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Use of a Taguchi Parameter Design to Optimize the Propagation of *Saccharomyces cerevisiae* to Produce Bioethanol

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The propagation of the yeast *Saccharomyces cerevisiae* was optimized using a Taguchi parameter design (TPD) $L_9(3^4)$ to produce bioethanol from an amylaceous material. The response factor selected was the specific growth rate of the yeast as calculated from the slope of the linear portion of its growth curve (neperian log cell concentration versus time). The reason is that the greater this rate, the higher the number of viable cells in the fermentation broth capable of ethanol production.

The control factors selected were the initial amount of inoculum in the medium, the amount of glucose, the temperature, and the shaking speed which are the chemical and physical variables that most affect the growth behavior of this yeast. The noise factor selected was the initial peptone concentration in the medium. Statistical analysis and factorial split-plots indicate that the factor that most affected the response was the inoculum concentration (50.79% contribution), followed by the glucose concentration (25.22%), and shaking speed (14.79%). The contribution of temperature to the response variable was small (2.85%). This result was independent of the uncontrolled variation in the percentage of peptone in the sample.

Finally, a confirmatory experiment was performed using two replicates of the optimal combination predicted by the design for each noise level: inoculum 0.1 g/100 mL, glucose 3 g/100 mL, temperature 30°C, and shaking speed 200 rpm. The specific growth rate of around 0.300 h⁻¹ obtained was the highest achieved in all the tests performed confirming this set of factors as the optimal combination.

Introduction

Fermentation is a catabolic process of incomplete oxidation undertaken by many microorganisms as reactions on organic compounds accompanied by energy release. Hundreds of species of yeasts, bacteria, and fungi are known to produce alcohol, but only two or three species of yeast are used to industrially obtain alcohol due to their capacity for rapid fermentation, their ability to tolerate high sugar and alcohol concentrations, and their high production yields. As the starting material, a nutrient solution (or broth) is used that contains the elements indispensable for the life of microorganisms: carbohydrates, nitrogen, and salts specific to each microorganism.^[1]

The microorganisms traditionally used in the process of fermentation for the production of ethanol are the yeast species *Saccharomyces cerevisiae* and *Saccharomyces uvarum*, although the use of some bacteria such as *Zymomonas* has also been proposed.^[2] *S. cerevisiae* is capable of converting into alcohol roughly 85% of substrate in 32 hours and 90% after 75 hours. This microorganism contains percentage weights of 45% carbon, 30.6% oxygen, 6.8% hydrogen and 9% nitrogen, and its energy requirements, in terms of grams of substrate needed for its growth, vary from 0.036–0.025 (g of cells/g of substrate/h) depending on whether the process is anaerobic or aerobic, respectively.^[3]

To achieve high alcoholic fermentation yields when using an amylaceous starting material and a yeast of the genus *Saccharomyces*,^[4–6] the first factor to be considered is the substrate concentration since carbon is supplied by the sugars in the culture medium such that the sugar concentration affects the fermentation rate, behavior and development of the yeast cells. A sugar concentration of 10–18% is usually satisfactory; the concentration most commonly used

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being around 12%. The size of the inoculum should also be considered. A relatively small amount of yeast in the mixture will render a low cell density at the end of the propagation stage with a consequent drop in the amount of ethanol produced through fermentation. An excess in the proportion of yeast cells will cause the rapid use of the sugars in the medium and also lead to a diminished alcohol yield.

Another factor that will substantially affect the efficiency of the process is the ethanol concentration, which severely affects productivity when in excess of 3%. If the ethanol level reaches 5%, both growth and fermentation will be affected while an ethanol concentration exceeding 10% will arrest growth altogether.

The working temperature is also a significant variable, since it affects physiological factors and also induces physical problems (losses due to ethanol evaporation at high working temperatures). Each yeast has an optimal development temperature at which it is most active. The optimal development temperature for *S. cerevisiae* is between 28 and 35°C, with some authors quoting an optimal value of 30°C.^[4-6]

The pH of the fermentation broth is also an important factor given its controlling actions on bacterial contamination, yeast growth, and alcohol production. The most adequate pH for the growth of *S. cerevisiae* is 4.4–5.0, with 4.5 generally quoted as the optimal pH.^[4-6]

Nutrients are a further factor to consider because the presence of adequate nutrients is necessary for the growth and development of the yeast, and sufficient concentrations of nutrients are essential for the vital activity of the yeast. The most influential nutrients are nitrogen, phosphorus, sulfur, vitamins, and some trace elements.

Finally, the aeration of the batch culture is yet another factor to consider. Air is a determining factor in any fermentation process and its presence promotes more vigorous yeast growth. Thus, contact between the cell membrane and the nutrient substrate should be constantly renewed through continuous mixing to ensure a free and constant supply of oxygen to each yeast cell in the substrate. Aeration ensures the rapid removal of CO₂ which reduces yeast growth and maintenance of the yeast cells in suspension at relatively low concentrations.

According to the literature,^[7-9] microbial propagation as a function of time can be described by a typical growth curve that can be classified into several easily distinguishable phases: lag, acceleration, exponential growth, stationary, and death. In the lag phase, the cells become adapted to the environmental conditions and growth rates are low because the microorganism is synthesizing enzyme resources to metabolize the different substrate components. The acceleration phase has not been mathematically defined but, in this stage, the proportion of progeny cells tends to reach 50% of the total cell population. In the exponential growth phase, each cell divides into two and they each divide again into a total of four through the same successive process. Exponential growth ceases in the stationary phase, essential nutrients run out, there is no increase or decrease in the number of cells or total mass, nutrients become limited, and toxic substances build up.

All of these processes lead to the death stage which usually occurs following continuous incubation since, although some cells may remain alive and continue to metabolize, chances are they will die. During this stage, the direct microscope count may remain constant but cell viability slowly diminishes. In some cases, death is accompanied by cell lysis leading to a drop in the viability count.

The present study was designed to gain insight into the propagation process of yeasts using amylaceous material as the starting product and *S. cerevisiae* as the yeast. Through a Taguchi parameter design, we determined the effects on the process of several chemical (inoculum, glucose, presence of proteins) and physical (temperature, shaking the medium) variables in order to identify the optimal values of the different variables that would determine the highest specific growth rate of the yeast.

The ideal starting material-to-yeast ratio should be such that the largest number of cells possible exists at the start of fermentation. This avoids long lag stages and thus leads to a more efficient use of the fermentation volume.

To calculate this, we used the growth curve, glucose consumption curve, specific growth rate curve, and the relationship between optical density and number of yeast cells.

Saccharomyces cerevisiae

MATERIALS AND METHODS

Experimental Design

To optimize the propagation process of the yeast *S. cerevisiae*, a Taguchi $L_9(3^4)$ design was used with four factors at three levels. The target or response function was the specific growth rate of *S. cerevisiae* ($\mu \text{ h}^{-1}$) which we tried to maximize. Table 1 lists the factors controlled and the levels of each one tested. With the noise factor, we considered the amount of protein added to the medium by varying the proportion of peptone. Accordingly, the design matrix consisted of nine experiments including the levels of the different factors that appear in the four columns of Table 2. Each of the nine experiments was performed in duplicate for each of the noise levels. All the materials used were previously sterilized in a Sanyo (Osaka, Japan) MLS-3780 autoclave and samples were obtained under sterile conditions in a Bio II-A safety cabinet.

Procedures

Preparing the Medium and Inoculum

For each of the nine experiments, the amount of glucose necessary was weighed out (see Table 2) and placed in the corresponding Erlenmeyer flask, adding 1.0 g of yeast extract to each flask. To two of the flasks, 1.0 g of peptone was added, 2.0 g of peptone were added to two others, and then 3.0 g were added to the remaining two (noise factor). After the addition of 50.0 mL of water, the flasks were placed on a heater with a magnetic stirrer until the medium had lost its turbidity. The mixtures were then transferred to volumetric flasks and made up to 100 mL with water. The contents of the volumetric flasks were returned to the conical flasks, stoppered with a hydrophobic cotton plug, and covered with aluminum foil. The flasks were then autoclaved at 121°C for 15 minutes.

To another six empty and autoclaved conical flasks

TABLE 1. Factors examined and levels tested.

FACTORS		LEVEL 1	LEVEL 2	LEVEL 3
Control Factors	Inoculum (g/100 mL)	0.20	0.10	0.15
	Glucose (g/100 mL)	1.00	2.00	3.00
	Temperature (°C)	32	30	34
	Shaking speed (rpm)	100	150	200
Noise Factors	Protein (g/100 mL)	1.00	2.00	3.00

TABLE 2. Design matrix and specific growth rate responses.

EXPERIMENT	INOCULUM (g/100 mL)	GLUCOSE (g/100 mL)	TEMPERATURE (°C)	SHAKING SPEED (rpm)	NOISE LEVELS		
					μ_1 (h^{-1})	μ_2 (h^{-1})	μ_3 (h^{-1})
1	0.20	1.00	32	100	0.169 0.171	0.167 0.160	0.162 0.159
2	0.20	2.00	30	150	0.192 0.214	0.213 0.208	0.216 0.216
3	0.20	3.00	34	200	0.248 0.244	0.261 0.266	0.270 0.273
4	0.10	1.00	30	200	0.308 0.304	0.309 0.306	0.279 0.285
5	0.10	2.00	34	100	0.280 0.269	0.276 0.291	0.284 0.285
6	0.10	3.00	32	150	0.274 0.301	0.299 0.273	0.312 0.317
7	0.15	1.00	30	200	0.183 0.182	0.187 0.177	0.184 0.189
8	0.15	2.00	34	100	0.238 0.246	0.270 0.284	0.248 0.249
9	0.15	3.00	32	150	0.266 0.277	0.269 0.273	0.249 0.252

(121°C for 15 min), the amounts of lyophilized yeast needed for each experiment were added (in the safety cabinet) along with 4.0 mL of autoclaved water. The flasks were placed in a Thermoshake THO 500 orbital incubator/shaker (Gerhardt Analytical Systems, Königswinter, Germany) setting the shaking speed and temperature indicated for each experiment over a 30 minute period and thus allowing the rehydration and activation of the yeast. Next, a sample was taken from each of the culture media to determine absorbance and subsequently poured into the conical flasks containing the hydrated yeast (always working in the safety cabinet). Finally, the Erlenmeyer flasks were covered with an autoclaved foam stopper. The six flasks containing the culture medium along with the hydrated yeast were arranged in the orbital incubator at the pre-established temperature and shaking speed for approximately 30 hours (photo below).

Obtaining Samples and Tests

Samples were taken at time zero and then every hour in the safety cabinet beside the flame, and the flasks were subsequently returned to the orbital incubator/shaker. Each sample was then subjected to three measurement procedures:

Optical Density. Using a micropipette with a sterile tip, 1.0 mL of sample was placed in a test tube containing 9.0 mL of autoclaved water (1:10 dilution). Absorbance was measured in a UV/Vis Varian Cary 50 spectrophotometer (Agilent Technologies, Madrid, Spain) at a wavelength of 600 nm. As the absorbance of the sample increases, the sample should be diluted before the optical density exceeds 1.0 and measures fall outside the linear range of the instrument.

Number of Cells per mL. Using a micropipette with a sterile tip, 1.0 mL of sample was placed in a test tube containing 9.0 mL of autoclaved water (dilution 1:10). The number of cells per mL was determined using a Thoma counting chamber and light microscope (Olympus CX41; Tokyo, Japan). Dilutions were prepared during the propagation cycle to ensure the number of cells in each square of the Thoma chamber was around 20.

Concentration of Glucose Consumed (g/L). Using a micropipette whose tip had been autoclaved, 1.0 mL of sample was placed in a conical vial. The concentration of glucose consumed (g/L) was determined using a biochemistry analyzer (2700 Select; YSI Life Sciences, Yellow Springs, Ohio USA). A 25.0 g/L glucose standard was used to check that the instrument was operating within its linear range. Sampling was stopped when the optical density and number of cells remained constant (*i.e.*, when the stationary phase had been reached).

Data Analysis

Using the data on optical density, number of cells, and glucose concentration obtained, different parameters such as the yeast growth

curve, glucose monitoring curve, specific growth rate, and the relationship between optical density and cell number were calculated to help understand the propagation process.

Growth Curve

Absorbance values and cell numbers were plotted against time to generate growth curves in which the different phases of the propagation process could be distinguished. The microorganism was thus characterized in terms of growth and the time taken to reach the late log, or exponential growth phase. It is at this stage that the yeast is used as inoculum to initiate the fermentation process at the highest cell density possible.

Glucose Consumption Curve

This curve was obtained by plotting the amount of glucose measured in the sugar analyzer against time since inoculation.

Specific Growth Rate

The rate of microbial growth is directly proportional to the cell concentration. The proportionality constant is the specific rate μ , which for each type of microorganism, depends mainly on the composition and concentration of the culture medium, presence of inhibitors, temperature, and pH.^[6, 10]

$$\frac{dx}{dt} = \mu \cdot x \quad (1)$$

where x is the cell concentration.

Graphically, μ represents the slope of the linear section of the growth curve and may be calculated by plotting the neperian logarithm of the cell concentration against elapsed time since inoculation as:

$$\ln \frac{x}{x_0} = \mu t \Rightarrow \mu = \frac{\ln \frac{x}{x_0}}{t} \quad (2)$$

There are several expressions for μ . The most widespread is the Monod equation^[12] that relates the value of μ to the concentration of a component of the culture medium that is diminished with respect to the microorganism's requirements: the limiting substrate. According to this equation, when the substrate concentration is high ($S \gg K_s$), the specific growth rate tends towards a maximum constant value, $\mu_{\max}$. Typical μ values for *S. cerevisiae* are 0.45 h⁻¹ for growth in anaerobic conditions and 0.25 h⁻¹ for aerobic growth.^[11-13]

Relationship Between Optical Density and Cell Number

Once the experiment was finished, absorbance values were plotted against the number of cells to establish a mathematical expression by linear fitting to a tendency line the relationship between absorbance and cell concentration.

RESULTS AND DISCUSSION

The last three columns in Table 2 show the specific growth rates obtained for each combination of factor levels (in duplicate) at the three noise levels. This is followed by a detailed description of Experiment 6 (I [inoculum]:2; G [glucose]:3; T [temperature]:1; and S [shaking speed]:2), which as may be inferred from the table provided the best results. Figure 1 shows the changes recorded in the three controlled responses (optical density, cell number, and glucose consumed) over time. As may be observed in Figure 2, the mean specific growth rate obtained in the presence of 1% peptone was 0.288 h^{-1} . This value is close to the mean value cited for yeasts of 0.250 h^{-1} [11-13].

The yeast growth curves plotted as optical density or cell concentration were similar in shape (Figure 1), although some points reflected some degree of experimental error. As observed in the glucose curve (Figure 1), this substrate diminished gradually with the number of cells per milliliter and also with optical density measured at 600 nm. The yeast needed seven hours to reach the stationary phase; the lag stage was fairly short since the lyophilized yeast was hydrated and activated for 30 minutes before its use to inoculate the medium. According to equation (2), the ordinate at the origin of the linear fitting used to calculate specific growth rate should be zero. In our case, a maximal value of 0.35 was recorded at the origin (Figure 2), which is around the expected figure.

Using the data from the whole set of experiments, we obtained a relationship between absorbance and cell concentration. The initial absorbances of the media differed for each level according to glucose concentration. Thus, absorbance should be measured at the start of each experiment before inoculation such that the results can be subsequently represented in the same graph.

Adjustment between optical density and cell number was conducted using a linear regression model although it was found that the residuals did not show the same variance. This means the condition of homoscedasticity was not fulfilled, which is an assumption required for the minimum squares method. To overcome this problem, cell numbers obtained experimentally were weighted according to their variance. This method gives more importance to the more precise measures. The results obtained may be seen in Figure 3. The error is mainly experimental and is essentially the outcome of errors easily made when conducting cell counts, and of the error introduced by the spectrophotometer. Thus, the relationship between the number of cells per milliliter and absorbance units may be expressed as:

$$\text{Number of cells (mL} \cdot 10^7) = 1.134 \cdot \text{Absorbance} \quad (3)$$

FIGURE 1. Changes recorded in the controlled variables over time in Experiment 6 (see Table 2).

FIGURE 2. Changes in specific growth rate over time recorded in Experiment 6 for noise level 1.

FIGURE 3. Mathematical relationship between optical density and number of cells.

Figure 4 shows the effects of the control factors on the specific growth rate of *S. cerevisiae*. It may be observed that the optimal set of control factors is level 2 for the percentage of inoculum, level 3 for the percentage of glucose, level 2 for temperature and level 3 for the shaking speed. It may also be noted that the first two factors, inoculum and glucose, contribute more to the variation in the response, or specific growth rate of the yeast, since differences between their levels are much greater than those of the other factors (temperature and shaking speed). The contribution of temperature to the variation in specific growth rate was the lowest of all.

Analysis of variance (ANOVA) was also performed on both the whole set of results and the signal-to-noise (S/N) ratio. ANOVA is a useful statistical tool for analyzing experimental data that depend on many types of effects simultaneously acting on the response of a given procedure. It estimates the size of

these effects and their contribution to the total variation in the data. The variability of experimental results is the outcome of the variation produced by the factors that affect the response and by random error. As may be observed in Table 3, in our case, the factors whose variation mostly affected the specific growth rate were: first, the inoculum which accounted for 50.79% of the variation followed by glucose which contributed 25.22%, and shaking speed, whose contribution was 14.79%. Temperature scarcely contributed at 2.85%. The percentage of variation due to error or to the residuals was 6.34%. This percentage includes factors that could affect the response but were not considered in the design, along with possible interactions among the control factors that were discarded by the hypothesis.

This analysis was followed by statistical analysis of the S/N ratio in terms of “the higher the ratio, the better the optimization.” Figure 5 indicates that the highest S/N ratios

SOURCE	POOL	Df	S	V	F	S'	rho
Inoculum	(N)	2	0.061	0.031	213.854	0.061	50.79
Glucose	(N)	2	0.031	0.015	106.418	0.031	25.22
Temperature	(N)	2	0.004	0.002	12.906	0.003	2.85
Shaking Speed	(N)	2	0.018	0.009	62.820	0.018	14.79
Noise Factor (Protein)	(Y)	2	4.060E-04	2.035E-04			
Inoculum × Protein	(Y)	4	6.476E-04	1.619E-04			
Inoculum × Protein	(Y)	4	0.001	2.801E-04			
Inoculum × Protein	(Y)	4	0.001	2.664E-05			
Inoculum × Protein	(Y)	4	0.002	4.294E-04			
e1	(Y)	0	0.000				
e2	(Y)	24	0.001	5.552E-05			
(e)	(Y)	45	0.006	1.435E-04		0.008	6.34
TOTAL		53	0.113	0.002			

ABBREVIATIONS:
degrees of freedom (Df), sum of squares (S), variance (V), variance ratio (F), pooled sum of squares (S'), percent of contribution (rho)

FIGURE 4. Specific growth rates recorded for the different factor levels.

FIGURE 5. Signal-to-noise ratio by factor level.

for each of the design's factors are level 2 for the inoculum, level 3 for glucose, level 2 for temperature, and level 3 for the shaking speed. These levels are consistent with those obtained from the mean response graph as well as with the contributions to the total and mean variation. They indicate the greatest contributions by the inoculum and glucose followed by the shaking speed, and finally by the temperature.

Table 4 shows the results of the ANOVA confirming the predictions of the S/N ratio in the factorial graph. The greatest contribution was that of the inoculum (45.38%) followed by glucose (26.43%) as the second factor with the most weight. The next factor in order of magnitude was the shaking speed

(12.79%). Temperature accounted for such a low variance that it was included with the residuals to give an overall value of 15.40% as the percentage contribution.

Once the results were statistically analyzed, a confirmatory experiment was performed using the following sequence of control factors predicted as optimal by the design (I:2, G:3, T:2 and S:3) for the three noise levels selected to give the results provided in Table 4. The specific growth rates obtained for *S. cerevisiae* in this experiment were the highest recorded in any of the experiments of the design. This in effect confirms the suitability of the experimental design selected for optimizing the propagation process.

TABLE 4. ANOVA of the response signal.

SOURCE	POOL	Df	S	V	F	S'	rho
Inoculum	(N)	2	13.194	6.597	12.788	12.162	45.38
Glucose	(N)	2	8.115	4.057	7.865	7.083	26.43
Temperature	(Y)	2	1.032	0.516			
Shaking Speed	(N)	2	4.461	2.230	4.323	3.429	12.79
e1	(Y)	0	0.000				
e2	(Y)	0	0.000				
(e)		2	1.032	0.516		4.127	15.40
TOTAL		8	26.801	3.350			

CONCLUSIONS

Our findings reflect the critical effects of chemical factors (inoculum size and glucose concentration) on the specific growth rate of the yeast *S. cerevisiae*. Among the physical factors, the shaking speed also had a significant effect on the target response. Temperature contributed modestly given that the range selected was within the optimal temperature range for the growth of the yeast. The contribution of uncontrolled factors (residuals) was 6.34% which, being lower than 15%, indicates that the factors were appropriately selected.

The combination of factor levels predicted by the Taguchi method: 0.1% inoculum; 3.0% glucose, 30°C temperature, and 200 rpm shaking speed provided the highest response of all, around 0.300 h⁻¹. This response could be reproduced independently of all uncontrollable external variations (noise factors).

The production of bioethanol from amylaceous material depends on the specific growth rate of the yeast *S. cerevisiae* used. According to the present results, this rate is maximal (0.298 h⁻¹) under the working conditions identified here (I:2; G:3; T:2; and S:3).

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