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# Influence of process factors interactions in enzyme catalyzed biodiesel obtainment

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## Abstract

*Transesterification of oils with various alcohols for the production of biodiesel is presently of great interest due to the advantages associated with the usage of this alternative fuel source. Lipases are adequate catalyzers for biodiesel production, being able to achieve both triglycerides alcoholysis and free fatty acids esterification in the same bioreactor. Biodiesel yield is influenced by the enzyme type, reaction systems and operational parameters. In the case of a two-level, five-factor experimental design, the relationship among the five reaction process variables – time, temperature, enzyme concentration, alcohol to oil molar ratio and water concentration – and the percent weight biodiesel conversion throughout the biodiesel obtainment process from soy bean oil with methanol in the presence of the lipase from Thermomyces lanuginosus, was determined. 96.22 and 17.63 were the maximum and minimum values obtained for biodiesel conversion, respectively. Also, a non-formalized, intuitive but theoretical-grounded statistical methodology of analysis of the factor interactions effect on outcome was described.*

**Keywords**: biocatalysis, experimental design, statistical analysis methodology, *Thermomyces lanuginosus,* transesterification, vegetable oils

## Introduction

Biodiesel has the highest energetic balance of all alternative fuels, being also biodegradable, $CO_2$ neutral and it conserves fossil fuels (Knothe & al [1]). Fatty acid methyl esters (FAME), known also under the name biodiesel, are presently of great interest as an alternative fuel source (Haas & al. [2]). Enzyme catalyzed transesterification is a very good alternative to all chemically catalyzed reactions (Kralova & al [3]), but it requires intensive research before commercialization; therefore, enzymatic biodiesel production through chemical reactions catalyzed by lipases under mild conditions has recently won an important commercial interest. Lipases used in biotechnology are usually of microbial origin and available on the market, sometimes as immobilized products. Immobilized enzymes offer two important advantages: on one hand the possibility of catalyst recovery, and, on the other hand, the possibility of continuous processes. The use of vegetable oils as renewable energy sources is an extremely viable alternative due to their liquid nature, high caloric power, low sulfur content and biodegradability. Substrates containing triglycerides differ according to the geographical and economical availability, but palm, soy bean and rape oil are most commonly used (Ribeiro & al [4]). Of the alcohols, methanol is usually used due to its low price.

Results have shown that the type of lipase, reaction systems and operational parameters (lipase load, reaction time, temperature and alcohol/oil molar ratio) have influenced biodiesel yield (Hernandez-Martin & al [5]; Ghiorghiță & al [6]). Regarding alcohol to oil molar ratio, the stoichiometric equation requires 3 moles of alcohol and one of triglyceride for the obtainment of 3 fatty acid methyl ester moles and 1 mole of glycerol. Experiments have shown that higher molar ratios have led to higher biodiesel yields. The use of solvents has proven to be necessary to maintain the miscibility between the methanol and triglycerides with the purpose of forming a monophasic system (Soumanou & al [7]). The water content is also an important parameter (Adlercreutz & al [8]; Salis & al [9]), although the presence of water seems to be the subject of a dispute. Some authors claim that certain quantities of water lead to large yields in biodiesel (Masaru & al [10]; Kamini & al [11]), while others claim just the opposite (Hsu & al [12]). The effect of water in the system depends on the enzyme, immobilization support and the medium (with or without solvent). Probably the main disadvantage in biocatalytic biodiesel obtainment is the cost of the enzyme. Enzymes present different capacities to maintain their activity after recovery and repeated use, probably due to catalyst inactivation in the oil phase, the type of carrier used in immobilization or enzyme sensitivity to long-term exposure (Soumanou & al [7]). In order to reduce the costs, there is also the possibility of using whole cells immobilized during cultivation, with no purification necessary (Fukuda & al [13]; Hama & al [14]).

Du et al. studied the effects of different process factors using Lipozyme TLIM and short chain alcohols and concluded that the optimum temperature is between 40-50$^{o}$C and oil/alcohol molar ratio should be 1:4 (Du & al [15]). In order to avoid enzyme inactivation by methanol (Salis & al [16]) the stepwise adding of methanol during the process has been proposed (Shimada & al [17]). Watanabe et al. developed a two-step process using Novozyme 435 by adding first 1/3 methanol molar equivalents and the 2/3 rest in the second phase, obtaining a 95% conversion (Watanabe & al [18]). Yoo et al used *Ralstonia* lipase to obtain biodiesel from palm and soy bean oil at 50-55$^{O}$C, pH 8.0, 5% methanol and 20% water. (Yoo & al [19]).Using lipase B from *Candida antarctica* at 32$^{O}$C, 5% lipase (g/g) from soy bean oil, 4% alcohol (v/v) Rosset et al obtained after 24 hours a biodiesel yield of 87% (Rosset & al [20]). De Oliveira et al took into account a Taguchi experimental model with the following variables: temperature (36-65$^{O}$C), water (0-10% from palm oil weight), enzyme (5-20% from palm oil weight). As biocatalyzers Novozyme 435 and Lipozyme IM were used and experimental conversions of 81.4% and 8%, respectively, were obtained. (De Oliveira& al [21]).

Response surface methodology has been used for interpreting the results obtained in experiments of optimization of the biodiesel production from both vegetal and animal fats, including waste oils (Ghadge & al [22]; Yuan & al [23]; Jeong & al [24]; Tiwari & al [25]).

The aim of this paper is twofold: 1) determining the relationships among the five reaction process variables – time, temperature, enzyme (% from weight of oil), alcohol to oil molar ratio and water (% weight of oil) – and the percent weight biodiesel conversion throughout the biodiesel obtainment process from soy bean oil with methanol in the presence of the lipase from *Thermomyces lanuginosus*; 2) describing a non-formalized, intuitive but theoretical-grounded statistical analysis methodology of the factor interactions effect.

## Materials and methods

Usually, for the optimization of the biodiesel obtainment process, the study of the highest number of experimental variables, depending on the factors that we are to work with, is necessary. The experiment number depends on the number of parameters whose influence is being studied, and because of that, the optimization of the process conditions requires an

optimization methodology, which allows the study of a larger number of factors and their interaction, optimizing one or more experimental responses (the response being the result of one experiment, biodiesel concentration, in our case) (Mansourpoor & al [26]).

Five key process parameters were chosen, together with an experimental design defined by a matrix with 16 experiments. For each parameter, 2 levels were chosen *a priori* (considered as under-optimal and over-optimal values for process evolution. Oil to biodiesel conversion (%) was considered as response. The experimental matrix used throughout this paper is the standard $2^{(5-1)}$ fractional factorial design. This is a part of a standard matrix of the five-level, five–factor central composite rotatable design (**5/5CCRD**). The interested reader might see an example (Chang & al [27]) or a more general approach in "Experiments with Mixtures: designs, models, and the analysis of mixture data" (Cornello & al [28]). More precisely, the 16 two–level factorial design experiments of the 5/5CCRD have been chosen, corresponding in usual notation to factor levels denoted -1 and 1 (Table 1). By using only these two levels (e.g. by ignoring the minimal, central and maximal values in the usual sequence of 5-level equally-spaced-sequence – usually denoted by -2, -1, 0, 1 and 2), the response surface methodology (RSM) cannot be properly applied. Nevertheless, the present paper's aim was not to estimate the optimal values of the considered factors, somewhere between minimal and maximal value, but to study the manner in which different combinations of the factor-levels influence the reaction response. The use of only 2 factor levels (instead of 5) simplified our analysis.

In order to avoid bias, each sample was repeated 3 times, to allow a statistic evaluation of the results.

The matrix (Table 1) was build based on the variation of 5 essential parameters, for which – as we mentioned above – two levels were chosen. The five parameters are: time ($x_1$), temperature ($x_2$), enzyme ($x_3$) (% from weight of oil), alcohol to oil molar ratio ($x_4$) and water ($x_5$) (% weight of oil).

Notation

On a common base, -1 and 1 are the symbols used for labeling two values symmetrically distributed around "a centre" (i.e. a regular value considered to be "the most usual"). In order to simplify the notation, we shall keep "1" for the higher value but we shall replace "-1" with "0" for the label of the lower value. Moreover, the five reaction parameters $x_1$, $x_2$, $x_3$, $x_4$ and $x_5$ will be denoted A, B, C, D and E, respectively. Taking into account these conventions, the following notation will be used.

**A1B0C1D0E1** = the experiment with A=1, B=0, C=1, D=0 and E=1, (namely $x_1$=1, $x_2$=-1, $x_3$=1, $x_4$=-1 and $x_5$=1) . There exist $2^5$ =32 such possible experiments, similarly defined: A0B0C0D0E0, A0B0C0D0E1, …, A1B1C1D1E1. We used only half of them, the 16 experiments listed in Table 1 and each one was replicated 3 times.

**S_A0** = the set of all experiments with A=0 (that is $x_1$=-1), irrespective the values of B, C, D, E. Similarly, **S_A1** is the set of all experiments with A=1. For the case of 5 parameters there exist $2^5/2$ =16 different experiments with A=0 and 16 with A=1. The experimental design presented in Table 1 uses 8 (half of them). The sets corresponding to the other parameters are similarly defined (there are 5x2=10 possible variants: S_A0, S_A1, S_B0, S_B1, ..., S_E1).

**S_A1B0** = the set of all experiments with A=1 and B=0 (namely $x_1$=1, $x_2$=-1), irrespective the values of C, D, E. For the case of 5 experimental parameters there exist $2^3$ =8 different experiments, corresponding to all possible combination of (C, D, E): (0, 0, 0), (0, 0, 1), (0, 1, 0), (0, 1, 1), (1, 0, 0), (1, 0, 1), (1, 1, 0), (1, 1, 1). But the experimental design

presented in Table 1 uses only 4 (namely half). The notation for the other parameter combinations is similarly defined (there are 10x4=40 possible variants: S_A0B0, S_A0B1, S_A1B0, S_A1B1, ..., S_D1E1).

**S_C1D0E1** = the set of all experiments with parameters C=1, D=0 and E=1, irrespective the values of D, E. For the case of 5 experimental parameters there exist $2^2$ =4 different experiments, corresponding to the four possible combination of (A, B). The design presented in Table 1 uses only 2 (namely half). The notation for the other parameter combinations are similarly defined (there are 10x8=80 possible variants: S_A0B0C0, S_A0B0C1, S_A0B1C0, S_A0B1C1, ..., S_C1D1E1).

**F_A0, F_A1, …, F_E1, F_A0B1,…, F_C1D1E1** are 0/1 dummy variables, corresponding to above mentioned sets, S_A0, S_A1, …, S_E1, S_A0B1,…, S_C1D1E1.

To be more specific: **F_XiYjZk** is defined as it follows (where i, j, k are 0 or 1):

F_XiYjZk =1 if and only if X=i, Y=j and Z=k; otherwise F_XiYjZk =0.

Examples: 1) F_A1 =1 if A=1 and F_A1=0 if A=0. 2) F_A0 =1 if A=0 and F_A0=0 if A=1. 3) F_C1D0 =1 if C=1 and D=0; F_C1D0 =0 if C≠1 or D≠0. 4) F_A0C1E0 =1 if A=0, C=1 and E=0; F_ A0C1E0=0 if A≠0 or C≠1 or E≠0.

Taking into account these definitions, the following useful relations are true:

F_XiYj= F_Xi ×F_Yj; F_XiYjZk= F_Xi ×F_Yj ×F_Zk (example: F_ A0C1E0= F_ A0× F_C1× F_E0).

Of course, there are many redundancies in our dummy variable set. Some examples: F_A0+F_A1=1; F_A1B0+F_A1B1= F_A1; F_A1B0C0+F_A1B1C0= F_A1C0, etc

Statistical model

The dummy variables were considered as independent variables in certain stepwise linear regression models in order to study the effect on the dependent variable (biodiesel production, in our case) of the factors and of the interactions of two factors. Definitely, the coefficients of the dummy variables describe the contribution of the factors or interactions attached to the respective dummy. This approach is similar to the GLM univariate procedure (see, as an example, the package SPSS). The main difference is the possibility of using some redundant dummies in a stepwise regression procedure. But, following the standard approach, we will avoid the use of redundant predictors.

Output linear equations which describe biodiesel production were used for factor interaction description.

Experiment description

The reaction mixture contained soy bean oil (2g) to which 4 portions of methanol were added throughout the process to avoid enzyme inactivation, 6mL *n*-hexane (Merck Chemical Co. Darmstadt, Germany), water (5% and 15% weight of oil) and the enzyme. TlL *(Thermomyces lanuginosus* lipase from CLEA - 50.000 U/g) was used as a catalyzer – 1% and 3% weight of oil (Table 1). The reaction was performed with agitation (250 rpm) at 35 and 55$^{O}$C, for 4 and 12 hours.

For obtaining biodiesel at laboratory level, a Heidolph Unimax 1010 reactor with a stirring unit and Heidolph Inkubator 1000 were used. The samples were vortexed with a Vortex Heidolph Reax Top, every time a methanol portion was added.

The sample analysis was performed by injecting a 1mm$^3$ aliquot in split less mode into a Hewlett Packard 6890 gas chromatograph (Avondale, PA, USA) equipped with a flame-ionization detector (FID), CP-Select CB for FAME 50m x 0.25mm x 0.25µm, 170$^{o}$C to 210$^{o}$C oven temperature, 300$^{o}$C detector temperature, 1 ml/min carrier gas flow. The calibration curve had 6 levels: from level c1≈16 µg/ml up to the level c6≈200 µg/ml, and for

both the calibration curve and the samples, internal standard solution 92.8 μg in concentration was added.

## Results and discussion

The purpose of the experiments was the study of biodiesel production with respect to the interactions of the factors. To this purpose, as independent process variables, 5 media components or key factors (time, temperature, enzyme (%), R-OH:R'-COOH and water (%)) were chosen. The rest of the factors remained unchanged. The values of biodiesel conversion for the 3 runs, as well as their means are presented (Table 1a).

**Table 1a -** Experimental matrix, experimental conditions and biodiesel outcome for the five-factor, two-level design

| Label | Experimental matrix | Experimental conditions* | Run1 | Run2 | Run3 | Mean |
|---|---|---|---|---|---|---|
| 1 | -1,-1,-1,-1,+1 | A0B0C0D0E1 | 79.3 | 81.07 | 80.2 | 80.19 |
| 2 | -1,-1,-1,+1,-1 | A0B0C0D1E0 | 18.34 | 17.14 | 17.41 | 17.63 |
| 3 | -1,-1,+1,-1,-1 | A0B0C1D0E0 | 50.73 | 42.4 | 49.36 | 47.50 |
| 4 | -1,-1,+1,+1,+1 | A0B0C1D1E1 | 55.59 | 58.58 | 57.9 | 57.36 |
| 5 | -1,+1,-1,-1,-1 | A0B1C0D0E0 | 35.23 | 45.32 | 38.02 | 39.52 |
| 6 | -1,+1,-1,+1,+1 | A0B1C0D1E1 | 34.1 | 31.98 | 33.04 | 33.04 |
| 7 | -1,+1,+1,-1,+1 | A0B1C1D0E1 | 90.3 | 86.96 | 88.63 | 88.63 |
| 8 | -1,+1,+1,+1,-1 | A0B1C1D1E0 | 56.26 | 56.35 | 56.3 | 56.30 |
| 9 | +1,-1,-1,-1,-1 | A1B0C0D0E0 | 33.17 | 39.5 | 43.69 | 38.79 |
| 10 | +1,-1,-1,+1,+1 | A1B0C0D1E1 | 38.8 | 37.32 | 38.5 | 38.21 |
| 11 | +1,-1,+1,-1,+1 | A1B0C1D0E1 | 95.92 | 96.99 | 95.74 | 96.22 |
| 12 | +1,-1,+1,+1,-1 | A1B0C1D1E0 | 55.92 | 52.23 | 57.33 | 55.16 |
| 13 | +1,+1,-1,-1,+1 | A1B1C0D0E1 | 39.42 | 38.72 | 39.07 | 39.07 |
| 14 | +1,+1,-1,+1,-1 | A1B1C0D1E0 | 32.51 | 32.77 | 32.64 | 32.64 |
| 15 | +1,+1,+1,-1,-1 | A1B1C1D0E0 | 87.79 | 88.21 | 88 | 88.00 |
| 16 | +1,+1,+1,+1,+1 | A1B1C1D1E1 | 56.32 | 56.31 | 56.3 | 56.31 |

* See Table 1b

**Table 1b. Specification of the two levels of the experimental conditions**

| $X_1$ (hours) | | $X_2$ (◦C) | | $X_3$ (% w/w) | | $X_4$ (molar ratio) | | $X_5$ (% w/w) | |
|---|---|---|---|---|---|---|---|---|---|
| Time | | Temperature | | Enzyme | | Alcohol/oil | | Water | |
| A0 | A1 | B0 | B1 | C0 | C1 | D0 | D1 | E0 | E1 |
| 4 | 12 | 35 | 55 | 1 | 3 | 3:1 | 5:1 | 5 | 15 |

Regression Model

A full non-redundant predictor set for interactions up to 2nd order interactions was considered in a linear regression model: F_A1, F_B1, F_C1, F_D1, F_E1, F_A1B1, F_A1C1, F_A1D1, F_A1E1, F_B1C1, F_B1D1, F_B1E1, F_C1D1, F_C1E1, F_D1E1.

The overall fit of the model was excellent: the significance value of the ANOVA test was $p<0,001$, while the determination coefficient is high, $R^2=0.99$. All coefficients of the

model were significantly different from 0 ($p<0.05$ for t-test), excepting coefficient of F_C1E1 (with $p=0.07$). The predictors were not independent, but the collinearity statistics coefficients were acceptable. The range of residuals were within acceptable limits (between -5.62 and 5.80). Hence, the model was accurate enough for a good prediction of the mean outcome.

The equation of the model was:

**Biodiesel%=31.90+6.89×F_A1+7.63×F_B1+15.6×F_C1–**
**14.27×F_D1+48.29×F_E1–**
**–6.79×F_A1B1+16.9×F_A1C1+2.94×F_A1D1–**
**20.76×F_A1E1+15.89×F_B1C1+**
**+4.35×F_B1D1–28.08×F_B1E1–4.79×F_C1D1–2.59×F_C1E1–**
**16.78×F_D1E1.**

The equation above is identical with the solution of ANOVA model provided by the univariate General Linear Model procedure of SPSS (to be more specific: UNIANOVA procedure, with DESIGN=F_A1 F_B1 F_C1 F_D1 F_E1 F_A1*F_B1 F_A1*F_C1 F_A1*F_D1 F_A1*F_E1 F_B1*F_C1 F_B1*F_D1 F_B1*F_E1 F_C1*F_D1 F_C1*F_E1 F_D1*F_E1).

The equation of the model was not friendly looking but, surprisingly, it was not difficult to be handled. Some interesting conclusions could be extracted from the above mentioned model equation.

*i) D0 was a better choice than D1*, irrespective of the selected fixed combination level for A, B, C and E. Indeed, D1 had a negative contribution to total outcome: it added at most 2.94+4.35 (because of the terms 2.94F_A1D1 and 4.35F_B1D1), but it diminished the total with 14.27 (because of the negative term –14.27F_D1). Conversely, D0 had a null contribution (all terms containing D1 disappeared).

*ii) For the choice D0, C1 was a better choice than C0*, irrespective of the selected fixed combination levels of A, B, E. Indeed, for the choice D0 we had F_D1=0, and, because F_XiYj= F_Xi×F_Yj, the regression equation become:

Proc=31.90+6.89×F_A1+7.63×F_B1+15.6×F_C1+48.29×F_E1–6.79×F_A1B1+
+16.9×F_A1C1–20.76×F_A1E1+15.89×F_B1C1–28.08×F_B1E1–2.59×F_C1E1.

The above relation showed that C0 was a bad choice (level C0 had a null contribution, but C1 had a positive contribution).

*iii) Hence, we concluded that the best factor combination must contain C1D0.*

*iv)* The equation of the model allowed estimating the outcome for all 32 possible tested and not tested combinations of the factors (see Table 2).

**Table2.** The best and the worst factor combinations

| The best five experimental combinations | | | The worst five experimental combinations | | |
|---|---|---|---|---|---|
| **Experiment** | **Biodiesel%** | **Label** | **Experiment** | **Biodiesel%** | **Label** |
| A1B0C1D0E1 | 96,23 | 11 | A0B1C0D1E0 | 29,61 | * |
| A0B0C1D0E1 | 93,2 | * | A0B0C1D1E0 | 28,44 | * |
| A0B1C1D0E1 | 88,64 | 7 | A1B0C0D1E0 | 27,46 | * |
| A1B1C1D0E0 | 88,02 | 15 | A0B0C0D1E0 | 17,63 | 2 |
| A1B1C1D0E1 | 84,88 | * | A1B1C0D1E1 | 15,32 | * |

*not tested combination

Rule of thumb methodology for interaction evaluation

We have sketched an empiric quick methodology for interaction evaluation for the case of a balanced design. The advantage of this approach is its straightforwardness. No sophisticated statistical device is necessary. Only the "Mean" column of Table 1a was be considered, excepting the ANOVA test to verify equality of two means, for the case of the presence/absence of a specified combination of factor levels (computed taking into account the values of Run1, Run2 and Run3 from Table 1a).

The idea of our *ad hoc* method was to use the synopsis table of each interaction. A synopsis table of the 3rd order interaction XYZ has 8 rows containing statistical information about the sets (remember the definition from "Notation") S_X0Y0Z0, S_X0Y0Z1, S_X0Y1Z0, S_X0Y1Z1, S_X1Y0Z0, S_X1Y0Z1, S_X1Y1Z0 and S_X1Y1Z1, respectively. The rows were arranged in the decreasing order of their means. Examples: for synopsis of 2nd interaction CD see the first 4 rows of Table 3, left; for synopsis of CDE see the first 8 rows of Table 3, right.

Without any general considerations, we have exemplified below the definition and how to manage the synopsis tables for our case.

**Table 3.** Empiric evaluation of the outcome depending on experimental factor levels

| Set | Mean | Max | min | p |
|---|---|---|---|---|
| S_C1D0[1)] | 80.09[2)] | 96.22[3)] | 47.50[4)] | 0.000[5)] |
| S_C1D1 | 56.28 | 57.36 | 55.16 | 0.697 |
| S_C0D0 | 49.39 | 80.19 | 38.79 | 0.419 |
| S_C0D1 | 30.38 | 38.21 | 17.63 | 0.000 |
| S_C1E1 | 74.63 | 96.22 | 56.31 | 0.000 |
| S_C1E0 | 61.74 | 88.00 | 47.50 | 0.177 |
| S_C0E1 | 47.63 | 80.19 | 33.04 | 0.263 |
| S_C0E0 | 32.15 | 39.52 | 17.63 | 0.000 |
| S_A1B0 | 57.09 | 96.22 | 38.21 | 0.596 |
| S_A0B1 | 54.37 | 88.63 | 33.04 | 0.953 |
| S_A1B1 | 54.01 | 88.00 | 32.64 | 0.996 |
| S_A0B0 | 50.67 | 80.19 | 17.63 | 0.559 |
| S_B0D0 | 65.67 | 96.22 | 38.79 | 0.039 |
| S_B1D0 | 63.81 | 88.63 | 39.07 | 0.085 |
| S_B1D1 | 44.57 | 56.31 | 32.64 | 0.096 |
| S_B0D1 | 42.09 | 57.36 | 17.63 | 0.034 |

Synopsis of biodiesel production depending on 2nd order interaction (selection, only for interactions CD, CE, AB and BC).

| Set | Mean | Max | min | p |
|---|---|---|---|---|
| S_C1D0E1 | 92.42 | 96.22 | 88.63 | 0.000 |
| S_C1D0E0 | 67.75 | 88.00 | 47.50 | 0.115 |
| S_C0D0E1 | 59.63 | 80.19 | 39.07 | 0.525 |
| S_C1D1E1 | 56.83 | 57.36 | 56.31 | 0.751 |
| S_C1D1E0 | 55.73 | 56.30 | 55.16 | 0.847 |
| S_C0D0E0 | 39.16 | 39.52 | 38.79 | 0.086 |
| S_C0D1E1 | 35.62 | 38.21 | 33.04 | 0.032 |
| S_C0D1E0 | 25.14 | 32.64 | 17.63 | 0.000 |
| S_A1C1D0 | 92.11 | 96.22 | 88.00 | 0.000 |
| S_A0C1D0 | 68.06 | 88.63 | 47.50 | 0.106 |
| S_A0C0D0 | 59.86 | 80.19 | 39.52 | 0.508 |
| S_A0C1D1 | 56.83 | 57.36 | 56.30 | 0.751 |
| S_A1C1D1 | 55.74 | 56.31 | 55.16 | 0.847 |
| S_A1C0D0 | 38.93 | 39.07 | 38.79 | 0.081 |
| S_A1C0D1 | 35.42 | 38.21 | 32.64 | 0.030 |
| S_A0C0D1 | 25.34 | 33.04 | 17.63 | 0.001 |

Synopsis of biodiesel production depending on 3rd order interaction (selection, only for interactions CDE and ACD).

Legend. Example: [1)]S_C1D0 is the set of all experiments with C1D0; [2)]80.09 is the mean production in S_C1D0; [3)]96.22 is the maximal mean production of an experiment from S_C1D0 (remind, each experiment is repeated three times); [4)]47.50 is the minimal mean production of an experiment from S_C1D0; [5)]p=0.000 (namely p<0.001) is the significance value for testing equality of mean of S_C1D0 with the joint mean of S_C1D1, S_C0D0 and S_C0D1.

Comments on Table 3

Table 3 is only a selection: no more than four 2nd order interactions and two 3rd order interactions were included.

The "most promising" synopsis table was that of the 2nd order interaction CD (see the first four rows of Table 3, left). Indeed, interaction CD showed the greatest difference between the best mean (80.09) and worst mean (30.38). Hence the levels of C and D had a dramatic influence on the outcome. The best level-combination of CD was C1D0 (Max= 96.22 and mean=80.09).

The following step was to add to CD a third good factor. The high difference between Max= 96.22 and min=47.50 on the row of C1D0 (see the first row of left side of Table 3) said that at least one from A, B, E had an important contribution. We found two 3rd order useful interactions: CDE and ACD (see the right side of Table 3). The best level choices for these interactions were C1D0E1 and A1C1D0, respectively. It was interesting to remark that the worst level combinations were the opposite of the best: C0D1E0 and A0C0D1, respectively. Hence, we had an empirical reason to suppose that the most promising combination was A1C1D0E1.

In the case of factor B, we had no valuable arguments to choose B0 or B1. Indeed, in the synopsis table BD (see the left side of Table 3), D significantly changed the means, but B seemed to be unimportant. Moreover, examining synopsis table AB (see the left side of Table 3) we concluded that there was no significant difference between the four variants (no significant p) and all Max values were good. Hence A and B were "not powerful enough" to significantly influence the outcome. Finally, the conservative conclusion was that the best choice for our experiments was C1D0E1. Concerning A and B it would be a good idea to revise the levels for A (4 and 12) and B (35 and 55).

## Conclusions

The aim of the present paper was to study the manner in which different factor-level combinations influence enzyme catalyzed biodiesel production. To this purpose a two-level-five factor experimental design was used.

The most important contribution to the yield has been shown by the alcohol to oil molar ratio, specifically a lower ratio is always better than the higher one, irrespective the experimental levels chosen for the other four variables. Also, when a lower molar ratio is chosen, the larger amount of enzyme proves to be more productive. Generally, the levels chosen for time and temperature seem not to be "powerful enough" for an important influence on the outcome. Still, in this particular set of experiments, the best combination is longer process time, larger amount of enzyme and lower molar ratio and the worst combination is: less process time, larger amount of enzyme and lower molar ratio, irrespective of the process temperature.

It can thus be concluded that a larger amount of biocatalyst, in our case *Thermomyces lanuginosus* lipase (3% weight of oil) will lead to a better yield, while water acts as a lubricant for the polypeptide chains and maintains the enzyme in an active conformation, which explains the better yields obtained when water 15% weight of oil were added. The better results obtained with a 3/1 alcohol to oil molar ratio could be related to an enzyme inhibition due to enzyme dehydration when higher methanol concentrations were used, in spite of its being added in portions throughout the experiment. The biocatalyst proved to be just as active at 35°C as at 55°C, but this outcome is of course useful only for this particular enzyme. The time factor also proved to be irrelevant in the case of our set of experiments, whether the process lasted for 4 or for 12 hours.

Our general conclusion is that a longer period of process time at a lower temperature, higher amounts of enzyme, and higher level of water and lower alcohol to oil molar ratio provide the best results (the estimated outcome value was 95.95%) and on the contrary, the

most damaging factor combination (with an estimated outcome of 7.94%) is short time, lower temperature, and lower added water concentration, together with higher enzyme input and higher alcohol to oil molar ratio.

Concerning the statistical analysis methodology which we applied, some brief conclusions could be of interest. 1) The definition of the dummy variables of factor interactions can provide a useful device for statistical analysis. 2) For the case of a balanced design, the use of dummies as predictors in linear regression models offer a valuable, more intuitive, alternative to ANOVA analysis. 3) Allowing for a redundant set of predictors it is possible to produce some alternative models for the same output. Unfortunately, this is a risky idea: we can obtain good descriptions of dependencies, but also misleading results. Therefore, in addition, the use of a set of non-redundant predictors is compulsory. 4) All regression models that were discussed confirm the conclusion that not only the factors, but also the 2$^{nd}$ and 3$^{rd}$ order interactions must be considered in order to obtain and understand a good prediction of the best factor combination. The same conclusion was drawn when using the rule of thumb methodology for interaction evaluation.

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## References

1. Knothe, G., Ed. The Biodiesel Handbook. Champaign, AOCS Press (2005).
2. Haas, M. J., Piazza, G. J., and Foglia, T. A., Enzymatic approaches to the production of biodiesel fuels, In Lipid Biotechnology, ed. T.M. Kuo and H. W. Gardner, Chapter 29, 587-598. Marcel Dekker, Inc., New York, NY (2002).
3. Kralova I. and J. Sj¨oblom, "Biofuels-renewable energy sources: a review" J Disper Sci Technol, 31, no. 3, p. 409–425, (2010).
4. Ribeiro Bernardo Dias, Castro AlineMachado de, Coelho Maria Alice Zarur and Freire DeniseMaria Guimaraes, Production and Use of Lipases in Bioenergy: A Review from the Feedstocks to Biodiesel Production, Enzyme Research, Article ID 615803, 16 pages (2011).
5. Hernandez-Martin, E., Otero, C., Different enzyme requirements for the synthesis of biodiesel: Novozyme 435 and Lipozyme TL IM, Bioresour. Technol. 99: 277–278, (2008).
6. Ghiorghiță, Alexandra, Câmpeanu Gheorghe, Popescu Mircea, Israel Florentina, Neață Gabriela, Enzymatic bioprocessing of vegetable oils for the production of biodiesel, International Conference "Agriculture for life, life for agriculture", Section 6 – Biotechnologies, vol. 16, 2012, ISSN 2285 – 1364, 4-6 Oct. 2012, Bucharest, Romania (2012).
7. Soumanou, M.M. and U.T. Bornscheuer Lipase-catalyzed alcoholysis of vegetable oils. Eur. J. Lipid Sci. Technol., 105, 656–660, (2003).
8. Adlercreutz, P. Biocatalysis in non-conventional media, p. 295–316. In: A.J.J. Straathof and P. Adlercreutz (eds.), Applied Biocatalysis, Harwood Academic Publishers(2000).
9. Salis, A., M. Monduzzi and V. Solinas, Enzymes for biocatalysis in non aqueous media. Biocatalysis: Chemistry and Biology. A. Trincone. Kerala, Research Signpost. 29–53, (2005).
10. Masaru Kaieda, Taichi Samukawa, Takeshi Matsumoto, Kazuhiro Ban, Akihiko Kondo, Yuji Shimada, Hideo Noda, Fumiki Nomoto, Koutaro Ohtsuka, Eiji Izumoto, Hideki Fukuda, Biodiesel fuel production from plant oil catalyzed by Rhizopus oryzae lipase in a water-containing system without an organic solvent, J. Biosci. Bioeng., 88, Issue 6, 627–631, (1999).
11. Kamini, N.R. and H. Iefuji, Lipase catalyzed methanolysis of vegetable oils in aqueous medium by *Cryptococcus* spp. S-2. Process Biochem 37, 405–410, (2001).
12. Hsu, A.-F., K. Jones, T.A. Foglia and W.N. Marmer, Immobilized lipase-catalysed production of alkyl esters of restaurant grease as biodiesel. Biotechnol. Appl. Biochem.. 36, 181–186, (2002).

13. Fukuda Hideki, Akihiko Kondo, Hideo Noda, Biodiesel fuel production by transesterification of oils, J. Biosci. Bioeng., 92, Issue 5, 405–416 (2001).
14. Hama, S., Tamalampudi, S., Fukumizu, T., Miura, K., Yamaji, H., Kondo, A., and Fukuda, H., "Lipase localization in *Rhizopus oryzae* cells immobilized within biomass support particles for use as whole-cell biocatalysts in biodiesel-fuel production", J. Biosci. Bioeng., 101, 328-333 (2006)
15. Du, W., Y. Xu and D. Liu Lipase-catalysed transesterification of soya bean oil for biodiesel production during continuous batch operation. Biotechnol. Appl. Biochem. 38, 103–106 (2003).
16. Salis, A., I. Svensson, M. Monduzzi, V. Solinas and P. Adlercreutz, The atypical lipase B from Candida antarctica is better adapted for organic media than the typical lipase from Thermomyces lanuginosa. Biochimica et Biophysica Acta 1646, 145–151, (2003).
17. Shimada, Y., Y. Watanabe, A. Sugihara and Y. Tominaga, Enzymatic alcoholysis for biodiesel fuel production and application of the reaction to oil processing. J. Mol. Catal. B: Enzym. 17, 133–142 (2002).
18. Watanabe, Y., Y. Shimada, A. Sugihara, H. Noda, H. Fukuda and Y. Tominaga, Continuous production of biodiesel fuel from vegetable oil using immobilized Candida antarctica lipase. J. Am. Oil Chem. Soc. 77, 355–360, (2000).
19. Yoo Hah-Young, Jaya Ram Simkhada, Seung Sik Cho, Don Hee Park, Seung Wook Kim, Chi Nam Seong and Jin Cheol Yoo, A novel alkaline lipase from Ralstonia with potential application in biodiesel production, Bioresource Technology, 102, Issue 10, p. 6104-6111, (2011).
20. Rosset Isac G., Maria Cecília H. Tavares, Elisabete M. Assaf and André Luiz M. Porto, Catalytic ethanolysis of soybean oil with immobilized lipase from Candida antarctica and 1H NMR and GC quantification of the ethyl esters (biodiesel) produced, App. Catal. A: General, 392, Issues 1-2, p. 136-142, (2011).
21. De Oliveira, D., M. Di Luccio, C. Faccio, C. Dalla Rosa, J.P. Bender, N. Lipke, S. Menoncin, C. Amroginski and J.V. De Oliveira, Optimization of enzymatic production of biodiesel from castor oil in organic solvent medium. Appl. Biochem. Biotechnol. 113–116, 771–780, (2004).
22. Ghadge SV, Raheman H, Process optimization for biodiesel production from mahua (Madhuca indica) oil using response surface methodology. Bioresource Technol. 97: 379-384 (2006).
23. Yuan X, Lui J, Zeng G, Shi J, Tong J, Huang G., Optimization of conversion of waste rapeseed oil with high FFA to biodiesel using response surface methodology. Renew Energy 33: 1678-1684 (2008).
24. Jeong GT, Yan HS, Park DH, Optimization of transesterification of animal fat ester using response surface methodology. Bioresource Technol 100: 25-30 (2009).
25. Tiwari AK, Kumar A, Raheman H, Biodiesel production from Jatropha oil with high free fatty acids: An optimized process. Biomass Bioenergy 31: 569-575 (2007).
26. Mansourpoor M, Shariati, A Optimization of Biodiesel Production from Sunflower Oil Using Response Surface Methodology. J Chem Eng Process Technol, 3:141 (2012).
27. Chang Hung-Min, Liao Hui-Fen, Lee Chin-Chia and Shieh Chwen-Jen, Optimized synthesis of lipase-catalyzed biodiesel by Novozym 435, Chem Technol Biotechnol, 80, 307–312 (2005).
28. Cornell John, A, Experiments with Mixtures: designs, models, and the analysis of mixture data, Willey, New York, Chichester, Brisbane, Toronto (1981).