Biomass and Bioenergy 151 (2021) 106166

Contents lists available at ScienceDirect

Biomass and Bioenergy

journal homepage: www.elsevier.com/locate/biombioe

Using Response Surface Methodology (RSM) to optimize 2G bioethanol production: A review

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ARTICLE INFO

Keywords:
Response surface methodology
Design of experiments
Bioethanol
Biomass pretreatment
Enzymatic hydrolysis
Fermentation

ABSTRACT

Environmental concerns arising from the release of greenhouse gases have stimulated the search for more sustainable technologies, including bioethanol production from lignocellulosic biomass. However, the rigid and complex structure of this biomass means that lignocellulosic biomass conversion to bioethanol requires numerous stages, such as pretreatment, enzymatic hydrolysis, and fermentation, not to mention the underlying costs and time, especially during enzymatic hydrolysis and fermentation. For all these reasons, this technology still is not attractive for industrial use. Response Surface Methodology (RSM) has helped to optimize the bioethanol production stages with a view to obtaining an economical process and to improving process efficiency. This review presents an overview of RSM and analyzes how this tool can be applied to study and to optimize the factors that influence lignocellulosic biomass pretreatment, enzymatic hydrolysis to obtain sugars, and sugar fermentation for ethanol production.

1. Introduction

Bioethanol is a biofuel that can be produced from different lignocellulosic biomass, including sugarcane bagasse, rice straw, corn straw, and wheat straw, all of which are considered agro-industrial waste. In this sense, bioethanol could become a sustainable alternative to fossil fuels [1,2]. In Brazil, sugarcane bagasse is the most promising substrate for this technology—large amounts of this biomass are generated during sugar and first-generation (1G) ethanol production; more specifically, around 270–280 kg of bagasse and 140 kg of sugarcane straw originate for each ton of processed sugarcane [3,4]. Brazil is the second largest producer of 1G ethanol (7060 million gallons of 1G ethanol were produced only in 2017), but the production of second-generation (2G) ethanol is still not competitive in the country due to the high operating costs and lack of robust technologies in the operating plants [1].

Lignocellulosic biomass is a heterogeneous polymer that consists mainly of cellulose (40–60%), hemicellulose (20–40%), and lignin (10–24%), whose intrinsic association provides a biomass with a recalcitrant structure [5]. This makes access to fermentable sugars difficult and constitutes the principal challenge regarding 2G ethanol production [5,6]. Cellulose is a highly stable polymer consisting of up to 12,000 glucose residues attached through linear chains. It is composed mostly of (1,4)-D-glucopyranose units that are attached by β-1,4 linkages and has an average molecular weight of around 100,000 [7,8]. Hemicellulose is the second most abundant heterogeneous polymer and comprises mainly glucuronoxylan and glucomannan, but it also contains trace amounts of other polysaccharides. Grasses and straws contain arabinan, galactan, and xylan, while mannan is a component of hardwood and softwood hemicellulose [8]. They are catalogued with sugar as a backbone; i.e., xylans, mannans, and glucans, being xylans and mannans the most common [9]. Lignin is a long-chain, heterogeneous polymer composed largely of phenyl-propane units that are most frequently linked by ether bonds [8]. Lignin acts like a glue that fills the voids between and around the cellulose and hemicellulose structures within the biomass. Lignin exists in all plant biomass, so it is considered a byproduct or residue of bioethanol production [8].

In the lignocellulosic structure, cellulose strains are bundled together to form the cellulose fibrils via hydrogen bonding, whereas hemicellulose serves as a connection between lignin and cellulose [10]. The rigid and complex structure resulting from the spatial interaction of cellulose, hemicelluloses, and lignin limits lignocellulosic biomass conversion to the desired product [11]. Therefore, lignocellulosic biomass conversion to bioethanol requires different stages, such as pretreatment, enzymatic hydrolysis, and fermentation [12].

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https://doi.org/10.1016/j.biombioe.2021.106166
Received 23 February 2021; Received in revised form 16 June 2021; Accepted 20 June 2021
Available online 25 June 2021
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The pretreatment step involves changing the cellulose-hemicellulose-lignin matrix structure, so that hydrolytic enzymes act on cellulose and convert it to fermentable sugars, finally allowing the fermentation process for bioethanol production to occur [13,14]. This is the stage that consumes the most energy, and it is considered one of the most expensive stages in 2G bioethanol production. Pretreatment can be classified as physical, chemical, physicochemical, and biological pretreatment. Physical pretreatment comprises techniques such as milling, extrusion, freezing, and microwave heating. Chemical pretreatment includes treatment with alkali or acid, dilute acid, ionic liquids, organic solvents, and ozonolysis. As for physicochemical pretreatment, explosion, fiber explosion, ammonium, CO_2 explosion, liquid hot water, and wet oxidation can be used. Lastly, biological pretreatment involves fungi [15]. Several factors can influence lignocellulosic biomass pretreatment; e.g., temperature, pressure, pH, high-temperature steam, and concentrations of H_2SO_4, H_3PO_4, and NaOH solutions, given that these factors affect lignin extraction and hydrolysis of hemicelluloses. Moreover, these variables impact the generation of inhibitors, thereby affecting sugar degradation [1,16].

To break down the polysaccharides and the crystalline cellulose of the lignocellulosic biomass, some enzymes are necessary, including cellulases like endoglucanases, cellobiohydrolases, and β-glucosidases. Endoglucanases hydrolyze β-1,4-glycosidic bonds in amorphous regions of the cellulose chains, to release cello-oligosaccharide. Cellobiohydrolases act on short cellulose molecules and cello-oligosaccharides, releasing cellobiose, whereas β-glucosidases cleave cellobiose into glucose [17]. In turn, lytic polysaccharide monooxygenases (LPMO) are copper-dependent enzymes that improve the hydrolytic performance of cellulases and increase accessibility to cellulose by degrading cellulose through an oxidative mechanism [17,18]. Given that enzymatic hydrolysis is the central step in 2G ethanol biorefineries, some factors, such as temperature, saccharification time, pH, enzyme load, substrate load, addition of chemical agents like surfactants, and mechanical agitation should be studied and optimized. A high concentration of reducing sugars, such as glucose, can increase the fermentation efficiency, which allows for higher conversion to ethanol [19,20].

Finally, there is the fermentation stage, during which fermentable sugars released in the previous stages are converted into bioethanol by action of fermenting microorganisms. *Saccharomyces cerevisiae*, which can produce ethanol with high productivity and yield, is the most widely used organism in fermentation [21]. This stage poses two main difficulties. The first is to obtain efficient conversion of xylose, which constitutes more than 35% of fermentable sugars in lignocellulosic biomass. The second difficulty is related to the presence of fermentation inhibitors (phenolic compounds, weak acids, and furan aldehydes) released during the pretreatment and hydrolysis stages [22]. Therefore, both the pretreatment and the hydrolysis steps are crucial for optimum fermentation to be achieved [23]. Some factors that are commonly evaluated in the fermentation stage include fermentation time, inoculum size, process temperature, agitation, supplement addition, and solid-liquid ratio [24–26].

Design of Experiment (DoE) tools and Response Surface Methodology (RSM) are great strategies that provide more accurate conclusions than the one-factor-at-time (OFAT) approach and reduce the number of experimental attempts and furnish more informative datasets. Moreover, DoE allows the interaction between several factors to be studied and the different tested conditions to be optimized, to improve process performance [27]. In this sense, DoE and RSM tools have been increasingly employed to optimize the various bioethanol production stages such as pretreatment, enzymatic hydrolysis, and fermentation stages —each step of the process has numerous factors that influence both the efficiency of biomass conversion to bioethanol and process costs, as mentioned previously. Thus, these tools can contribute to the development of feasible and efficient processes for bioethanol production from lignocellulosic biomass for economically viable application in the industry [28–31]. Review papers on the use of DoE and RSM tools in the three main stages of ethanol production have not been found in the literature.

In this context, this review aims to contextualize and to discuss the use of DoE and RSM tools in the study of the factors and the optimization of the 2G bioethanol production stages. The sections that follow will address some literature research papers that have applied DoE and RSM tools in the context of ethanol production over the last seven years. We believe that the contexts covered in this article are of interest to the scientific community and can contribute to future research into 2G bioethanol production.

2. Design of experiment (DoE)

Design of Experiment is a set of methods and procedures that are mainly used to analyze data about specific variables regarding a specific research problem. There are various types of DoE such as Full Factorial Design, Fractional Factorial Design, Plackett-Burman Design, Central Composite Rotational Design, and Box-Behnken Design, among others. All these designs have advantages and disadvantages, so which one should be chosen depends mostly on the aim of the research and available resources [32]. The first step in using DoE is to define the independent and dependent variables. The independent variables, or factors, can be altered in different levels or values, while the dependent variable, or response, is influenced by the factors [33]. To validate the mathematical model obtained in the DoE and to determine a set of conditions, one must calculate the residue, which is the difference between the experimental result and the estimated result. A good mathematical model has a low residue value [33].

When the significance of the mathematical model is evaluated, *t*-test and Analysis of Variance (ANOVA) are the most often applied statistical methods. *t*-Test is used to compare two samples or treatments, whereas ANOVA is employed when there are more than two treatments. For both analyses, the level of significance must be fixed; for example, 5% ($p < 0.05$), to indicate that the treatments have statistically significant difference. The level of significance is also called "α", which is the probability of a null hypothesis being rejected. Hence, when "p" calculated by ANOVA is lower than the level of significance, the results can be considered significant, whilst "p" greater than the level of significance means that the null hypothesis is real and that the results are not significantly different, so there are no evidences to reject the null hypothesis [34].

2.1. Full Factorial Design

Full Factorial Design is generally employed in RSM. The factors (k) and their levels are combined in a way that the design has all the possible combinations. This type of DoE provides the main effects and the interaction effects. Factor is the parameter that the researcher wishes to evaluate experimentally, and level is a value that a factor can assume [35]. The Full Factorial Design can be symmetric, when the number of levels is the same for all the studied factors, or asymmetric, when each factor has a distinct number of levels [36].

Designs with two levels, 2^k, are the simple forms of an orthogonal design. They are usually used to screen variables and to analyze their factors, and they encompass analysis of k factors in two levels: high (+) and low (−). Designs with three or more levels are more frequently applied to construct the response surface because they show the effects of not only linear factors, but also quadratic factors [36].

The Full Factorial Design may not be viable for a large number of factors because implementing many experimental conditions is costly and complex. In these cases, the researcher can consider a Fractional Factorial Design as an alternative [37].

2.2. Fractional Factorial Design

A Fractional Factorial Design involves using a subset selected from

the experimental conditions of a Full Factorial Design; in other words, just some conditions of a Full Factorial Design will be employed [38]. This is more economical because it reduces the number of experiments. However, decreasing the number of experiments makes it impossible for some effects of the study factors to be distinguished, so the degree of fractionation will depend on the resolution that one wants to get. Low-resolution designs are normally applied to define the principal effects and disregard the interactions, while higher-resolution designs indicate both the principal effects and the interactions [36].

The notation that is frequently used for this type of design is I^{k-p}, where I is the number of levels that is analyzed in each factor; k is the number of factors that is employed in the analysis, and p is the size of the fraction of the applied full factorial [39].

2.3. Plackett-Burman Design

A Plackett-Burman Design (PBD) experiment uses N number of experimental runs and allows up to N-1 factors to be tested (N is defined as a multiple of 4) [40]. PBD does not take possible interactions between the factors into account. Therefore, this tool is only useful to estimate the main effect of the factors that are involved in the process, and it cannot be used to obtain the response surface during optimization of said process [36].

Like the 2^k design, PBD allows two levels for each one of the factors of the control k (−1, +1), but it requires a much smaller number of experiments. To construct PBD in k factors, the first line in which the elements are equal to −1 or +1 must be selected, so that the number of 1's is (k+1)/2, and the number of -1's is (k-1)/2. In the next step, k-1 lines are generated from the first line by cyclically shifting from a place to the right k-1 times. Then, the last line of the design is only added with -1's [41]. For PBD to be used correctly, Rodrigues and Lemma [42] suggested choosing a matrix with four tests or more than the number of factors to be studied.

Wu [43] employed PBD to evaluate how seven factors, namely initial pH, $(NH_4)_2SO_4$, KH_2PO_4, inoculation amount, fermentation time, temperature, and rotation speed, influenced the mixed fermentation of *Aspergillus niger* and *Candida shehatae*, which allowed the author to choose the most significant factors in this process by using just 12 experiments. Compared to a full design 2^7, this was a more economical strategy that resulted in 128 experiments [43].

2.4. Central Composite Rotational Design

The Central Composite Rotational Design (CCRD) is most often used to obtain the response surface because it allows a second-order (quadratic) model for the response to be constructed [39]. This kind of design occurs when the distances to both the axial points, as the factorial points of the Central Composite Design, to the center are the same central distance, so that chances in the available response are the same in all the points of a centralized sphere at the origin. Equation (1) shows the second-order mathematical model [36]:

$$y = \beta_o + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1}^{k} \sum_{j=1}^{k} \beta_{ij} x_{ij} + \varepsilon \quad (1)$$

where y is the response, β_0 is the intercept, β_i values represent the coefficient of the main effects, β_{ij} are the coefficients of interaction effects, β_{ii} are the second-order terms and ε is the random error component that is determined by fitting the model to the data [36]. The design consists of factorial (−1, +1) and axial (-α, + α) points and of repetitions at the central point, which provides properties such as orthogonality and rotativity to the adjustment of quadratic polynomials. The axial points (2k) enable the curvature to be evaluated and to estimate new extremes for all the study factors in high and low configurations. This design is useful because it provides full knowledge of responses with the smallest number of experiments [36,44]. The number of experiments for this design can be obtained from Equation (2).

$$N = k^2 + 2k + C_p \quad (2)$$

where k represents the number of factors, and Cp represents the number of replicates of the central point [45].

2.5. Box-Behnken Design

The difference between this design and CCRD is that it uses three levels for the study factors (−1, 0, +1), while CCRD uses five levels (-α, −1, 0, +1, +α). Equation (3) gives the number of experiments (N) that is required for the Box-Behnken Design [46], where k is the number of factors and Cp is the number of replicates of the central point:

$$N = 2k(k-1) + C_p \quad (3)$$

This design is also employed in RSM to obtain the second-order mathematical model (Equation (1)), which allows the studied process to be optimized. The advantage of the Box-Behnken Design is that it points out where the problems of the experimental limits are and avoids unnecessary combinations of treatments [47].

Despite the advantages of employing RSM for analyzing experimental data, the technique does not specify which design type should be used to collect and to analyze the information [48]. The Central Composite Design and the Box-Behnken Design are the two most commonly employed in studies of 2G ethanol production. The Box-Behnken Design has been the most employed to study and to optimize the several stages of 2G bioethanol production. This can be justified by the fact that the Box-Behnken Design requires only three levels for each factor: 1, 0, and +1, while CCRDs require five levels (-α, −1, 0, +1, and α). All the design points (except at the center) have length two; that is, they all lie on the same sphere. These designs are particularly suitable for spherical regions. Because of the spherical property, there should be at least three to five runs at the central point. For example, in the case of a Box-Behnken Design for k = 3, all the points are located in the edge region, and all of them are at a distance of $\sqrt{2}$ from the design center. There are no factorial points or face points. Despite covering the edge points, the Box-Behnken Design does not include the entire cube because it does not have any corner points even at a distance of $\sqrt{3}$ from the design center. In this design, the combination of its spherical nature with the rotatable designs (or near-rotatable designs) indicates that broad center runs should be used. For instance, for k = 3, three to five runs should be used in the central point [49,50].

Table 1 summarizes the different types of Experimental Design and their advantages and disadvantages.

3. Response surface methodology for process optimization

Response surface methodology (RSM) is a statistical technique that effectively optimizes complex processes by using an approach of many factors and responses [54]. The first step of RSM is to determine which factors will be employed in the study. This step can be carried out through a fractional design or PBD, as described previously, thereby allowing the factors that are statistically significant for the responses to be evaluated by using a smaller number of assays.

Optimization by the RSM method involves three major steps: statistically designing the experiments, estimating the coefficients in a mathematical model, and predicting the response and checking the adequacy of the model (Equations (1) and (4)) within the setup of the experiments [55].

$$Y = f(X_1, X_2, X_3, \ldots..X_n) \quad (4)$$

where Y is the system response, and X_n is the factor of action called factors. Y can be obtained from the second-order mathematical model shown in Equation (1). ANOVA analysis of each term of this equation

Table 1
Types of experimental design.

Types of Design	Function	Advantages and disadvantages	References
Full Factorial Design	It is convenient for a small number of factors if the resources are available.	Advantages: It is the only design that allows for categorical variables with 3 or more levels to be used for screening.	[51,52]
	It investigates the effects of factors and their interactions on the dependent variable.	Disadvantages: It cannot be used for optimization by RSM.	
Fractional Factorial Design	It only takes into account a small number of main effects and lower-order interactions. The higher- order interactions are neglected due to their negligible effects on the response variable.	Advantages: It is very economical and can be used for screening.	[51,52]
	The number of factors to be studied ranges from 3 to 13.	Disadvantages: The researcher must determine which variables can be ignored. Not all the interactions can be evaluated.	
Plackett-Burman Design	It is an economical alternative to the Fractional Factorial Design, but it only studies the main effects of factors.	Advantages: It is used for screening.	[53]
	The number of factors to be studied ranges from 8 to 35.	Disadvantages: It is not used for optimization.	
Central Composite Rotational Design (CCRD)	It determines the optimal levels of design variables by including a few more experiments to a Full Factorial Design.	Advantages: It is used for optimization.	[51–53]
	The number of factors to be studied ranges from 2 to 6.	Disadvantages: It requires five levels for the studied factors.	
Box-Behnken Design	It is an alternative to Central Composite Designs when preceding results from a factorial design are not available and when the optimum response is not located at the extremes of the experimental region.	Advantages: It is used for optimization.	[51–53]
	The number of factors to be studied ranges from 3 to 6.	Disadvantages: It requires more central points (03–05).	

will determine whether the two-factor interactions and the higher-order interactions can be neglected.

The graphical representation of the model is a suitable way to find the optimum location. Two types of graphs may be helpful: the response surface in the three-dimensional space (Fig. 1a) and the graph of contours, which is the projection of the surface on a plane, represented as lines of constant response (Fig. 1b). Each contour corresponds to a specific height of the surface. In these graphs, the response is represented as a function of two factors. According to the established optimization criterion, the optimum value that is sought may correspond to a maximum, a minimum, or a specific value that can be found by simple visual inspection of the graph. When more than two factors are studied, the values that are not plotted must be set at a constant value, so a limited part of the experimental domain is shown, and the optimum is not necessarily seen in the graph. For this reason, the value of the fixed factor must be selected very carefully. Overlaying the contour plots constructed with pair combination of three factors allows the best compromise region to be visually searched, satisfying response requirements.

The desirability function can be applied to optimize one or multiple responses, thereby maximizing or minimizing the studied object and transforming the values of the obtained responses into values of desirability functions that range between 0 and 1. The value 0 is designated when the values do not have a desirable response, and the value 1 is designated for the ideal performance of the study factors. This combination of RSM with the desirability function is called optimization methodology of desirability, or DOM [56]. When the optimization of process conditions depends on several responses, the optimization criteria are often contradictory. The overall solution must be comprehended in an optimum region, leading to an accord solution. In this sense, the desirability function defined by Harrington [57] and Derringer and Suich [58] solves optimization of multiple responses [58]. The general approach involves transforming each response y_i into an individual desirability function d_i that varies over the following interval:

$$0 = \leq di \leq 1 \tag{5}$$

If the obtained response (y_i) is at the study goal, d_i is equal to 1; if the response is outside the region that is considered acceptable by the study, di is equal to 0. Then, the factor parameters are chosen in order to maximize the overall desirability [57].

$$D = (d_i, d_2, \ldots . d_m)^{1/m} \tag{6}$$

where m is the number of responses. If the goal T for the response **y** is a maximum value,

$$d = \begin{cases} 0 & y < L \\ \left(\dfrac{y-L}{T-L}\right)^r & L \leq y \leq T \\ 1 & y > T \end{cases} \tag{7}$$

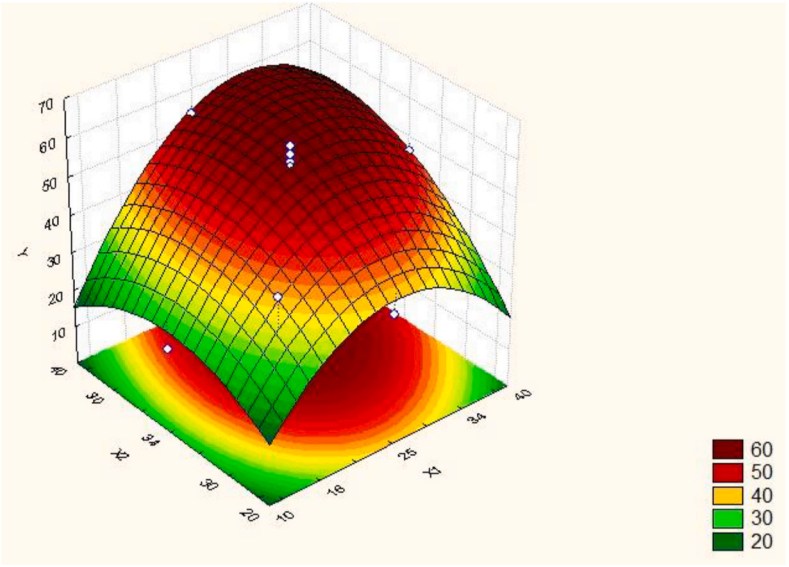
If the weight **r** is equal to 1, the desirability function is linear. When r $>$ 1, proximity to the goal value is emphasized; when r falls between 0 and 1, this is less important.

When the desirable target for the response is a minimum value, Equation (7) is changed to Equation (8):

$$d = \begin{cases} 1 & y < T \\ \left(\dfrac{U-y}{U-T}\right)^r & T \leq y \leq U \\ 0 & y > U \end{cases} \tag{8}$$

4. Using RSM in lignocellulosic biomass pretreatment

Among the bioethanol production stages, pretreatment is the most crucial step because it greatly impacts the general bioconversion efficiency. In lignocellulosic biomass, cellulose and hemicellulose are densely compacted with lignin, which has various functions including protecting the plant against enzymatic hydrolysis [59]. Pretreatment methods alter the structure and chemical composition of the lignocellulosic matrix in different ways; for example, they increase the porosity and accessible surface area of the enzymes, thus modifying substrate hydrophilicity; remove hemicellulose and lignin; or decrease the cellulose degree of polymerization and crystallinity [10,30]. An ideal pretreatment is economically viable; that is, it consumes less energy, has shorter operational time, produces fewer effluents, and avoids degradation of carbohydrates and production of enzymatic inhibitors and products that are toxic to fermentation microorganisms [30].



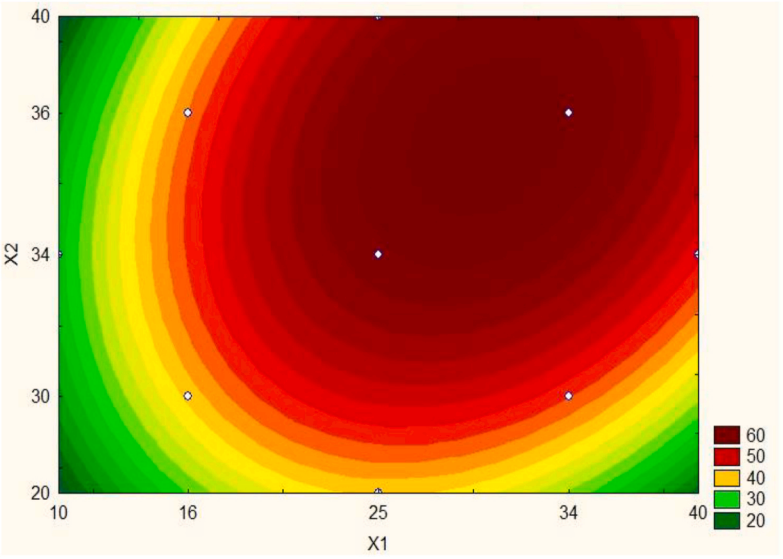


Fig. 1. Representation of Response Surface (a) Response surface in the three-dimensional space, (b) Graph of contours. Y: response, X_1 and X_2: factors. Adapted from Rodrigues and Lemma [42].

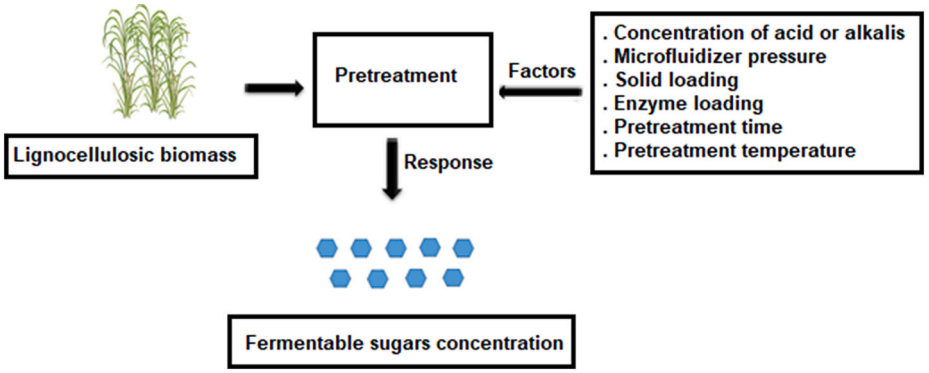


Fig. 2. Representative scheme of the variables that were studied and optimized in the pretreatment stage by using DoE tools and RSM.

Fig. 2 illustrates the pretreatment process and the main factors that are involved in this stage:

Some authors have employed RSM by using either CCRD or the Box-Behnken Design to optimize lignocellulosic biomass pretreatment (Table 2). Researchers have considered different factors such as the concentration of acid or alkali or bleaching agent, solid biomass load, chemical treatment temperature and time, and concentration of enzyme. These variables and their values can be chosen in preliminary tests or by using screening designs such as the Fractional Factorial Design or PBD.

Turhan et al. [60] used the Box-Behnken Design to optimize ethanol production by considering the factors of pretreatment by microfluidization (pressure: 500, 1000, and 1500 bar and solid load: 1, 2, and 3%), enzymatic hydrolysis at different enzyme loads (5, 10, and 15 FPU.g^{-1} of dry wheat straw), and simultaneous saccharification and co-fermentation (SSCF) experiments with addition of a mutant *Saccharomyces cerevisiae* (ATCC 20618) yeast, which can ferment both xylose and glucose. The responses were glucose, xylose, and ethanol yields. The authors observed that, regardless of the applied pressure, wheat straw pretreated at 1% solid load gave the maximum delignification. ANOVA showed that the solid concentration of biomass slurry was the most important parameter and significantly impacted the glucose and xylose yields. Thus, a reduction in solid load while keeping the other process variables constant increased the glucose and xylose yields. For the ethanol yield, the authors observed that the solid load and enzyme concentration had statistically significant negative and positive impacts, respectively. The authors obtained the second-order regression models for the glucose, xylose, and ethanol yield by including all the terms, but the microfluidization pressure did not significantly affect any response variables. The authors did not show the p-value of the model coefficients. By means of RSM analysis, they found that the optimum conditions were pressure of 1500 bar, 1% solid load, and 15 FPU.g^{-1} of enzyme load, which was experimentally validated. The predicted responses were 82% glucose yield, 94% xylose yield, and 65% ethanol yield.

Lavudi et al. [61] studied the sweet sorghum bagasse pretreatment stage by CCRD. Initially, the authors analyzed two types of pretreatments, acid and alkaline, and verified that alkaline pretreatment increased the glucose concentration the most efficiently. Therefore, the authors decided to continue optimizing the alkaline pretreatment by using CCRD. The factors (and their levels) were alkali concentration (0.65–4.85%), pretreatment temperature (119.89–145.11 °C), and pretreatment time (3.18–36.82 min); the evaluated responses were glucose and xylose content. The experiments performed according to CCRD 2^3 afforded values in the range of 36.11–59.83 g L^{-1} for glucose and 5.91–12.61 g L^{-1} for xylose. All the factors significantly influenced glucose production ($p < 0.05$). RSM allowed the authors to optimize the pretreatment conditions, which were 67.24 g L^{-1} glucose and 10.14 g L^{-1} xylose when the pretreatment was conducted with 4% sodium hydroxide solution at 140 °C for 30 min.

Ramaraj and Unpapromm [62] investigated the potential of small-flowered nutsedge for ethanol production. For this purpose, the authors optimized the alkaline pretreatment stage by using the Box-Behnken Design and by considering four factors: solid.liquid^{-1} ratio (0.05–0.3), NaOH concentration (1–2%), H_2O_2 concentration (0.5–1.5%), and time (24–72 h). The authors successfully selected the ranges of the studied factors and established the optimum values, which were 0.05 solid.liquid^{-1} ratio, 1% NaOH, 1% H_2O_2, and 72 h. The optimized conditions furnished 0.194 g g^{-1} of total sugars

Table 2
Response Surface Methodology (RSM) applied in the pretreatment stage of lignocellulosic biomass.

Type of Design	Numbers of experiments	Independent variable	Measured response	Optimized conditions	Optimized response	Ref.
Box-Behnken	15	Microfluidizer pressure (bar), solid load of wheat straw solution (%), and enzyme dosage (FPU.g^{-1} of pretreated biomass)	Glucose, xylose, and ethanol yields from the pretreatment of wheat straw with microfluidizer	Microfluidizer pressure (500–1000 bar), solid load of wheat straw solution (1–3%) and enzyme dosage (5–15 FPU.g^{-1})	82% glucose, 94% xylose, and 65% ethanol	[60]
Central Composite Rotational Design (CCRD)	27	Alkali concentration (%), temperature (°C), and time (min)	Glucose and xylose yield from the pretreatment of sweet sorghum bagasse	Alkali concentration (0.65–4.85%), temperature (119.9–145.1 °C), and time (3.2–36.8 min)	57.24 g L^{-1} glucose and 10.14 g L^{-1} xylose	[61]
Box-Behnken	27	Solid.liquid^{-1} ratio, NaOH concentration (%), H_2O_2 concentration (%), and time (h)	Sugar concentration obtained from the pretreatment of nutmeg	Solid.liquid^{-1} ratio (0.05–0.3), NaOH concentration (1–2%), H_2O_2 concentration (%) (0.5–1.5%), and time (24–72 h)	0.183 g. g^{-1} sugar concentration	[62]
Box-Behnken	17	Temperature (°C), time (min), and organic acid concentration (%)	Reducing sugars concentration from the pretreatment stage of palm oil stem biomass	Temperature (120–140 °C), time (30–60 min), and organic acid concentration (5–15%)	For acetic acid: 1.0336 mg mL^{-1}. For citric acid: 1.2302 mg mL^{-1}. For oxalic acid: 1.7975 mg mL^{-1} of reducing sugars	[63]
Box-Behnken	15	HNO_3 concentration (%), pretreatment temperature (°C), and time (min)	Xylose yield from the pretreatment of rice straw	HNO_3 concentration (0.2–1.0%), pretreatment temperature (140–180 °C), and time (1–20 min)	87.3% xylose	[64]
Central Composite Rotational Design (CCRD)	20	HCl concentration (%), incubation time (min), and biomass load (%)	Reducing sugars from dilute acid pretreatment of pine needles	HCl concentration (0.5–1.5%), incubation time (20–30 min), and biomass load (5–10%)	0.2 g g^{-1} reducing sugars	[65]
Central Composite Rotational Design (CCRD)	17	Temperature (°C), time (min), and substrate load (% w.v^{-1})	Glucose concentration from water pretreatment of wheat straw	Temperature (176.36–246.69 °C), time (3.18–36.82 min), and substrate load (0.80–9.20% w.v^{-1}).	30.24 g L^{-1} glucose	[66]
Central Composite Rotational Design (CCRD)	29	Temperature (°C), NaOH concentration (%), and time (days)	Sugar concentration of sorghum stalk (SS) and sugarcane leaf (SL) by chemical pretreatment	Temperature (30–40 °C), NaOH concentration (1–3% w/v), and days (1–3)	38.977 and 32.621 g L^{-1}, respectively, of SS and SL of reducing sugars	[67]

concentration, which was close to the value predicted by the regression mathematical model (0.183 g g^{-1} of total sugars), indicating that the model had low residue value.

Rattanaporn et al. [63] optimized the acid pretreatment conditions of palm oil trunk biomass by using RSM along with the Box-Behnken Design. They examined three different acids—acetic acid, citric acid, and oxalic acid—in an attempt to improve enzymatic saccharification and bioethanol production from palm oil trunk biomass. They evaluated the influence of factors such as temperature (120–140 °C), time (30–60 min), and organic acid concentration (5–15%) on reducing sugars released during enzymatic hydrolysis for each acid in separate. They conducted enzymatic hydrolysis of the pretreated sample by using 20 FPU of Celluclast ® and 100 CBU of cellobiase at 45 °C and 200 rpm for 72 h. ANOVA showed that the regression models were statistically significant with coefficients of determination (R^2) of 0.7890, 0.9293, and 0.8255 for pretreatments with acetic acid, citric acid, and oxalic acid, respectively. All the terms of the second-order regression model were statistically significant ($p < 0.05$) for pretreatment with citric acid, whereas pretreatment with acetic acid and oxalic acid afforded reduced models. The authors generated contour plots from regression models to identify the optimum conditions for each type of acid, which were 107.3 °C, 30 min, and 8.3% organic acid concentration for acetic acid; 131.92 °C, 58.92 min, and 13.92% organic acid concentration for citric acid; and 100 °C, 60 min, and 15% organic acid concentration for oxalic acid. The optimal conditions were experimentally tested, which resulted in reducing sugars concentrations close to the values predicted by the regression models.

To optimize the pretreatment of rice straw, Kim et al. [64] applied the Box-Behnken Design with RSM to assess how the HNO_3 concentration (0.2–1.0%), pretreatment temperature (140–180 °C), and reaction time (1–20 min) affected the xylose yield. The coefficients of the second-order model such as the HNO_3 concentration, temperature, interaction of these factors, interaction of HNO_3 concentration x time, and interaction of temperature x time were statistically significant ($p < 0.05$). Even though the time factor was not statistically significant, the authors maintained it in the model due to the high adjusted determination coefficient (Adj. $R^2 = 0.9673$) of the full model. The pretreatment optimum conditions to obtain the maximum xylose yield (87.3%) were 0.65% HNO_3, 158.8 °C, and 5.86 min of reaction. The predicted yield was validated experimentally, resulting in 86.5% xylose yield, which was close to the predicted value.

Slathia et al. [65] applied RSM to optimize the dilute acid pretreatment of pine needles of *Pinus roxburghii* for bioethanol production. The authors initially employed one variable at time (OVAT); they used dilute hydrochloric acid (HCl) at 0.5%, 1%, 1.5%, and 2%, and biomass load of 5%, 10%, and 15%. The maximum reducing sugars yield was 96 mg g^{-1} of biomass, which was achieved with 1% HCl and 5% biomass load. To optimize the pretreatment, they used a CCRD with 20 experiments to evaluate the HCl concentration (0.5–1.5%, incubation time (20–30 min), and biomass load (5–10%). ANOVA was used to evaluate how the studied factors affected the reducing sugars concentration. The statistical significance was established by F test. The second-order model was significant ($F < 0.05$), but the lack of fit was not statistically significant ($F > 0.05$), which indicated that the model can be used to predict the responses and to establish the optimum conditions. Thus, 0.5% ($v.v^{-1}$) HCl, 30 min, and 5% biomass load were chosen as the optimum conditions that allowed the reducing sugars concentration to be increased from 0.1 g g^{-1} (obtained by OVAT) to 0.2 g g^{-1} of biomass.

In the pretreatment stage of wheat straw for bioethanol production, Chen et al. [66] used CCRD with RSM, to perform 17 experiments. The authors evaluated three factors: temperature (176.36–246.69 °C), time (3.18–36.82 min), and substrate load (0.80–9.20% $w.v^{-1}$). On the basis of ANOVA, the second-order model was statistically significant ($p < 0.05$), but the lack of fit was not significant, which indicated that the model was statistically valid and predictive. The R^2 value was 0.9076, demonstrating that the results were well fitted to the second-order model. The optimum conditions (220.51 °C, 22.01 min, and 2.50% w. v^{-1} substrate load) decreased the hemicellulose content by 18.37% and increased the cellulose and lignin contents by 25.92% and 8.81%, respectively, yielding maximum glucose concentration (30.24 g L^{-1}).

Manmai et al. [67] studied the chemical pretreatment of sorghum stalk (SS) and sugarcane leaf (SL) for bioethanol production. The authors carried out RSM by using CCRD, which generated 29 experiments. They analyzed three variables: temperature (30–40 °C), NaOH concentration (1–3% $w.v^{-1}$), and time (1–3 days). Statistical analysis by ANOVA showed that the second-order model was statistically significant (F test and P-value < 0.05), but the lack of fit was not significant, with R^2 values of 0.9981 and 0.9917 for SS and SL, respectively. This indicated that the results were well fitted to the model, and that the model can be used to obtain the optimal conditions. Thus, the optimal conditions were 2% NaOH, 40 °C, and 3 days, which yielded the highest total sugar concentrations (38.977 and 32.621 g L^{-1} for sorghum stalk and sugarcane leaf, respectively).

5. Using RSM in enzymatic hydrolysis

During enzymatic hydrolysis, cellulases and hemicellulases depolymerize cellulose and hemicellulose to hexoses (glucose, galactose, and mannose) and pentoses (xylose and arabinose), respectively. The three main groups of cellulases involved in the hydrolysis reaction are endoglucanases, 4-β-D glucan cellobiohydrolases, and β-glucosidases [68,69]. Compared to acid hydrolysis, enzymatic hydrolysis is more advantageous because it requires less energy and milder experimental conditions and is less corrosive and toxic [68].

Enzymatic hydrolysis is the central step in 2G ethanol biorefineries, so optimization of this stage has been investigated to increase the efficiency of biomass conversion to bioproducts. A high concentration of reducing sugars, such as glucose, can increase the fermentation efficiency, which allows for higher conversion to ethanol to be achieved [19,20].

Fig. 3 represents the enzymatic hydrolysis process and shows the main factors and responses that are involved in this stage:

Because factors or independent variables such as temperature, pH, reaction time, and substrate concentration can affect enzymatic digestion, the effect of all the factors and their interactions must be assessed when enzymatic hydrolysis is optimized. RSM has frequently been used for this purpose (Table 3) together with CCRD and the Box-Behnken Design.

Lavudi et al. [61] optimized the second stage of ethanol production from pretreated sweet sorghum by using RSM and CCRD. More specifically, they studied and optimized substrate concentration (10–15%), concentration of the enzymatic cocktail Celluclast (10–20 IU.g-dwt^{-1}), incubation temperature (40–60 °C), and incubation time (24–60 h). The statistical analyses of the data showed that the four factors significantly affected ($p < 0.05$) sugar production. Thus, the authors obtained the regression mathematical model by considering these variables, which allowed them to build the response surface and to obtain the optimum conditions (15% substrate concentration, incubation at 60 °C, 20 IU. g-dwt^{-1} Celluclast, and incubation time of 58 h). Enzymatic hydrolysis in the optimum conditions allowed them to obtain 68.41 g mL^{-1} glucose and 6.1 g mL^{-1} xylose.

Ben Taher et al. [69] used the Box-Behnken Design and RSM to optimize potato peel enzymatic hydrolysis for ethanol production in 27 runs. The studied factors were temperature (30–60 °C), pH (5–8), substrate concentration (2–10%), and surfactant surface (0–1%). The data were fitted to a second-order model ($p < 0.05$), which was built with the regression coefficients of the significant variables. Only the interaction pH x substrate concentration was not statistically significant ($p > 0.05$), so the authors did not include this coefficient in the model. The response surface was generated from the model, and the optimum conditions–45 °C, pH 5, 10% substrate, and 0.5% surfactant–resulted in 77.1 g L^{-1} reducing sugars. The authors validated the model

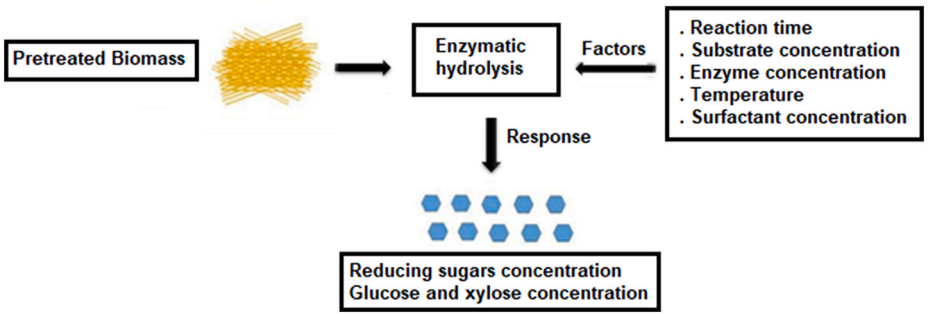


Fig. 3. Representative scheme of the variables that were studied and optimized in the enzymatic hydrolysis stage by using DoE tools and RSM.

Table 3
Response Surface Methodology (RSM) applied in the enzymatic hydrolysis stage.

Type of design	Number of experiments	Independent variables	Measured responses	Optimized conditions	Optimized response	Ref.
Central Composite Rotational Design (CCDR)	20	Substrate concentration, temperature, Celluclast concentration, and incubation time	Glucose and xylose concentration ($g.L^{-1}$) from saccharification of pretreated sweet sorghum bagasse	Substrate concentration (15%), temperature (60 °C), Celluclast concentration (20 IU.g-ds-$^{1)}$, and incubation time (58 h)	68.41 g L^{-1} glucose and 6.1 g L^{-1} xylose	[61]
Box-Behnken	27	Temperature, pH, substrate concentration, and surfactant concentration	Reducing sugars concentration ($g.L^{-1}$) from saccharification of potato peel	Temperature (45 °C), pH (5.0), substrate concentration (10%), and surfactant concentration (0.5%)	77.1 g L^{-1} reducing sugars	[69]
Box-Behnken	29	Temperature, pH, enzyme load, and substrate load	Reducing sugars concentration ($mg.g^{-1}$) from saccharification of pretreated *Parthenium hysterophorus* biomass	Temperature (30 °C), pH (4.5), enzyme load (0.25%), and substrate load (0.25%)	550.6 ± 1.20 mg g^{-1} reducing sugars	[70]
Box-Behnken	15	Substrate concentration, enzyme concentration, and incubation time	Percentage (%) of saccharification of wheat straw	Substrate concentration (2%), enzyme concentration (0.5%), incubation time (6 h), and temperature (50 °C)	40.12% wheat straw saccharification	[71]
Central Composite Rotational Design (CCRD)	27	Cellulase enzyme load (FPU. g^{-1} of substrate), β-glucosidase enzyme load (U.g^{-1} of substrate), hydrolysis temperature (°C), and hydrolysis time	Glucose and xylose concentration ($mg.g^{-1}$ of substrate) from saccharification of narrow-leaf cattail	Cellulase enzyme load (13.50 FPU/g of substrate), β-glucosidase enzyme load (16.50 U/g of substrate), hydrolysis temperature (50 °C), and hydrolysis time (24 h)	Glucose (552.9 mg g^{-1} of substrate) and xylose (74 mg g^{-1} of substrate)	[72]
Box-Behnken	29	Temperature (°C), reaction time (h), pH, and enzyme load (mL)	Reducing sugars yield (%) from saccharification of corn and rice straw	Reaction time of 3.84 h and temperature of 51.45 °C at high enzyme dosage (2 mL)	1.42% of reducing sugar yield for saccharification of corn straw 1.61% of reducing sugar yield for saccharification of rice straw	[73]
Box-Behnken	15	Time (h), substrate load (%), and enzyme load	Reducing sugars concentration ($mg.g^{-1}$)	8 h, 2% substrate load, and 4% enzyme load	116.93 mg g^{-1} reducing sugars for saccharification of wheat bran	[74]
Central Composite Rotational Design (CCRD)	29	Cellulase load (U.g^{-1}), xylanase load (U.g^{-1}), pH, and temperature (°C)	Reducing sugars concentration ($g.L^{-1}$)	9 U g^{-1} cellulase load, 9 U g^{-1} xylanase load, pH 5, and incubation temperature at 30 °C	29.20 g L^{-1} reducing sugars for saccharification of pretreated *Cedrus deodara* (deodar) sawdust	[75]
Box-Behnken	15	Enzyme concentration (% w. w^{-1}), reaction time (h), and liquid solid ratio (v.w^{-1})	Dry basis glucose ($g.L^{-1}$) and wet basis glucose (g. L^{-1})	For dry basis saccharification: 5% w. w^{-1} enzyme, reaction for 51 h, and liquid solid ratio of 5:1 v.w^{-1}. For wet basis saccharification: 5% w.w^{-1} enzyme concentration, reaction for 48 h, and liquid solid ratio of 5:1 v. w^{-1}	For dry basis saccharification: 125.2 g L^{-1} glucose. For wet basis saccharification: 130 g L^{-1} glucose	[76]

experimentally and verified that the experimental data agreed with the predicted values.

Bhagwat and Kumar [70] optimized pretreated *Parthenium hysterophorus* biomass saccharification. The study comprised two steps. First, the authors carried out PBD to evaluate the effect of five factors, namely temperature (40–60 °C), pH (2–7), moisture content (60–100%), substrate load (1–5 g), incubation time (24–96 h), and enzyme load (0.1–1 mL) by using only 12 experiments. The results showed that the temperature, pH, enzyme load, and substrate load significantly affected sugar yield ($mg.g^{-1}$ of dry biomass weight). In the second step, they accomplished an optimization study for which they used RSM and considered these four factors. To this end, they carried out Box-Behnken Design, which allowed them to find the ideal conditions to obtain high released sugars yield (550.6 ± 1.20 mg g^{-1} of dry biomass), namely 30 °C, pH 4.5, 0.25% enzyme load, and 0.25% substrate load.

Irfan et al. [71] also used the Box-Behnken Design with RSM to

optimize alkali pretreated wheat straw saccharification. The pretreated wheat straw contained 83% cellulose, 10.5% hemicelluloses, and 4.5% lignin. The authors considered three factors for the optimization: substrate concentration (2, 2.5, and 3.0%), commercial cellulase enzyme concentration (0.25, 0.50, and 0.75%), and incubation time (6, 8, and 10 h). They kept the reaction temperature at 50 °C. The dataset was fitted to a second-order mathematical model, which was statistically significant ($p < 0.05$), but the coefficient of determination of model was low ($R^2 = 0.778895$). The authors did not show the lack of fit of the mathematical model, but the residues revealed that the observed values might differ from the predicted values by up to 69.77%. The optimum conditions given by the response surfaces were 2% wheat straw, 0.5% enzyme, incubation for 6 h, and 50 °C, which gave maximum saccharification of 40.12%.

For ethanol production from narrow-leaf cattail, Ruangmee and Sangwichien [72] used RSM to optimize enzymatic hydrolysis. Previously, they treated narrow-leaf cattail with 5% $w.v^{-1}$ NaOH, which resulted in a material containing 65.8% cellulose, 16.2% hemicellulose, 12.1% lignin, and 5.9% ash. The authors accomplished CCRD by considering four factors with five levels: cellulase enzyme load (5–25 $FPU.g^{-1}$ of substrate), β-glucosidase load (0–20 U g^{-1} of substrate), hydrolysis temperature (30–50 °C), and hydrolysis time (24–96 h). They obtained a second-order model or glucose and xylose release as a function of the studied factors. They evaluated these models in two states: full and reduced. The authors demonstrated that the F-value for the reduced models was higher after backward elimination of non-significant terms. The p-value of lack of fit of the reduced models was also greater ($p > 0.05$) and was considered more accurate for prediction. The optimum conditions were obtained from the glucose production model, which had higher R^2 than the xylose production model. By using the optimized conditions (13.50 $FPU.g^{-1}$ of substrate for the cellulase enzyme, 16.50 U g^{-1} of substrate for β-glucosidase, hydrolysis temperature of 50 °C, and hydrolysis time of 24 h), enzymatic hydrolysis of narrow-leaf cattail yielded 552.9 and 74 mg of released $glucose.g^{-1}$ of substrate (45.6% saccharification), respectively.

Chen et al. [73] recently optimized enzymatic hydrolysis of rice and corn straw by using RSM with the Box-Behnken Design, which enhanced the reducing sugars yield of a unique GH5 cellulase, AgCMCase from *Aspergillus glaucus*. The studied factors and their levels were temperature (50, 60, and 70 °C), time (2, 3, and 4 h), pH (5, 6, and 7), and enzyme dosage (1, 1.5, and 2 mL). The authors obtained second-order mathematical models with high regression coefficients for rice and corn straw (0.9402 and 0.93, respectively). The linear coefficients for reaction time and enzyme dose, the quadratic coefficients for temperature and reaction time, and the interaction terms (reaction time x enzyme dose) were statistically significant ($p < 0.05$). The reaction time and temperature had greater significance for the enzymatic activity and, consequently, for the reducing sugars production. The enzyme lost its activity at high temperature, whereas a mid-level temperature increased the reducing sugars production. Thus, the optimum condition involved milder reaction conditions: 3.84 h and 51.4 °C, which resulted in reducing sugars production of 1.61% for rice straw degradation. By comparing this result with the value obtained in the initial condition (reaction time of 3 h and temperature of 60 °C), the authors concluded that RSM allowed straw degradation to be increased by 1.52-fold.

Silva et al. [74] employed the Box-Behnken Design and RSM to optimize the saccharification of wheat bran; they used the endoglucanase from *Botrytis ricini* URM 5627 and analyzed the following factors and their levels: time (4, 8, and 12 h), percent substrate load (1, 2, and 3%), and percent enzyme load (3, 4, and 5%). They built a predictive mathematical model from the regression coefficient. The Pareto graph showed that both the linear and quadratic enzymatic load and quadratic time influenced saccharification the most. The second-order model was statistically significant and did not have lack of fit according to ANOVA. Therefore, the model fit the experimental results well. The authors built the response surface from a reduced second-order model ($R^2 = 0.9983$) and found that the optimum conditions to achieve the highest concentration of reducing sugars (116.93 mg g^{-1}) were reaction time of 8 h, 2% substrate load, and 4% enzyme load.

Raina et al. [75] used RSM based on Central Composite Design (CCD) to optimize the saccharification of pretreated *Cedrus deodara* (deodar) sawdust. After determining the optimized conditions for acid pretreatment (1.5% HCl concentration, 10% biomass load, and 30-min incubation time), the authors optimized the enzymatic hydrolysis of the pretreated biomass. They performed 29 experiments to evaluate the following factors and their levels: cellulase load (5–13 U g^{-1}), xylanase load (5–13 U g^{-1}), pH (4.5–5.5), and temperature (28–32 °C). The obtained second-order model was statistically significant ($p < 0.0001$) according to ANOVA and was confirmed by the correlation between the experimental and theoretical results, which gave a correlation factor R^2 (0.9801) close to the adjusted value ($R^2 = 0.9602$). The maximum value of reducing sugars (29.20 g L^{-1}) was obtained with 9 U/g cellulase load, 9 U g^{-1} xylanase load, pH 5, and incubation at 30 °C, which was close to the predicted value of 28.08 g L^{-1}.

Guarneros-Flores at al [76]. used RSM and the Box-Behnken design to optimize the enzymatic saccharification of sweet sorghum bagasse (dry basis and wet basis), a biomass composed of 34–44% cellulose, 25–27% hemicellulose, and 18–20% lignin. The authors evaluated 03 factors with 03 levels: enzyme concentration (5, 6, and 7% $w.w^{-1}$), reaction time (24, 48, and 72 h) and liquid solid ratio (5, 7, and 9 $v.w^{-1}$). They generated the regression coefficient of the second-order model for both responses, dry and wet basis glucose. The statistical significance of the coefficients was evaluated by a Student's *t*-test with p value at a confidence level of 95%. For the dry and wet basis saccharification, the coefficient of determination (R^2) was 0.9505 and 0.926, respectively, which showed that both models fit the experimental data well. The authors mentioned that the lack of fit was not significant in any of the cases, but ANOVA analysis was not shown. For dry basis glucose, the optimum conditions were 5% (w/w) enzyme concentration, reaction for 51 h, and 5:1 $v.w^{-1}$ liquid solid ratio, which yielded the highest glucose concentration (125.2 g L^{-1}). For wet basis saccharification, the optimum conditions were 5% $w.w^{-1}$ enzyme concentration, reaction for 48 h, and 5:1 $v.w^{-1}$ liquid solid ratio, which gave the highest glucose concentration (130 g L^{-1}).

6. Using RSM in the fermentation stage

Sugar fermentation by fermenting microorganisms can be conducted via different processes, such as simultaneous saccharification and fermentation (SSF), enzymatic hydrolysis and separate fermentation (SHF), and simultaneous saccharification and co-fermentation (SSCF) [77]. Starch SSF is an efficient and economical method to produce ethanol because it requires less costly equipment. *Saccharomyces cerevisiae* and *Zymomonas mobilis* are the two main species used on the industrial scale of bioethanol production [78]. Lignocellulosic biomass saccharification can release several sugars, like glucose, cellodextrins, and pentoses. *Saccharomyces cerevisiae* preferentially uses glucose as a primary carbon source because this yeast does not contain specific natural transporters of xylose or cellobiose and cannot use these sugars as carbon sources [79]. However, some authors have tested mutant yeasts that can ferment both xylose and glucose.

The fermentation process is recognized as a limiting factor for large-scale production and commercialization of bioethanol, so it is important to evaluate and to understand the effect of different parameters involved in this process. Some parameters that are commonly evaluated to achieve the optimum and most economically viable production are shown in Fig. 4.

RSM techniques have been applied to optimize factors such as temperature, sugar concentration, pH, fermentation time, agitation rate, medium composition, and inoculum size, among others, and to enhance alcohol production [80]. Table 4 lists some works that have optimized fermentation process conditions to improve ethanol production. Some

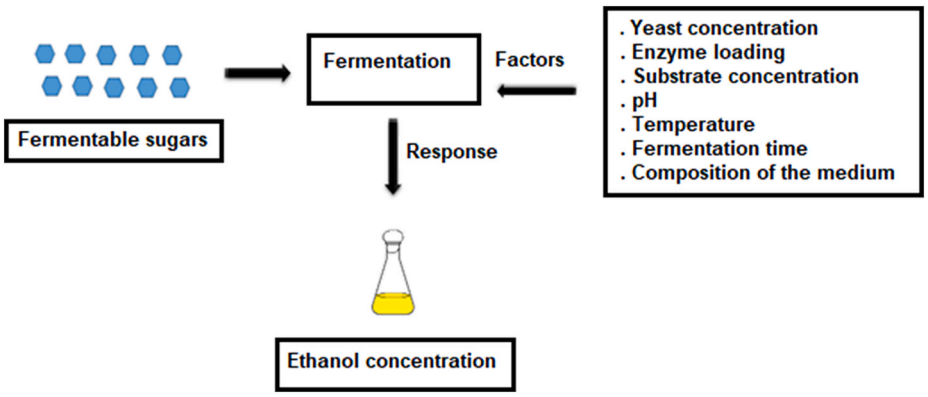


Fig. 4. Representative scheme of the variables that were studied and optimized in the fermentation stage by using DoE tools and RSM.

Table 4
Response Surface Methodology applied in the fermentation stage.

Type of Design	Number of experiments	Independent variables	Response	Optimized conditions	Optimized response	Ref.
Central Composite Rotational Design (CCRD)	20	pH, temperature (°C), and substrate concentration (%)	Ethanol concentration (g. L^{-1}) from damaged corn grains	pH (5.6), temperature (31 °C), and substrate concentration (12%)	4.24 g.(100 mL)$^{-1}$ ethanol ethanol productivity 0.88 (g L^{-1} h^{-1}).	[81]
Box-Behnken	15	Malt extract (g.L^{-1}), Tryptone (g.L^{-1}), and KH_2PO_4 concentration (g.L^{-1})	Ethanol concentration (g.L^{-1}) from potato peel waste	Malt extract (25 g L^{-1}), Tryptone (0 g L^{-1}), and KH_2PO_4 (2.5 g L^{-1})	21.3 g L^{-1} ethanol	[82]
Box-Behnken	15	Yeast extract (g.L^{-1}), Malt extract (g. L^{-1}), and $MgSO_4{\cdot}7H_2O$ concentration (g.L^{-1})	Ethanol concentration (g.L^{-1}) from industrial potato waste	Malt extract (50.0 g L^{-1}), $MgSO_4{\cdot}7H_2O$ concentration (4.84 g L^{-1}), and yeast extract (0.0 g L^{-1})	24.6 g L^{-1} ethanol	[83]
Box-Behnken	27	Substrate concentration (g.L^{-1}), pH, fermentation time (h), and Na_2HPO_4 concentration (g.L^{-1})	Ethanol concentration (g.L^{-1}) from sugarcane bagasse with hydrolysate	Substrate concentration (40 g L^{-1}), pH (4.5), fermentation time (48 h), and Na_2HPO_4 (0.15 g L^{-1})	14.44 g L^{-1} ethanol	[84]
Fractional Factorial	27	Temperature (°C), substrate concentration (%), enzyme load (FPU.g-glucan^{-1}), and yeast concentration (g. L^{-1})	Ethanol concentration (g.L^{-1}) from sweet sorghum bagasse	Temperature (35 °C), enzyme load (29 FPU.g-glucan^{-1}), substrate concentration (10%), and yeast concentration (1.4 g L^{-1})	39 g L^{-1} ethanol	[85]
Central Composite Rotational Design (CCRD)	50	Initial sugar concentration (g.L^{-1}), bacterial dry weight (g), peptone weight (g), yeast extract weight (g), and time (h)	Ethanol concentration (g.L^{-1}) from potato peel (PP) wastes	Initial sugar concentration (61.3 g), bacterial dry cell (0.024 g), meat peptone (0.35 g), yeast extract (0.35 g), and fermentation time (31 h).	23.3 g L^{-1} ethanol	[86]
Box-Behnken	17	Yeast titre, temperature (°C), and enzyme load (U.g^{-1})	Ethanol concentration (g.L^{-1}) from sugarcane bagasse	yeast titre (1 time), enzyme load (100 U g^{-1}) and temperature (39 °C).	4.88 g L^{-1} ethanol	[87]
Central Composite Rotational Design (CCRD)	31 to SHF and 32 to SSF	For SHF: temperature (°C), incubation time (h), inoculum volume (v.v^{-1}) and inoculum age (h). For SSF, the same, with the addition of the substrate load (w.v^{-1}) variable	Ethanol concentration (g.L^{-1}) from a mixture of lignocellulosic biomass	For SSF: inoculum volume (8% v. v^{-1}), temperature (38.18 °C), inoculum age (44.84 h), incubation time (30 h), and substrate load (w. v^{-1}). For SHF: inoculum volume (8% v. v^{-1}), temperature (38.18 °C), inoculum age (24 h), and incubation time (27.33 h)	41.9 g L^{-1} ethanol from SSF and 25.49 g L^{-1} ethanol from SHF	[88]

SHF: Separate hydrolysis and fermentation, SSF: Simultaneous saccharification and fermentation.

authors initially performed a screening design such as PBD to select the factors that they later optimized by using RSM with CCRD or the Box-Behnken Design.

Gawande and Patil [81] studied ethanol production from damaged corn grain in a process involving SSF and an *A. niger* and *S. cerevisiae* co-culture. The authors evaluated three factors (and their levels), pH (5–6), temperature (28–32 °C), and substrate concentration (8–16%), by CCRD. ANOVA of the results evidenced that only the linear and quadratic coefficients of pH and temperature were statistically significant ($p < 0.05$). The authors did not show the reduced model and did not determine the lack of fit of the model. Response surface analyses provided the optimized conditions that afforded the maximum predicted ethanol production yield (4.24 g.(100 mL)$^{-1}$) after 48 h: pH of 5.6, 31 °C, and 12% substrate. Ethanol productivity was 0.88 g L^{-1} h^{-1}. The authors did not test these conditions experimentally, which is an important step to evaluate the validity of model.

Hossain et al. [82] initially employed PBD followed by the Box-Behnken Design to optimize bioethanol production from potato peel residues. For the screening design, the analyzed factors (and levels) were related to the composition of the fermentation medium, namely malt extract (0.5–5 g.L^{-1}), tryptone (0.2–2 g.L^{-1}), $MgSO_4{\cdot}7H_2O$ (0.005–0.05 g L^{-1}), $(NH_4)_2SO_4$ (0.2–2 g.L^{-1}), KH_2PO_4 (0.1–1 g.L^{-1}), $CaCl_2{\cdot}2H_2O$ (0.005–0.05 g L^{-1}), Na_2HPO_4 (0.25–2.5 g L^{-1}), and NaCl (0.1–1 g.L^{-1}). The results showed that only the factors malt extract, tryptone, and

KH_2PO_4 significantly impacted bioethanol production ($p < 0.05$). In the next step, the authors optimized these factors on the basis of RSM with the Box-Behnken Design. Only malt extract had a positive linear influence on ethanol production when potato peel waste was used as carbon source. The optimum medium composition to maximize ethanol production by using *Wickerhamia* sp. was 25 g L^{-1} malt extract, 0 g L^{-1} tryptone, and 0 g L^{-1} KH_2PO_4, which resulted in the predicted value of 21.05 g L^{-1} ethanol. The experimental value was close to the model-predicted value (21.7 g L^{-1} ethanol after fermentation for 96 h).

Izmirlioglu and Demirci [83] studied another potato residue for bioethanol production by *S. cerevisiae*. They optimized industrially hydrolyzed potato waste fermentation for bioethanol production by employing a strategy similar to the one used by Hossain et al. [58]. Initially, they conducted PBD to evaluate the influence of the factors (and their levels) yeast extract (0.5–5 g.L^{-1}), malt extract (2–20 g L^{-1}), $(NH_4)_2SO_4$ (2–6 g.L^{-1}), $MgSO_4{\cdot}7H_2O$ (0.2–2 g.L^{-1}), KH_2PO_4 (0.5–3 g. L^{-1}), $CaCO_3$ (0.2–2 g.L^{-1}), $FeSO_4{\cdot}7H_2O$ (0.01–0.1 g L^{-1}), and $CaCl_2{\cdot}2H_2O$ (0.3–3 g.L^{-1}) on ethanol production from glucose, as substrate. Medium supplementation with yeast extract (g.L^{-1}), malt extract (g.L^{-1}), and $MgSO_4{\cdot}7H_2O$ (g.L^{-1}) affected ethanol production positively, while KH_2PO_4 and $CaCl_2{\cdot}2H_2O$ exerted a negative effect. Therefore, the authors selected the first three variables to optimize ethanol production from potato waste by using RSM along with the Box-Behnken Design, which allowed them to obtain the second-order polynomial equation for ethanol production and cell population data. To obtain the model, the authors excluded the non-significant terms from the equation. The linear coefficients of malt extract and $MgSO_4{\cdot}7H_2O$ and the quadratic coefficient of $MgSO_4{\cdot}7H_2O$ were significant for ethanol production, whereas only the linear and quadratic coefficients of malt extract were significant for cell population. The ideal medium composition was 50.0 g L^{-1} malt extract and 4.84 g L^{-1} $MgSO_4{\cdot}7H_2O$ without yeast extract, which yielded 24.6 g L^{-1} ethanol.

Dasgupta et al. [84] optimized sugarcane bagasse fermentation by *Kluyveromyces* sp. IIPE453 (MTCC 5314) to maximize ethanol production by using a strategy that resembled the strategies employed by Izmirlioglu and Demirci [59] and Hossain et al. [58]. Initially, the authors performed PBD to estimate whether the factors pH (4.5–5.5), fermentation time (24–48 h), substrate concentration (20–40 g L^{-1}), yeast extract (1–5 g.L^{-1}), $MgSO_4$ (0.06–0.12 g L^{-1}), $(NH_4)_2SO_4$ (1–5 g. L^{-1}), Na_2HPO_4 (0.1–0.45 g L^{-1}), KH_2PO_4 (0.15–0.45 g L^{-1}), and inoculum volume (5–10% v.v^{-1}) impacted bioethanol production significantly. In the following step, they conducted the Box-Behnken Design to optimize the pH, substrate concentration, sodium di-hydrogen phosphate, and fermentation time to maximize ethanol production. Statistical analyses of the data showed that the linear coefficients and the interactions between pH and substrate concentration, substrate concentration and Na_2HPO_4 concentration, and fermentation time and Na_2HPO_4 concentration were statistically significant ($p < 0.05$). The quadratic coefficients were not significant. ANOVA of the unreduced regression model showed that the model was statistically significant ($p < 0.05$) and had high R^2_{pred} (0.96). The authors did not show the reduced regression model after backward elimination of non-significant terms. By using the full model, the authors found the optimal conditions—40 g L^{-1} substrate, pH 4.5, fermentation for 48 h, and 0.15 g L^{-1} Na_2HPO_4—that yielded the maximum ethanol concentration predicted by the model (17.39 g L^{-1}). The model was experimentally validated by employing the optimum conditions for fermentation, yielding a final ethanol concentration of 17.44 g L^{-1}, which was close to the value predicted by the model.

Ethanol production by SSF (Simultaneous saccharification and fermentation) of pretreated sweet sorghum bagasse was optimized by using a fractional 3^4 factorial experimental design with a total of 27 experiments [85]. The effect of the factors (at three levels) temperature (35, 40, and 45 °C), sorghum bagasse concentration (4, 7, and 10%), enzyme load (10, 20, and 30 FPU.g-glucan^{-1}), and yeast concentration (0.5, 1, and 1.5 g L^{-1}) on ethanol yield, production rate, and concentration were evaluated. By considering all the terms (significant and non-significant), a second-order polynomial equation was obtained for each analyzed response However, statistical analyses demonstrated that only the linear and quadratic coefficients of temperature and the quadratic coefficient of yeast concentration were statistically significant ($p < 0.05$). In addition to the coefficient of the interaction cellulase loading x yeast concentration, similar coefficients also had significance for the ethanol production rate model ($p < 0.05$). As for ethanol concentration, the linear and quadratic coefficients of temperature, the linear coefficient of bagasse solid concentration, the quadratic coefficient of yeast concentration, and the interaction temperature x bagasse solid concentration were statistically significant ($p < 0.05$). To find the optimum conditions, the response surfaces were generated from the full models for each response. Thus, the optimal conditions for maximum ethanol yield (98.8%) were 37 °C, 25 FP.g^{-1} of glucan enzyme load, 7% solid bagasse, and 1.3 g L^{-1} yeast, whereas the maximum ethanol concentration (39 g L^{-1}) was achieved at 35 °C, 29 FPU.g^{-1} of glucan enzyme load, 10% solid bagasse, and 1.4 g L^{-1} yeast. The maximum expected rate of ethanol production was 6.42 g L^{-1} h^{-1}, which was obtained with the following conditions: 45 °C, 30 FPU.g^{-1} of glucan enzyme load, 4% solid, and 1.5 g L^{-1} yeast. Other authors have used optimization of multiple responses to obtain a desirability function from the ethanol yield, concentration, and production rate models [57,58]. Screening design has also been employed to optimize the process, which is not recommended because important information can be lost. In addition, for 3^4 experiment, 81 experiments should have been carried out, but the authors only conducted 27 experiments. Screening design is only used to study how factors affect the responses. However, the authors generated response surfaces by employing the mathematical model they obtained during screening design.

Mazaheri and Pirouzi [86] investigated *Zymomonas mobilis* to produce bioethanol from potato peel (PP) wastes by optimizing the fermentation process. Initially, the authors performed an enzymatic hydrolysis on potato peel for the fermentable sugars to be released. After that, they used the hydrolysate for ethanol fermentation by *Z. mobilis*; they employed the experimental conditions (50 runs) according to CCRD and optimized the fermentation process by RSM. The authors optimized the following factors: initial sugar concentration (40–70 g L^{-1}), bacterial dry weight (0.010–0.030 g), peptone weight (0.0–0.5 g), yeast extract weight (0.0–0.5 g), and time (24–48 h). According to ANOVA, the p value was <0.0001, demonstrating that the model was significant. The model included the factors initial sugar concentration (A), bacterial dry weight (B), peptone weight (C), yeast extract weight (D), and their interactions AB, AC, and BC; the quadratic coefficients A^2 and C^2 were also significant ($p < 0.05$). The authors obtained maximum ethanol concentration of 23.3 g L^{-1} for 61.3 g L^{-1} initial sugar concentration, 0.024 g of bacterial dry cell, 0.35 g of meat peptone, 0.35 g of yeast extract, and fermentation time of 31 h.

Jugwanth at al [87]. applied RSM with the Box-Behnken design (BBD) to optimize the simultaneous saccharification and fermentation of salt-alkali pretreated sugarcane bagasse (SCB). The studied parameters and their levels were yeast titre (1,3 and 5 times), temperature (30, 40 and 50 °C), and enzyme loading (20, 60, and 100 U g^{-1}) in a total of 17 experiments. The authors obtained the second-order model from ANOVA, which showed high F value (5.21) and low p-value (0.0203), indicating that the model was significant. Moreover, the lack of fit was not significant, demonstrating that the experimental values fit the model well. The predictability of the model proved to be adequate, as judged from the high value of the coefficient of determination ($R^2 = 0.87$). The authors achieved the highest ethanol concentration (4.88 g L^{-1}) under the conditions of 1 time of yeast titre, 100 U g^{-1} enzyme load, and temperature of 39 °C.

Althuri and Banerjee [88] accomplished the Central Composite design (CCD) with RSM to obtain the optimum conditions of separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF), to enhance ethanol productivity by using

Saccharomyces cerevisiae. The authors designed a CCD with 31 and 32 experiments to optimize SHF and SSF, respectively. For SHF, they tested the following conditions: temperature (30–40 °C), incubation time (18–30 h), inoculum volume (8–12% $v.v^{-1}$), and inoculum age (24–72 h). For SSF, they analyzed the same conditions as well as substrate load (15–25% $w.v^{-1}$). According to ANOVA, the model had F values of 32.07 and 387.42 for SHF and SSF, respectively, with $p < 0.001$ in both cases, indicating that the model was statistically significant. They achieved the highest ethanol productivity in SSF (1.396 g L^{-1} h^{-1}), at 80 U g^{-1} cellulase load, 8% ($v.v^{-1}$) inoculum volume, temperature of 38.18 °C, 25% substrate load, inoculum age of 44.84 h, and incubation time of 30 h. However, SHF provided lower ethanol production (0.929 g L^{-1} h^{-1}) at 8% ($v.v^{-1}$) inoculum volume, inoculum age of 24 h, temperature of 38.18 °C after 27.33 h, and 139.9 U g^{-1} cellulase load. The ethanol concentration was 41.9 g L^{-1} for SSF versus 25.49 g L^{-1} for SHF.

7. Conclusion

On the basis of this review, researchers investigating 2G ethanol production have been widely using Response Surface Methodology (RSM) with DoE tools to improve the efficiency of the ethanol production stages: pre-treatment, enzymatic hydrolysis, and fermentation. DoE tools such as the Central Composite Design and Box-Behnken Design have been the most employed to find the optimum conditions to maximize sugar release during pretreatment or enzymatic hydrolysis or to enhance ethanol production during fermentation. We consider that, to achieve the desired success, researchers must choose the factors and their levels correctly, which can be accomplished by means of screening designs, such as the Fractional Factorial Design and Plackett Burman Design. Few studies used a screening design when more than five factors are being evaluated; the Placket-Burman Design was the most applied for this purpose. Screening designs also allow the levels of factors to be identified and changed when a CCRD or the Box-Behnken Design is applied in the optimization study by RSM. On the other hand, the use of screening design to optimize the process is not recommended because a reduction in the number of experiments affects the design resolution and causes important information to be lost. Another fact to consider is that correct statistical analysis of the data is necessary when the second-order polynomial model achieved by using CCRD or the Box-Behnken Design is used to predict a response in other non-studied conditions. In this review, we have seen that many authors included the statistically significant and non-significant coefficients in the models and generated response surfaces by using these models. Few authors correctly followed the procedure to obtain the reduced mathematical model to predict the response. In addition, some authors did not conduct the determination of model lack of fit or experimentally validate the optimum conditions. These analyses must be carried out to evaluate the prediction of the mathematical models. Finally, the works presented here did not use analyses of multiple response to determine the optimum conditions when they analyzed more than one response. We hope this tool will be used in future works.

Acknowledgements

The authors would like to thank Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - CAPES (Coordination for the Improvement of Higher Education Personnel) and Fundação de Amparo à Pesquisa do Estado de São Paulo (São Paulo Research Support Foundation, FAPESP) for financial support. The authors wish to express gratitude and recognition to Profa. Dra. Maria Isabel Rodrigues for the hard work and contribution to knowledge and dissemination of DoE tools in the Brazilian institutions.

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