (CCD), mixture designs, Box-Behnken designs (BBD) among others explained in detail elsewhere [1]. When many factors are expected to influence the process, the I- optimal design can be considered to reduce the number of experiments. The DS is then defined based on a design whose goal is optimization.

OPTIMIZATION OF CULTURE MEDIA

In biotechnological processes with industrial purposes the most important analyzed parameter is culture medium composition because the growth media could account for approximately 30 – 40% of the total production costs [30]. Many applications of DoE involve the optimization of production and growth of culture medium. The main aim is to determine the best selection and quantitative culture medium CPPs like glucose, temperature, yeast extract, pH, agitation speed among others influencing CQAs like cell yield, cell density, cell quality, protein yield, enzyme production and production of antibodies, among others. Table 1 gives examples of medium optimization with the use of DoE methodology.

Generally, most studies for culture medium optimization start with screening potential medium components using a screening design, then the medium components identified to be significant are used for the optimization design. The most used DoE for screening potential medium components is the PBD which has been successfully applied in several studies [31–35] to screen several components and identify the most important ones to be used for optimization. One good example where the ability of the PBD can be exemplified is in the screening of 22 medium components that were reduced to only

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nine medium components including lactose, yeast extract, peptone, meat extract, urea, $(NH_4)_2SO_4$, $MnSO_4$ and $ZnSO_4$, molasses significantly influencing the production of threonine by *P. pentosaceus* TL-3 [31]. Later the nine components were used in a CCD combined with RSM which identified four significant medium components namely; $MnSO_4$, meat extract, $(NH_4)_2SO_4$ and molasses whose optimum concentrations were 0.098 g/L, 25.30 g/L, 8.59 g/L and 30.79 g/L respectively. The production of threonine by *P. pentosaceus* TL-3 in the optimized medium was doubled compared to the control and the predicted threonine production was 123.07 mg/L.

Taguchi design has been used to screen several culture components believed to influence phytase production by *Aspergillus oryzae* SBS50. The medium components determined to significantly influence phytase production were starch, beef extract, $MgSO_4$, $FeSO_4$ and Tween 80. The five identified significant CPPs were further optimized using a second Taguchi design at four different levels. The maximum phytase production was 47.432 U/L at optimum conditions of 1% starch, 2% beef extract, 3% Tween 80, 0.1% $MgSO_4$ and 0.225% $FeSO_4$ [36]. Taguchi experiments led to a 14.9-fold increase in the production of phytase in comparison with the medium optimized by the OFAT methodology.

Not all culture medium optimization processes begin with a screening design and end with an optimization design. In situations where the CPPs to be considered are not very many and the scientist already has good knowledge about the process, it is sufficient to directly carry out an optimization design. One elucidating example of successful medium component optimization that did not begin with a screening design is that aimed at optimizing the production of recombinant laccase by *Y. lipolytica* YL4. A Taguchi's experimental design with the help of Qualitek-4 software was utilized and four culture components including sucrose, NH_4Cl, yeast extract and thiamine were optimized. An increase to 900 U/L in recombinant laccase production by *Y. lipolytica* YL4 strain was observed after medium optimization. Large production in a 5 L bioreactor reached up to 6760 U/L indicating the importance of DoE methodology in optimization of culture media [37]. Culture conditions influencing pigment production were statistically optimized in shake flask experiments. L-tryptophan, lactose and KH_2PO_4 were the CPPs for culture medium identified to significantly affect pigment production. To investigate the interactions among CPPs and find the optimal values for maximum pigment production, the BBD in combination with RSM were utilized. The optimal production of pigment was 521.64 mg/L in a 50 L bioreactor with optimal culture conditions of 11.25 g/L of lactose, 6 g/L of L-tryptophan and 650 ppm of KH_2PO_4 [34].

TABLE 1

Objective	Screening DoE	Screening CPPs	Optimisation DoE	Optimisation CPPs	Optimal conditions	Reference
Production of BC production by *Komagataeibacter hansenii* AS.5	Glucose, yeast extract, KH_2PO_4, $MgSO_4$, ethanol, pH, inoclume size, temperature, incubation time	PBD	Three-level and four-factor BBD	$MgSO_4$, ethanol, pH and yeast extract	Based on the optimized media BC yield was 6.30 g/l	[32]
Optimization of a recombinant laccase production in *Yarrowia lipolytica* yeast	NA	NA	Taguchi's experimental design	Sucrose, ammonium chloride, yeast extract, thiamine	Recombinant laccase production by *Y. lipolytica* YL4 strain increased to 900 U/L, the production rate reached 6760 U /L in a 5 L bioreactor	[37]

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Optimal medium for butyric acid production by *Clostridium thermobutyricum*	NA	NA	Three-level and four-factor BBD	K_2HPO_4, Glucose, acetate, yeast extract.	A maximum butyric acid yield of 12.05 g/L was obtained at K_2HPO_4 7.2 g/L, 34.9 g/L glucose, 20 g/L yeast extract, and 15 g/L acetate, which compared well to the predicated production of 12.13 g/L	[45]
Develop a serum free medium for the expansion of human lymphocytes from PBMCs	Phosphatidyl choline, polyamine supplement, antioxidant supplement, and cholesterol	FFD	Polyamine supplement and cholesterol	RSM	Cholesterol was 13.2 mg/L and polyamine supplement was 0.1-fold increase.	[46]
Enhance the threonine production by *P. pentosaceus* TL-3	Glucose, sucrose, fructose, lactose, molasses, yeast extract, peptone, meat extract, K_2HPO_4, KH_2PO_4, urea, $(NH_4)_2SO_4$, $(NH_4)_2HC_6H_5O_7$, NaOAc, $MgSO_4$, $MnSO_4$, Tween 80, $FeSO_4$, $ZnSO_4$, $CuSO_4$ and biotin	PBD	Lactose, Yeast extract, Peptone, Meat extract, Urea, $(NH_4)_2SO_4$, $MnSO_4$ and $ZnSO_4$	CCD identified 4 significant CPPs, molasses, meat extract, $(NH_4)_2SO_4$ and $MnSO_4$	The optimum concentrations were 30.79 g/L for molasses, 25.30 g/L for meat extract, 8.59 g/L for $(NH_4)_2SO_4$ and 0.098 g/L $MnSO_4$. The predicted net threonine production was 123.07 mg/ L	[31]
Optimization for enhanced phytase production by *Aspergillus oryzae* SBS50	Temperature, incubation period, starch, beef extract, NH_4NO_3, Tween 80, K_2HPO_4, $FeSO_4$, KCl, $MgSO_4$ and pH	Taguchi design	Starch, beef extract, Tween 80, $MgSO_4$ and $FeSO_4$	Second Taguchi design	1% starch, 2% beef extract, 3% Tween 80, 0.1% $MgSO_4$ and 0.225% $FeSO_4$ supported maximum phytase production of 47.432 U/L.	[36]
Production of cyclic adenosine 3′,5′- monophosphate (cAMP) with *Microbacterium* sp. no. 205	Glucose, K_2HPO_4, KH_2PO_4, $MgSO_4$, urea, biotin, $CoCl_2$, NaF, peptone, hypoxanthine and initial pH	FFD (2^{11-7}).	K_2HPO_4, $MgSO_4$ and NaF.	Steepest ascent method used in combination with CCD and RSM	12.78 g/L for K_2HPO_4, 3.53 g/L for $MgSO_4$ and 0.18 g/L for NaF. The maximum cAMP production was 8.50 g/L	[28]
Production of alkaline protease by *Bacillus* sp. BGS	Dextrose, peptone, K_2HPO_4, molasses, $ZnSO_4$, and Tween 60, pH and inoculum size	PBD	Molasses, peptone, pH, and inoculum size	FCCCD in combination with RSM	Optimal concentrations 16.827 g/L of peptone, 1.128% of molasses, pH value of 11, and 2% of inoculum size. The optimal protease 2,992.75 U/mL which is a 6.36-fold increase compared to the one obtained with the original medium (470.35 U/mL)	[47]
Glutaminase free L- asparaginase production by *Streptomyces olivaceus* NEAE-119	Temperature, pH, incubation time, inoculum size, inoculum age, agitation speed, dextrose, starch, L-asparagine, KNO_3, yeast extract, K_2HPO_4, $MgSO_4{\cdot}7H_2O$, NaCl, and $FeSO_4{\cdot}7H_2O$	PBD	Temperature, inoculum age, and agitation speed	FCCD	The measured L- asparaginase activity obtained under the optimal conditions obtained from FCCD was 68.59 U/mL	[48]
Optimization of crude oil biodegradation by a local marine bacterium isolate *Pseudomonas* sp. sp48	Glucose, peptone, yeast-extract, NH_4Cl, $CaCl_2$, $MgSO_4$, arabic gum, triton X-100, fertilizer (K_2HPO_4 + urea), pH and inoculum size	PBD	Glucose, $MgSO_4$, triton X- 100 and inoculum size	RSM using BBD	The predicted optimum oil removal was 89%, which is 2.4 times more than the basal medium	[29]
Optimize the ribonuclease production	Culture temperature, initial pH, inoculum size, sucrose, yeast extract, $MgSO_4{\cdot}7H_2O$ and KNO_3	PBD	Sucrose, yeast extract, $MgSO_4{\cdot}7H_2O$, and KNO_3	2^4 factorial CCD	The optimal medium for ribonuclease production was determined as 8.50 g/L sucrose, 9.30 g/L yeast extract, 2.00 g/L $MgSO_4{\cdot}7H2O$ The optimized medium produced 29.85 U/ mL of ribonuclease	[49]
Production of halophilic cellulases by *A. terreus* UniMAP AA- 6	KH_2PO_4, KOH, yeast extract, $MgSO_4{\cdot}7H_2O$, $FeSO_4{\cdot}7H_2O$, NaCl, peptone, CMC, temperature, agitation speed and inoculum size	PBD	NaCl, CMC and $FeSO_4{\cdot}7H_2O$	FCCCD	Halophilic cellulase production increased from 0.029 U/mL to 0.0625 U/ mL, which was approximately 2.2-times greater than before optimization	[50]
Optimisation of red pigment yield by the monoculture of P.	Depth of fermentation broth, membrane diameter, rotary	FFD	Glucose concentration and membrane diameter are	CCD	Maximum yield of red pigment in shake flask reached 4.25 g/L	[51]

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novae- zeelandiae HSD07B and *Candida tropicalis*	speed, glucose concentration, temperature and pH		important factors influencing the yield of red pigment			
Optimization of cholesterol oxidase production by *Streptomyces cavourensis* strain NEAE- 42	Temperature, incubation time, inoculum size, agitation speed, pH, glucose, starch, cholesterol, yeast extract, peptone, $(NH_4)_2SO_4$, K_2HPO_4, NaCl, $MgSO_4$ and $FeSO_4$	PBD	Cholesterol, initial pH and $(NH_4)_2SO_4$	CCD	The level of cholesterol oxidase production obtained was 20.521 U/mL	[52]
Uricase production by *Aspergillus welwitschiae*	Temperature, pH, inoculums size, inoculums age, incubation time and medium volume) and chemicals factors like (sucrose, uric acid, peptone, yeast extract, $NaNO_3$, K_2HPO_4, NaCl, $MgSO_4{\cdot}7H_2O$ and $FeSO_4{\cdot}7H_2O$)	PBD	Incubation time, inoculum size, medium volume and yeast extract	CCD	The maximum uricase production was achieved at 5 days of incubation time, 2 g/L of yeast extract, 4 mL of inoculum size and 50 mL medium volume. Optimum uricase production was 60.03 U/mL. This was a 3.02-fold increase compared to the unoptimized medium of 19.87 U/mL	[35]
Production of laccase by fungal strain *Marasmiellus palmivorus* LA1	NA	NA	Temperature, pH, $NH_4H_2PO_4$, galactose, cupric sulphate, inoculum concentration and substrate length	Taguchi design	The optimum production of laccase was found to be 667.4 ± 13 IU/mL. Overall yield increase of 17.6-fold was obtained after optimization	[53]
Optimisation of culture medium for the expansion of mESC	L-cysteine, C1-metabolites, transferrin, LIF, insulin, calcium, zinc, defined lipids, BMP4, transferrin supplement and L-carnosine	PBD	L-cysteine, transferrin, LIF, insulin, BMP4, calcium and lipids	MinRes IV design	LIF was the main factor for the survival and proliferation of mESCs	[12]
Optimising β-glucosidase production	Temperature, pH, incubation time, inoculum size, moisture content, substrate concentration, $NaNO_3$, KH_2PO_4, $MgSO_4{\cdot}7H_2O$, KCl, $CaCl_2$, yeast extract, $FeSO_4{\cdot}7H_2O$, Tween 80, and $(NH_4)_2SO_4$	PBD	BBD	$NaNO_3$, KH_2PO_4 and Tween 80	The maximum β-glucosidase production was 4457.162 U/g	[57]
Increasing spore production of the plant growth.	Glucose, $MgSO_4{\cdot}7H_2O$ KH_2PO_4 meat extract, yeast extract special peptone, $(NH_4)_2SO_4$, and $MnCl_2{\cdot}4H_2O$	PBM	Glucose, $MgSO_4{\cdot}7H_2O$	full factorial and CCD	High final spore cell density of 8.78×10^9 CFU/mL and a high sporulation efficiency of 94.2% at bioreactor level.	[54]
Production of recombinant HIV1 gp41 by E. coli	Growth, initial medium pHs, IPTG concentrations, induction times, temperature, yeast extract, tryptone, glucose NaCl, betaine and ampicillin	FFD	pH, induction time, temperature, IPTG concentrations, Growth initial medium	BBD and RSM	High yield in protein (0.63–0.72 mg/L) and cell (1.7–2 g/L) of the desired product in four litter fermentations attained	[23]
Optimize culture medium for increased production of EPS	Sucrose, $NaNO_3$, K_2HPO_4, KCl, $MgSO_4$, $FeSO_4$ and *Ashbya gossypii* extract	PBD	Sucrose, $NaNO_3$ and *A. gossypii* extract	RSM	Maximum production of 29 g/L EPS achieved during 84 h batch fermentation	[55]

Fractional factorial design (FFD); not available (NA); central composite design (CCD); Peripheral blood mononuclear cells (PBMCs); Box-Behnken design (BBD), exopolysaccharide (EPS); Mouse embryonic stem cells (mESCs); response surface methodology (RSM); Leukaemia inhibitory factor (LIF); biocellulose (BC); Plackett–Burman Design (PBD); CMC (carboxymethylcellulose); Face-Centered Central Composite Design (FCCD), Isopropyl β- d-1-thiogalactopyranoside (IPTG); Minimum run resolution IV (MinRes IV); Bone morphogenic protein 4 (BMP4).

OPTIMIZATION OF PROCESS PARAMETERS

Bioprocesses are complex and involve many process parameters that need to be optimized with the most cost effective and efficient methodology. Parameter optimization involves control parameters including the feeding strategy, the main aim of process parameter optimization is to be able to define a better physical environment for cell growth, protein production, enzyme production and antibody production among other CQAs.

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A two-factor three level (3^2) full factorial DoE has been utilized to detect significant interactions between two factors (inoculation density, agitation rate) in culturing human embryonic stem cells (hESCs). Results from the full factorial DoE were used to define the optimal conditions and significant interactions between the two factors were found [38]. The optimal conditions were maximum agitation rate of 100 rpm and a low inoculation density of 2.0 x10^4 cells/mL. A validation of the optimal results was done on two cell lines (H1 and H9) in duplicate bioreactors and similar growth rates between the two cell lines were observed. Hunt *et al.* concluded that the hESCs remained pluripotent in the different cell lines under optimal conditions and maintained their differentiation capacity.

Several examples of optimizing process variables with the aid of DoE methodology can be found in Table 2. The PBD has proved to be robust in being able to screen important process CPPs expected to influence a particular CQA like antibody production, cell production, protein production among others from several candidate CPPs in order to have a smaller, manageable and cost-effective process [33,35,39]. The PBD has been used to screen a total of eight CPPs including vector type, bacterial strain, culture medium (dissolved oxygen), expression temperature, shaking speed, Isopropyl β-d-1-thiogalactopyranoside (IPTG), glucose and antibiotic concentrations influencing the yield recombinant protein production by *Escherichia coli (E. coli)* with a total of only twelve runs. From the eight variables three variables including temperature, IPTG concentration and dissolved oxygen were the most significant CPPs influencing the yield of recombinant protein. The RSM based on the BBD was further used with the three CPPs to optimize the process and the optimal conditions for the maximum production of recombinant protein were determined to be 100 µM of IPTG, 30% of dissolved oxygen and 20 ˚C of temperature [39]. The estimated maximum yield of recombinant protein production by *E. coli* was estimated to be 45 µg/L. The PBD was clearly able to reduce the process from eight to three significant CPPs to use for the optimization and a higher yield was achieved.

An economical and feasible biopolymer production process to produce polyhydroxyalkanoate (PHA) by *Acinetobacter junii* BP 25 has been successfully optimized with the aid of DoE methodology. The PBD was used for screening of a total of thirteen CPPs influencing the process including glycerol, KH_2PO_4 and incubation time were identified to significantly influence the process. The BBD was used further for optimization and the maximum PHA production was determined to be 3.04 g/L which was a 5.84-fold increase compared to the PHA concentration before optimization (0.52 ± 0.05 g/L) [40].

To optimize the production of recombinant cyclodextrin glucanotransferase (CGTase) by *E. coli*. the CCD was used with three CPPs including concentration of IPTG, concentration of arabinose, post-induction temperature and the maximum CGTase production was predicted to be 68.76 U/mL under the parameters optimized at 25.76 µM IPTG, 1.0% arabinose and 34.7 ˚C post-induction temperature. Validation experiments resulted into 69.15 ± 0.71 U/mL. The use of DoE methodology resulted into a 3.45-fold increase in CGTase production compared to initial process parameter conditions before optimization (0.58 mg/L) [41]. To enhance the production of cellulose by *Botryosphaeria rhodina* a two-level FFD has been used to screen several CPPs to determine the most significant ones. The CPPs identified to significantly influence cellulose production include initial moisture content, amount of substrate, and initial pH of nutrient supplied in the SSF system. To optimize the process, a RSM in accordance with the CCD and the optimal environmental conditions for cellulose production were found to be 24.32% of initial moisture content, 5.96 g of initial pH of nutrient and 3.98 g of substrate,

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these optimal conditions increased the enzyme activity to 17.95 U/g from 3.26 U/g (5.49-fold increase) [42].

TABLE 2

Objective	Screening CPPs	Screening DoE	Significant CPPs	Optimization DoE	Optimization Condition	Reference
Optimize yield recombinant protein production by *Escherichia coli*	Vector type, bacterial strain, culture medium (dissolved oxygen), expression temperature, shaking speed, IPTG, glucose and antibiotic concentrations	PBD	Temperature, IPTG, dissolved Oxygen	BBD combined with RSM.	The optimal conditions were 100 µM of IPTG, 30% of dissolved Oxygen and 20 °C of temperature. The optimal yield of recombinant protein produced was 45 µg/L	[39]
Maximize production of IgM McAb by hybridoma M1A2 cells	NA	NA	FBS concentration, cultivation time, temperature levels (low and high) and type of medium (DMEM, and RMPI 1640)	CCD combined with RSM	At optimum 12% of FBS concentration, 33 °C of temperature and 3.5 days of incubation the optimum McAb production was 1132.69 µg/mL in DMEM. For RPMI maximum McAb production was 1105.12 µg/mL achieved at optimum conditions of 33 °C of temperature, 11% of FBS and 4 days of incubation	[56]
Optimize the production of FTase by SSF	Inoculum rate, incubation temperature, initial pH and packing density	BHHD	Inoculum rate, temperature and Packing density	BBD combined with RSM	Maximum FTase activity was 1347 U/L and obtained at 32 °C, using packing density of 0.7 g/cm^3. Inoculum rate was not significant	[57]
Optimizing the production of PHA by *Acinetobacter junii* BP 25	Peptone, Na_2HPO_4, $(NH_4)_2HPO_4$, glycerol, incubation time, NaCl, pH, inoculum size, temperature, $MgSO_4{\cdot}7H_2O$, KH_2PO_4, yeast extract and agitation speed	PBD	Glycerol, KH_2PO_4 and incubation time	BBD combined with RSM	The maximum PHA production was 3.04 g/L which is a 5.84-fold-increase compared to the PHA concentration before optimization (0.52 ± 0.05 g/L).	[40]
Optimization of pectin extraction from muskmelon	NA	NA	Time, pH and temperature	BBD and RSM	Pectin extracted from muskmelon peel was classed as high methoxy pectin with the equivalent weight of 384.5 g/mol.	[58]
Maximum extraction yield of pectin from waste *Artocarpus heterophyllus*	NA	NA	Liquid-solid ratio, pH, sonication time and extraction temperature	Four factor three level FCCCD and RSM	Liquid-solid ratio of 15:1 mL/g, pH of 1.6, sonication time of 24 min and temperature of 60 °C	[59]
Optimal extraction of pectin from industrial waste of *Musa balbisiana*	NA	NA	Ultrasound power, pH, time and liquid–solid ratio	CCD	The optimal extraction process condition was ultrasound power of 323 w, pH of 3.2, extraction time of 27 min and solid–liquid ratio of 1:15 g/mL. The mean experimental yield of pectin was 8.99 ± 0.018%	[27]
Production of lipase from *Staphylococcus arlettae*	NA	NA	Temperature, oil concentration, inoculum size, incubation time and pH	CCD and RSM	An optimum lipase yield of 6.5 U/mL has been obtained with 9.39% inoculum with the oil concentration of 10.285% in 2.99 h using pH of 7.32 at 38.8 °C	[60]
Optimization of molar hydrogen yield by a genetically optimized *Escherichia coli* strain, DJT135.	NA	NA	3^K full factorial BBD and RSM	pH, temperature and glucose concentration	A maximum molar hydrogen yield of 1.69 mol /mol of glucose was obtained under the optimal conditions of 75 mM glucose, 35 °C and pH of 6.5	[61]
Improve the yield of filamentous phage produced in *Escherichia coli* strain	NA	NA	Full factorial CCD	Temperature, dissolved oxygen, and pH	The predicted phage was 2.86 × 1011 TU/mL. A validation experiment resulted into phage production of 3.49 × 1011 TU/mL	[62]

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DOI: 10.xxxxxxxxxx
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Optimization of the surface expression yield of the model protein SefA, a Salmonella enterica fimbrial subunit with potential for use in vaccine applications,	NA	NA	Two-level with three center points	Medium pH, cultivation temperature, and inducer concentration	Optimal surface expression yield of SefA was increased by 200%. At the same time, the yield of full-length protein was increased by 300%, indicating a 33% reduction in proteolysis	[63]
Optimization of Bromelain production in *Escherichia coli*, BL21-AI clone	Induction time, L- arabinose concentration, harvest time and temperature	NA	FCCCD and RSM	Temperature, L-arabinose concentration and harvest time	Optimal conditions were 0.2% L-arabinose, 8 h harvest time and 25 °C temperature produced bromelain activity of 9.2 U/mg while validation experiments gave bromelain activity of 9.6	[64]
Optimize preparation method of PEI for production of biomass.	NA	NA	RSM with two-level- two-factor (2^2) full factorial CCD.	Amounts of PEI and cross-linker GA.	Optimum conditions were found to be 4.29 g of PEI and 0.15 mL of GA, with 10 g of the biomass, where the sorption capacity was enhanced by 4.52-fold compared to that of the raw biomass.	[65]
Optimization of efficient protein secretion in *Escherichia coli*	NA	NA	CCD	Concentration of IPTG, concentration of arabinose and post-induction temperature	Optimum conditions for maximum recombinant CGTase production were found to be 25.76 µM IPTG, 1.0% arabinose and 34.7 °C post-induction temperature, with a predicted extracellular CGTase activity of 68.76 U/ ml. Validation of the model gave an extracellular CGTase activity of 69.15 ± 0.71 U/ mL,	[41]
Optimize cellulase production from OPEFB by *Botryosphaeria rhodina*	Temperature, initial pH of nutrient, initial moisture content, no of mycelium plug and substrate	Two-level factorial design	CCD	Moisture content, amount of substrate and initial pH of nutrient supplied	Enzyme production increased from 3.26 to 17.95 U/g	[42]
Optimization of tannase production by *Lactobacillus plantarum* CIR1	NA	NA	Taguchi methodology	pH, temperature, tannic acid, phosphate, nitrogen and Mg^{2+}	Tannase optimal production achieved with a 2.52-fold increase	[66]
Optimization of culture conditions for flexirubin production by *Chryseobacterium artocarpi*	NA	NA	BBD and RSM to visualize the design space.	Lactose, L- tryptophan and KH_2PO_4	Optimum conditions for maximum production of pigment 521.64 mg/L in 50 L bioreactor were lactose 11.25 g/L, L-tryptophan 6 g/ L and KH_2PO_4 650 ppm	[34]
Optimize levansucrase production by *Bacillus subtilis* NRC1aza.	pH, moisture content, sucrose, glucose, yeast extract, peptone, $(NH_4)_2SO_4$, KNO_3, K_2HPO_4, KH_2PO_4, $MgSO_4$, $CuSO_4$, $FeSO_4$, $ZnSO_4$ and $MnSO_4$	PBD	FFD	Yeast extract, and pH	Maximal enzyme productivity of 170 U/g	[67]

Not available (NA); central composite design (CCD); human embryonic stem cells (hESCs); Box-Behnken design (BBD), Fetal bovine serum (FBS); Fructosyltransferase (FTase); Solid-State Fermentation (SSF); Box-Hunter and Hunter design (BHHD); polyhydroxyalkanoate (PHA); polyethyleneimine (PEI); glutaraldehyde (GA); fractional factorial design (FFD); Face centered central composite design (FCCCD); oil palm empty fruit bunch (OPEFB); Roswell Park Memorial Institute Mledium (RPMI 1640); Dulbecco's Modified Eagle Medium (DMEM); Isopropyl β- d-1-thiogalactopyranoside (IPTG).

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Critical discussion of DoE-DS approach for bioprocess optimization

The complexity of most biotechnology unit operations has been partly the reason the biopharmaceutical industry has not been so competitive in the past decades. Bioprocess operations require analysis of numerous variables, feed material attributes, and raw material attributes and this presents varied challenges [14]. The solution to this conundrum and to allow for an efficient estimation of both the main effects and interactions while ensuring minimal experiments are used with the limited resources available is to employ the DoE-DS approach. The DoE-DS approach is one of the most valuable techniques for bioprocess development and optimization due to the ability to refine and improve the many interdependent parameters involved in the bioprocess sequence [43]. In view of the emerging regulatory demands on pharmaceutical manufacturing processes which is exemplified by the PAT initiative of the United States FDA, the use of DoE approach to improve process development for sustainable production is becoming increasingly important [44].

The use of random experimentation is not cost effective and efficient in biopharmaceutical manufacturing because of the fact that resources are limited and bioprocesses are expensive [11]. In addition, random experiments rely on trial and error with little or no statistical scrutiny. When the process is straightforward random experiments does shed light on the process but in complex processes like bioprocesses observation of results in absence of quantitative data could lead to a wrong path thus process failure. There is need for a structured and organized methodology which is based on sound statistics and thus the DoE approach. The DoE approach uses statistical algorithms to design the most efficient experiments. The statistics produced using DoE provide the basis for bioprocess optimization and becomes the ultimate method in biopharma. Technically in biopharmaceutical process development, most responses are CQAs while factors are the CPPs. With the increasing competitiveness and regulations in the biotech industry, the use of DoE approach becomes increasingly important. For a successful DoE, good planning is important because a poorly planned DoE does not teach us something about the CPPs influencing a particular process. Several tips for a successful experimentation to avoid mistakes include;

- Choosing the right software and learning how to use it properly, several software for designing experiments exist on the market but care needs to be taken in the selection as some software will actually help you to select the wrong CPPs.
- It is important to ensure that the equipment on which the experiment is to be performed has been well calibrated and the timely maintenance has been done and is up to date.
- When selecting ranges of the CPPs care needs to be taken as a narrow range from low to high may give a result that concludes the chosen CPPs do not influence the process, but in reality the selected CPPs do not influence the process in the range selected. In addition, selecting very wide ranges from low to high may give results where some combinations of the factors yield unstable results.
- Finally, always do verification runs do not base the final results on predicted software values.

As all methods applied to bioprocess the DoE-DS methodology also has several drawbacks when used for bioprocess optimization. One is the approach heavily depends on statistics of which it requires complex calculations that may not be appealing to many with limited knowledge of statistics. Another

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main set back of the method is that to get statistical significance one needs a large number of experimental runs because for each combination of CPPs you need several data points, the requirements for testing increases rapidly and thus an evaluation of a DoE for a process takes quite a substantial investment. Other difficulties encountered with the DoE-DS methodology include problems with finding all the variables that influence a particular process, not able to control certain variables like room temperature thus causing more variability and also some CPPs are nonlinear making it difficult to identify the optimal conditions giving an irregular DS. Nevertheless, DoE-DS methodology plays a crucial role in bioprocess optimization and is responsible for the success of the biopharmaceutical industry and if all the above mentioned tips are utilized in the DoE methodology and proper planning is done the highest percentage of failures in bioprocess can be avoided.

Conclusion

The DoE-DS methodology is becoming a valuable and important tool for bioprocess optimization in the biopharmaceutical industry because it gives maximum information about the bioprocess while utilizing a few experiments saving time and financial resources. A review of publications in bioprocess optimization that used the DoE-DS methodology indicates that it has been utilized efficiently at several stages (both screening and optimization), CPPs influencing the process identified at the screening stage and a fewer CPPs used in the optimization greatly saving on time and costs. The DoE methodology is an efficient way to optimize bioprocess and this will guarantee the consistency of the final bioprocess output like cell yield and antibody production to be used in drug testing by pharmaceutical companies. It is also worth mentioning that, for the DoE method to be efficient in bioprocess development it is important that scientists take care and utilize their extensive knowledge to ensure that the CPPs selected for the experimental design and the results make practical sense and are reproducible.

Future perspective

Design of experiments has proven to be an excellent tool that gives scientists the opportunity to systematically manipulate factors in-line with a predefined design taking into consideration budget limitations. Currently the exploration of the DS is mainly done with the RSM that is based on mean response optimization and ignores uncertainties. This may lead to a high probability of giving unexpected results for future production. The adoption of a predictive approach to DS computation would lead to a risk-based DS, which gives guarantee of quality, and assurance that the final product will be within specifications. A Bayesian predictive approach to DS computation considers past knowledge and uncertainties of the CQAs and CPPs. It is worth noting that PAT enables us to achieve a full QbD approach where process control and monitoring is built into the system in-line with a predefined design to ensure process quality. Being able to effectively control and monitor bioprocess in real time gives a guarantee of product quality and reproducibility. The main advantage of the QbD methodology is the ability to apply in-line bioprocess monitoring and control of processes to avoid the bottlenecks associated with off-line methods. Integration of PAT into the bioprocess would enable us

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to move easier from a QbT approach to a QbD strategy that is less conservative [20]. Implementation of the QbD methodology using PAT has the advantage of real-time process monitoring. Consequently, reliable quality control coupled with the application of multivariate data analysis methods and mathematical modeling, can be useful in predicting the final outcome [21]. In addition, PAT enables online prediction of the CQAs that are required to maintain product quality and thus gives the opportunity for real time product quality monitoring and control [17]. This gives the opportunity to obtain better process understanding and a robust process where quality would be guaranteed. PAT is defined as a system for designing, analyzing, and controlling pharmaceutical manufacturing processes by measuring the CPPs influencing the CQAs in real time [15]. The use of PAT was initiated by the FDA and is also promoted by European Medicines Agency (EMA). Real time process monitoring gives us the opportunity to make an informed decision on what action to undertake in a situation when the process performance deviates from the optimal conditions or expected product quality of the desired attributes [13]. For successful PAT applications to bioprocess monitoring there are three main tools used today and they include; Multivariate data acquisition and data analysis tools, Process analytical chemistry (PAC) tools and Continuous improvement tools.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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