

Feasibility of bioethanol production from rice bran

Viabilidade da produção de bioetanol a partir do farelo de arroz

Francieli Begnini Siepmann¹; Daneysa Lahis Kalschne^{2*}; Caroline Zabotti³;
Eder Lisandro de Moraes Flores⁴; Cristiane Canan⁵; Eliane Colla⁵

Highlights:

Rice bran protein hydrolysis allowed an increase in the final bioethanol production.
Temperature and inoculum concentration affect the bioethanol production.
Optimized temperature and an inoculum allowed to produce 40.70 g L⁻¹ of bioethanol.
Validation in benchtop bioreactor produced 40.0 g L⁻¹ of bioethanol from rice bran.
Sequential design and protease addition improve bioethanol production from rice bran.

Abstract

Rice bran is a by-product of rice production with a high carbohydrate and starch content and the potential for bioethanol production by alcoholic fermentation. This article describes bioethanol production by *Saccharomyces cerevisiae* from hydrolyzed defatted rice bran (DRB) a rice by-product applying ultrasonic treatment and protease addition, as well as a sequential strategy of experimental design (SEED). In the first Central Composite Rotatable Design (CCRD), the temperature (25-30 °C) and inoculum concentration (0.5-50 g L⁻¹) had positive effects on bioethanol production, while the effect of pH (4.0-6.0) was not significant. In the second CCRD, the temperature (28-35 °C) and inoculum concentration (10-70 g L⁻¹) had negative and positive effects on bioethanol production (p < 0.05). Protease addition (15 µL g⁻¹) increased the conversion of substrate into bioethanol by 76%. The optimized conditions for the production of 40.7 g L⁻¹ bioethanol were a temperature of 31.5 °C and an inoculum concentration of 70 g L⁻¹. Validation in a benchtop bioreactor produced 40.0 g L⁻¹ of bioethanol from hydrolyzed DRB, and the SEED was characterized as a useful tool to improve bioethanol production from DRB. Furthermore, the DRB proved to be a by-product with great potential for bioethanol production, derived from alternative sources not commonly used in human food.

Key words: Enzymatic hydrolysis. Ultrasonic treatment. Protease. Response surface methodology. Alcoholic fermentation.

Resumo

O farelo de arroz é um subproduto com alto teor de carboidratos e amido, com potencial para produção de bioetanol por fermentação alcoólica. O presente artigo descreve a produção de bioetanol pela ação da *Saccharomyces cerevisiae* no farelo de arroz desengordurado hidrolisado (DRB) - um subproduto

¹ Discente do Curso de Mestrado do Programa de Pós-Graduação em Tecnologia de Alimentos, Departamento de Alimentos, Universidade Tecnológica Federal do Paraná, UTFPR, Medianeira, PR, Brasil. E-mail: franbegnini@gmail.com

² Pós-Doutoranda do Curso de Mestrado do Programa de Pós-Graduação em Tecnologia de Alimentos, Departamento de Alimentos, UTFPR, Medianeira, PR, Brasil. E-mail: daneysa@hotmail.com

³ Discente do Curso de Graduação em Engenharia de Alimentos, Departamento de Alimentos, UTFPR, Medianeira, PR, Brasil. E-mail: carolzabotti@hotmail.com

⁴ Prof., Curso de Mestrado do Programa de Pós-Graduação em Tecnologias Ambientais, Departamento de Química, UTFPR, Medianeira, PR, Brasil. E-mail: eder@utfpr.edu.br

⁵ Prof^{as}, Curso de Mestrado do Programa de Pós-Graduação em Tecnologia de Alimentos, Departamento de Alimentos, UTFPR, Medianeira, PR, Brasil. E-mail: canan@utfpr.edu.br; ecolla@utfpr.edu.br

* Author for correspondence

do arroz - com a aplicação do tratamento ultrassônico e adição de protease, e estratégia sequencial de planejamento experimental (SEED). No primeiro Delineamento Composto Central Rotacional (CCRD), a temperatura (25-30 °C) e a concentração de inóculo (0,5-50 g L⁻¹) tiveram efeitos positivos na produção de bioetanol, enquanto o pH (4,0-6,0) não foi significativo. No segundo CCRD, a temperatura (28-35 °C) e a concentração do inóculo (10-70 g L⁻¹) tiveram efeitos negativo e positivo, respectivamente, na produção de bioetanol ($p < 0,05$). A adição de protease (15 µL g⁻¹) aumentou a conversão do substrato em bioetanol em 76%. Na temperatura de 31,5 °C e concentração de inóculo de 70 g L⁻¹ obteve-se a condição otimizada para produção de bioetanol, com a produção de 40,7 g L⁻¹. Na validação, realizada em um fermentador de bancada, foram produzidos 40,0 g L⁻¹ de bioetanol a partir de DRB hidrolisado; e o SEED foi caracterizado como uma ferramenta útil para otimizar a produção de bioetanol a partir de DRB. Além disso, o DRB provou ser um subproduto com grande potencial para a produção de bioetanol, derivado de fontes alternativas normalmente não utilizadas na alimentação humana.

Palavras-chave: Hidrólise enzimática. Tratamento ultrassônico. Protease. Superfície de resposta. Fermentação alcoólica.

Introduction

Bioethanol, a sustainable and renewable fuel used for transportation, aids 21st century society in its endeavor to meet the growing energy demand for transportation and industrial processes. A large portion of the bioethanol currently produced in the world is derived from sugar or starch crops and therefore competes directly with food sources. Bioethanol production requires the development of a sustainable process using renewable by-products composed of lignocellulose and starch to remain economically viable (Das et al., 2013; Hayes, 2009).

Brazil is the largest producer of rice (*Oryza sativa*) outside Asia. The estimate for these countries' rice crop in 2019/2020 reached 10,563 thousand tons in a cultivated area of 1,685 ha (CONAB, 2020). One of the by-products generated during the production of polished rice is rice bran. This by-product represents between 8% and 11% of the total rice grain weight (Parrado et al., 2006) and presents a high content of protein (15%), fat (21%), total carbohydrates (52%) and starch (15%) (Amagliani, O'Regan, Kelly, & O'Mahony, 2017). The concentrations of carbohydrates justify the use of rice bran in fermentative processes as for bioethanol production. However, the presence of nutrients such as protein and lipids may reduce the yeast's performance (Watanabe, Honda, Kashiwamura, Sasano, & Watanabe, 2009a). Defatted rice bran

(DRB) has lower concentrations of fat (<2%) and higher protein (18%), total carbohydrates (60%), and starch (21%) contents (Watanabe et al., 2019). Thus, the hydrolysis of DRB proteins is an option to increase the bioethanol yield considering that several studies (Johnston & McAloon, 2014; Lei, Zheng, Wang, Zhao, & Zhao, 2013; Watanabe et al., 2009b; Yao, Lee, Wang, Moura, & Johnson, 2012) demonstrated that the addition of a protease resulted in an increase in the final bioethanol concentration.

The amino acids released during protein hydrolysis increase the concentration of nitrogen, an essential macronutrient for the action of yeast, in the medium. Some studies (Mendes-Ferreira, Mendes-Faia, & Leão, 2004) evaluated the growth of *S. cerevisiae* and fermentation of wines using different initial concentrations of nitrogen (16.5 to 805 mg L⁻¹) supplemented by the addition of diammonium sulfate or a mixture of amino acids and observed an increase in yeast growth (biomass and conversion of the substrate into the product) when a nitrogen supplement was used.

Therefore, the aim of this study was to examine bioethanol production from hydrolyzed DRB by *S. cerevisiae* alcoholic fermentation employing a sequential strategy of experimental design. Two Central Composite Rotatable Designs (CCRD) were employed in order to optimize the variables of temperature, pH, and inoculum concentration with

respect to the response of bioethanol production. Ultrasonic treatment and protease addition were also evaluated in order to increase bioethanol production.

Materials and Methods

Raw material, enzymes, and microorganism

DRB, provided by Riograndense Rice Institute (IRGA-Pelotas/RS), was ground in a knife mill (SL31, Solab, Piracicaba, Brazil) and frozen at -12 °C until analysis. The DRB was submitted to enzymatic hydrolysis by amylolytic enzymes used industrially in the production of rice vinegar thermostable α -amylase (Termamyl 2X, Novozymes, Bagsværd, Denmark) and amyloglucosidase (AMG 300L, Novozymes) and protease (Alcalase 2.4 L FG, Novozymes), all provided by LNF - Latin America. After hydrolysis, alcoholic fermentation was carried out using *S. cerevisiae* (commercial lyophilized yeast, Saf-instant, Campinas, Brazil).

Amylolytic enzymatic hydrolysis of DRB

DRB was hydrolyzed enzymatically as previously described by Siepmann, Canan, Jesus, Pazuch, & Colla (2018) in order to release fermentable sugars. Then α -amylase (30 $\mu\text{L g}^{-1}$ DRB) was added to the DRB suspension (200 g L^{-1}), and pH 6.0 and a temperature of 90 °C were maintained for 2 h. For amyloglucosidase (40 $\mu\text{L g}^{-1}$ DRB) hydrolysis, the suspension was cooled, and the temperature and pH were adjusted to 55 °C and 4.7, respectively and maintained for 3 h. Subsequently, the sugar profile of hydrolyzed DRB was determined according to Stobienia et al. (2020). An ultra-high-performance liquid chromatograph (Ultimate 3000, Thermo Scientific, Germering, Germany), equipped with an

automatic sample injector, quaternary pump, oven, and refraction index (RI) detector and controlled by Chromeleon 7.0 software was used. The extract was filtered (0.45 μm ; Millipore, Massachusetts, USA) and injected (20 μL) into the chromatograph. A Bio-Rad Aminex HPX-87H (300 mm \times 7.8 mm, 9 μm particle size) column and a mobile phase composed of H_2SO_4 5.0 mM at a flow rate of 0.6 mL min^{-1} were used. The oven temperature was set at 50 °C, and detection was performed by IR at 210 nm. The following standards were used: glucose, fructose, xylose, arabinose, mannose, maltose, and galactose, all from Sigma-Aldrich (Saint Louis, USA). The sugar profile of hydrolyzed DRB evidencing a glucose content of 58.04 g L^{-1} , a maltose content of 3.08 g L^{-1} , and a mixture of galactose, xylose, mannose, and fructose of 4.58 g L^{-1} , arabinose was not detected.

Sequential strategy of experimental design for the alcoholic fermentation of DRB

Alcoholic fermentation was carried out in three steps (Figure 1). The first one was performed by CCRD in order to evaluate the effect of temperature, pH, and inoculum concentration on bioethanol production (CCRD-A). Subsequently, an ultrasound and protease (US+P), and a protease only (P) treatment were tested in order to increase the conversion of substrate (DRB) into bioethanol. Lastly, a second CCRD was performed considering the variables temperature and inoculum concentration on bioethanol production (CCRD-B) taking into account a previous treatment of substrate with protease (P). CCRD-A and B were performed using three central points, without repetition; however, the response variables were determined in triplicate. CCRD-A and B followed a generic model detailed in Equation 1 and 2, respectively.

$$y = \beta_0 + \beta_1 \cdot x_1 + \beta_1 \cdot x_1^2 + \beta_2 \cdot x_2 + \beta_2 \cdot x_2^2 + \beta_3 \cdot x_3 + \beta_3 \cdot x_3^2 + \beta_{12} \cdot x_1 \cdot x_2 + \beta_{13} \cdot x_1 \cdot x_3 + \beta_{23} \cdot x_2 \cdot x_3 \quad (\text{Eq. 1})$$

$$y = \beta_0 + \beta_1 \cdot x_1 + \beta_1 \cdot x_1^2 + \beta_2 \cdot x_2 + \beta_2 \cdot x_2^2 + \beta_{12} \cdot x_1 \cdot x_2 \quad (\text{Eq. 2})$$

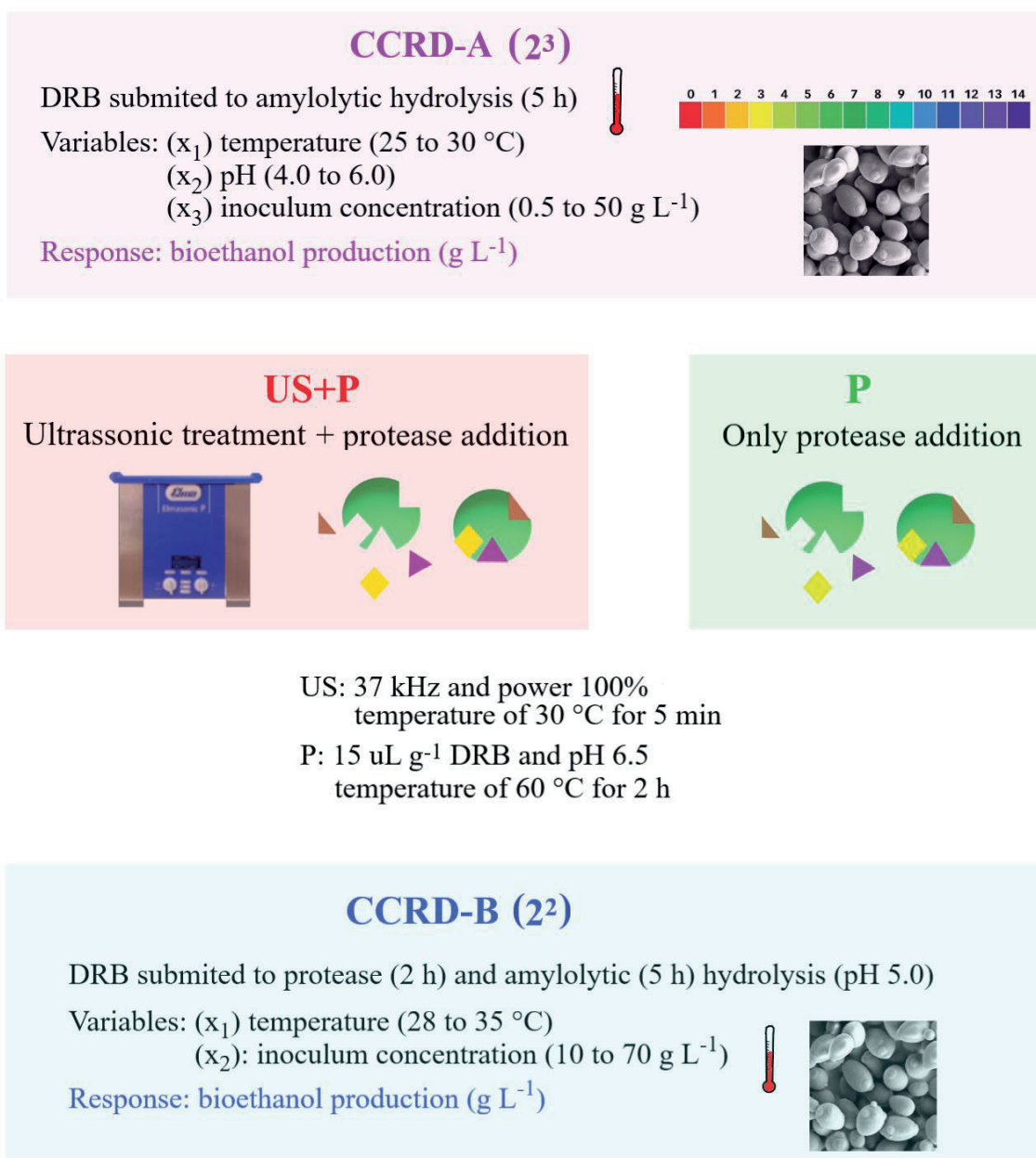


Figure 1. Flowchart sequence of tests carried out during the study of the hydrolyzed defatted rice bran alcoholic fermentation.

CCRD-A

First, a CCD (2³ plus star configuration, 3 central points, total of 17 runs) referred to as CCD-A, was applied. The effect of temperature (25 to 30 °C) (x₁), pH (4.0 to 6.0) (x₂), and inoculum concentration (5.0 to 50.0 g L⁻¹) (x₃) were studied in order to evaluate the response of bioethanol concentration (g L⁻¹) (Table 1). The variables used

in the process and their levels were defined by preliminary tests (data not shown). All runs were conducted in 500 mL Erlenmeyer flasks. After the enzymatic hydrolysis, DRB suspension temperature and pH were adjusted before the inoculum addition following the experimental design (Table 1). All runs were incubated in a shaker (SL 221, Solab) under a stationary state.

Application of ultrasonic treatment and protease addition

In order to evaluate the possibility of increasing bioethanol production by CCRD-A, ultrasonic treatment (Elmasonic P 120 H, Singen, Germany) and protease addition prior to amylolytic hydrolysis were performed in two sets of experiments. In the first (US+P), the DRB suspension was exposed to ultrasonic treatment (frequency 37 kHz, power 100%, temperature 30 °C for 5 min) following protease addition (15 µL g⁻¹ DRB, pH 6.5, temperature 60 °C

for 2 h). In the second (P), protease was added to the DRB suspension under the same conditions as in the first, without ultrasonic treatment. After the US+P and P treatment, sugar hydrolysis was performed as described previously, followed by alcoholic fermentation. The parameters used in alcoholic fermentation were defined by the results obtained in CCRD-A (temperature 30 °C, pH 5.0, and inoculum concentration 50 g L⁻¹), conducted in a shaker (SL 221, Solab) in a stationary state.

Table 1

Matrix of CCRD-A with coded and real values (in parenthesis) of the variables studied and responses of reducing sugar, biomass, and concentration of bioethanol - after 48 h of alcoholic fermentation process

Run	x ₁ ^a	x ₂ ^b	x ₃ ^c	Reducing sugar (0 h) (g L ⁻¹) ^d	Reducing sugar (48 h) (g L ⁻¹) ^d	Biomass (g L ⁻¹) ^d	Bioethanol (g L ⁻¹) ^d
1	-1 (26.0)	-1 (4.4)	-1 (14.0)	67.25±1.89	0.46±0.21	34.74 ± 2.09	16.10±0.24
2	+1 (29.0)	-1 (4.4)	-1 (14.0)	66.23±2.71	0.22±0.08	34.37 ± 4.08	18.10±0.18
3	-1 (26.0)	+1 (5.6)	-1 (14.0)	64.82±2.64	0.31±0.15	31.55 ± 4.33	17.20±0.35
4	+1 (29.0)	+1 (5.6)	-1 (14.0)	64.23±1.09	0.16±0.12	37.93 ± 0.57	15.40±0.31
5	-1 (26.0)	-1 (4.4)	+1 (41.0)	66.90±0.97	0.53±0.42	80.16 ± 6.87	16.60±0.06
6	+1 (29.0)	-1 (4.4)	+1 (41.0)	63.76±1.24	0.15±0.08	59.23 ± 4.30	19.40±0.32
7	-1 (26.0)	+1 (5.6)	+1 (41.0)	66.50±1.23	0.27±0.20	67.91 ± 10.27	19.50±0.14
8	+1 (29.0)	+1 (5.6)	+1 (41.0)	65.09±1.55	0.24±0.11	60.70 ± 3.68	20.40±0.14
9	-1,68 (25.0)	0 (5.0)	0 (28.0)	66.46±1.01	0.23±0.15	50.31 ± 2.87	18.25±0.07
10	+1,68 (30.0)	0 (5.0)	0 (28.0)	66.31±0.59	0.31±0.23	67.96 ± 0.64	19.10±0.09
11	0 (27.5)	-1,68 (4.0)	0 (28.0)	66.42±0.77	0.55±0.21	59.67 ± 1.05	14.10±0.11
12	0 (27.5)	+1,68 (6.0)	0 (28.0)	68.42±1.43	0.35±0.23	75.12 ± 5.23	16.82±0.14
13	0 (27.5)	0 (5.0)	-1,68 (5.0)	67.13±0.67	0.33±0.02	19.59 ± 0.96	15.30±0.08
14	0 (27.5)	0 (5.0)	+1,68 (50.0)	66.19±1.09	0.57±0.08	113.34 ± 9.11	17.47±0.21
15	0 (27.5)	0 (5.0)	0 (28.0)	66.86±1.52	0.28±0.16	62.37 ± 2.31	15.95±0.11
16	0 (27.5)	0 (5.0)	0 (28.0)	67.21±0.73	0.34±0.18	69.16 ± 0.94	16.43±0.14
17	0 (27.5)	0 (5.0)	0 (28.0)	66.07±0.76	0.29±0.03	62.28 ± 4.83	16.21±0.21

^a Temperature (°C); ^b pH; ^c Inoculum concentration (g L⁻¹); ^d Result expressed by mean ± standard deviation (n = 3).

CCRD-B

In order to improve bioethanol production, after the CCRD-A and the US+P and P treatment, a second CCRD, referred to as CCRD-B was

conducted. The DRB suspension with protease was applied (15 µL g⁻¹ DRB, temperature 60 °C and 2 h), without exposure to ultrasonic treatment. Sugar hydrolysis was performed as described previously, followed by alcoholic fermentation.

For the CCRD-B, the significant variable ranges applied in CCRD-A were adjusted. Thus, in the CCRD-B (2^2 plus star configuration, 3 central points, total of 11 runs) the effect of temperature (28 to 35 °C) (x_1) and inoculum concentration (10 to 70 g L⁻¹) (x_2) were studied in order to evaluate the response of bioethanol concentration (g L⁻¹)

(Table 2). All runs were conducted in 500 mL Erlenmeyer flasks. Before inoculum addition, the pH of the DRB suspension was adjusted to 5.0 (fixed variable), and the temperature was adjusted following the experimental design (Table 2). All runs were incubated in a shaker (SL 221, Solab) in a stationary state.

Table 2

Matrix of CCRD-B with coded and real values (in parenthesis) of the variables and responses of reducing sugar, biomass, and concentration of bioethanol observed and predicted - after 36 h of alcoholic fermentation process

Run	x_1^a	x_2^b	Reducing sugar (0 h) (g L ⁻¹) ^c	Reducing sugar (36 h) (g L ⁻¹) ^c	Biomass (g L ⁻¹) ^c	Observed bioethanol (g L ⁻¹) ^c	Predicted bioethanol (g L ⁻¹) ^d	Relative deviation (%) ^e
1	-1 (29.0)	-1 (18.5)	67.25±1.89	0.35±0.21	20.88±1.38	39.20±0.35	39.19	0.03
2	+1 (34.0)	-1 (18.5)	63.23±2.71	0.80±0.08	20.44±3.64	36.50±0.28	35.99	1.40
3	-1 (29.0)	+1 (61.5)	64.82±2.64	0.41±0.15	102.80±2.40	38.70±0.21	38.69	0.03
4	+1 (34.0)	+1 (61.5)	64.23±1.09	0.45±0.12	116.48±2.45	40.40±0.06	39.89	1.26
5	-1.41 (28.0)	0 (40.0)	66.90±0.42	0.36±0.41	97.93±0.21	37.90±0.12	37.83	0.19
6	+1.41 (35.0)	0 (40.0)	63.76±1.24	0.41±0.08	93.84±1.54	35.80±0.30	36.42	-1.73
7	0 (31.5)	-1.41 (10.0)	66.50±1.23	0.61±0.02	37.04±1.73	38.30±0.09	38.57	-0.70
8	0 (31.5)	+1.41 (70.0)	65.01±1.55	0.34±0.01	185.89±2.56	40.70±0.21	40.97	-0.65
9	0 (31.5)	0 (40.0)	66.46±0.99	0.46±0.23	97.53±2.72	39.38±0.08	39.29	0.23
10	0 (31.5)	0 (40.0)	66.31±0.59	0.54±0.21	101.09±1.33	39.07±0.04	39.19	-0.56
11	0 (31.5)	0 (40.0)	66.43±0.76	0.56±0.11	102.57±0.60	39.43±0.11	35.99	0.36

^a Temperature (°C); ^b Inoculum concentration (g L⁻¹); ^c Results expressed by mean ± standard deviation (n = 3); ^d Bioethanol values predicted by the model (g L⁻¹); ^e Relative deviation = ((Y - \hat{Y})/Y)*100; where Y= observed bioethanol and \hat{Y} = predicted bioethanol by the model.

Validation of alcoholic DRB fermentation

Validation of the optimized conditions for the step of alcoholic fermentation was carried out in a benchtop bioreactor (Bioreactor Tec-Bio 4,5 L, Tecnal, Piracicaba, Brazil), using 3.0 L of DRB suspension and fixing the temperature (31.5 °C), pH (5.0), and inoculum concentration (50 g L⁻¹).

Sampling interval

For CCRD-A, CCRD-B, US+P, and P experiments, samples were collected every 12 h to determine the bioethanol concentration and every

6 h to monitor the biomass and reducing sugar (RS) concentration. In the validation step of DRB alcoholic fermentation, all analyses (biomass, RS, and bioethanol concentration) were performed at intervals of 12 h. All analyses were conducted in triplicate.

Determination of reducing sugars and biomass (dry cell weight)

The concentration of RS was determined using the method of Somogyi (1945) and Nelson (1944), and the results were expressed in g L⁻¹.

The concentration of cellular biomass was determined as dry cell weight in g L^{-1} . The optical density (OD) of culture medium aliquots was read in a spectrophotometer (Lambda XLS, Perkin Elmer, Beaconsfield, United Kingdom) at 600 nm using calibration curves of OD *versus* dry mass in g L^{-1} .

Determination of bioethanol concentration

Bioethanol concentration was determined in an ultra-high-performance liquid chromatograph (UPLC) (Ultimate 3000, Thermo Scientific Germering, Germany), equipped with an automatic sample injector, quaternary pump, oven, and refraction index (RI) detector, controlled by Chromeleon 7.0 software. The samples were filtered in a $0.45 \mu\text{m}$ nylon membrane (Millipore, Massachusetts, USA) and injected ($20 \mu\text{L}$) into an UHPLC. A Rezex[®] ROA-Organic Acid H+ (8%) column (Phenomenex, Torrance, USA) and an oven temperature of $30 \text{ }^\circ\text{C}$ were employed. An isocratic elution with $5 \text{ Mm H}_2\text{SO}_4$ at a flow rate of 0.6 mL min^{-1} was applied (Stobienia et al., 2020).

Yield and kinetic parameters of alcoholic fermentation

The yield (Y) of the alcoholic fermentation of CCRD-B was calculated using Eq. 3.

$$Y (\%) = \frac{[E]_f - [E]_0}{([RS]_0 - [RS]_f) \times 0.511} \times 100 \quad (\text{Eq. 3})$$

Where: [E] and [RS] are the bioethanol and reducing sugar concentrations at a given time (f = final; 0 = initial). The value 0.511 g g^{-1} corresponds to the stoichiometric conversion of RS into bioethanol for *S. cerevisiae*.

The conversion factors of ethanol yield from sugar ($Y_{P/S}$) and into biomass yield ($Y_{X/S}$) and of ethanol yield from biomass ($Y_{P/X}$) were calculated using Eq. 4, 5, and 6.

$$Y_{P/S} = \frac{P_f - P}{S_0 - S_f} \quad (\text{Eq. 4})$$

$$Y_{X/S} = \frac{X_f - X}{S_0 - S_f} \quad (\text{Eq. 5})$$

$$Y_{P/X} = \frac{P_f - P_0}{X_f - X_0} \quad (\text{Eq. 6})$$

Where: P_0 and P_f are the initial and final bioethanol concentrations (g L^{-1}); X_0 and X_f are the initial and final biomass (g L^{-1}); and S_0 and S_f are the initial and final substrate (RS) concentrations (g L^{-1}).

Statistical treatment of the data

CCRD-A and CCRD-B runs were conducted randomly, and the data were analyzed by the Experimental Design procedure of Statistica 8.0 software (Statsoft Inc., Tulsa, USA). The adequacy of the second-order models was expressed by the determination coefficient (R^2) and adjusted R^2 ($R^2\text{-adj}$). Statistical significance was determined by ANOVA. A significance level of 5% was considered ($p < 0.05$). Moreover, the kinetic parameters were compared by Tukey's test ($p < 0.05$) using the abovementioned software.

Results and Discussion

Optimization of temperature, pH, and inoculum concentration for bioethanol production from DRB - CCRD-A

Alcoholic fermentation was carried out for 72 h; however, it was observed that in 48 h, 99% of the substrate was consumed. The initial (0 h) RS concentration (between 63.76 and 68.42 g L^{-1}) reached levels from 0.15 to 0.57 g L^{-1} after 48 h of alcoholic fermentation (Table 1), allowing a bioethanol concentration between 14.10 (run 11) and 20.40 g L^{-1} (run 8) (Table 1). The results for biomass concentration varied between 19.59 and 113.34 g L^{-1} . It became apparent that the final concentration of biomass varied according to the initial addition of inoculum. It is important to emphasize that the

initial concentration of *S. cerevisiae* was between 9.25×10^7 and 1.65×10^9 cells mL⁻¹ for inoculum concentrations of 5 and 50 g L⁻¹, respectively.

Table 3 shows the effects of the variables studied in CCRD-A. The quadratic term of temperature (x_1) and the linear term of inoculum concentration (x_3) had a significant positive

effect on bioethanol production by *S. cerevisiae* from DRB. The results indicate that an increase in these variable ranges resulted in a bioethanol concentration increase. In contrast, the pH had a non-significant effect on the response ($p > 0.05$) in the range studied.

Table 3
Effects of the variables studied on CCRD-A 2³ and CCRD-B 2² for the responses concentration of bioethanol (g L⁻¹) and analysis of variance (ANOVA) of the quadratic model for prediction of bioethanol (g L⁻¹) of the CCRD-A and CCRD-B

Parameter	Effect	Effects			Source of variation	Analysis of variance				
		Standard error	t(7)	p-value		Sum of square	Degrees of freedom	Mean square	p-value	F ratio ^a
CCRD-A						CCRD-A				
Mean	16.13	0.50	31.99	< 0.000*	Regression	42.93	8	5.37	0.004*	7.97
x_1 (L)	0.78	0.47	1.65	0.143	Residual	5.39	8	0.67		
x_1 (Q)	2.18	0.52	4.18	0.004*	Lack of fit	5.27	6	0.88	0.354	14.64
x_2 (L)	1.01	0.47	2.12	0.071	Pure error	0.12	2	0.06		
x_2 (Q)	-0.09	0.52	-0.18	0.861	Total	48.32	16			
x_3 (L)	1.87	0.47	3.94	0.006*				R ² = 89%		R ² -adj = 77%
x_3 (Q)	0.56	0.52	1.07	0.319				F _{0.05; 8, 8} = 3.44		F _{0.05; 6, 2} = 19.33
x_1 by x_2	-1.43	0.62	-2.30	0.055						
x_1 by x_3	0.878	0.62	1.41	0.200						
x_2 by x_3	1.38	0.62	2.22	0.062						
CCRD-B						CCRD-B				
Mean	39.29	0.27	143.41	< 0.000*	Regression	19.60	5	3.92	0.004*	17.41
x_1 (L)	-0.99	0.34	-2.96	0.032*	Residual	1.13	5	0.23		
x_1 (Q)	-2.19	0.40	-5.46	0.003*	Lack of fit	1.05	3	0.35	0.310	9.20
x_2 (L)	1.70	0.34	5.06	0.004*	Pure error	0.08	2	0.04		
x_2 (Q)	0.48	0.40	1.19	0.287	Total	20.73	10			
x_1 by x_2	2.20	0.47	4.64	0.006*				R ² = 95%		R ² -adj = 90%
								F _{0.05; 5, 5} = 5.05		F _{0.05; 3, 2} = 19.16

CCRD-A: x_1 = Temperature (°C); x_2 = pH; x_3 = Inoculum concentration (g L⁻¹); * $p < 0.05$.

CCRD-B: x_1 = Temperature (°C); x_2 = Inoculum concentration (g L⁻¹); * $p < 0.05$.

^a F ratio = model significance (F ratio = Mean Square regression/Mean Square residual or Mean Square Lack of fit/Mean Square Pure error); * $p < 0.05$.

A valid quadratic model (with $R^2 = 0.89$ and $R^2\text{-adj} = 0.77$) was obtained for bioethanol production (Table 3). Non-significant effects were incorporated into the residual of the model, except in cases in which the effect influenced the $R^2\text{-adj}$ negatively. The $F_{\text{calculated}}$ was significant for the regression ($p = 0.004$) and was not significant for the Lack of Fit ($p = 0.354$), indicating that the results are explained by the regression and not by the variations in the process. In addition, the percentage of the variation

explained by the model was adequate ($R^2 = 89\%$), considering the inherent variability of bioprocesses (Rodrigues & Iemma, 2014). Thus, the model was considered well-adjusted to the experimental data, and the response surface is presented in Figure 2a. It may be observed from the response surface and contour plot that the highest concentrations of bioethanol were obtained by employing the maximum temperature and inoculum concentrations studied.

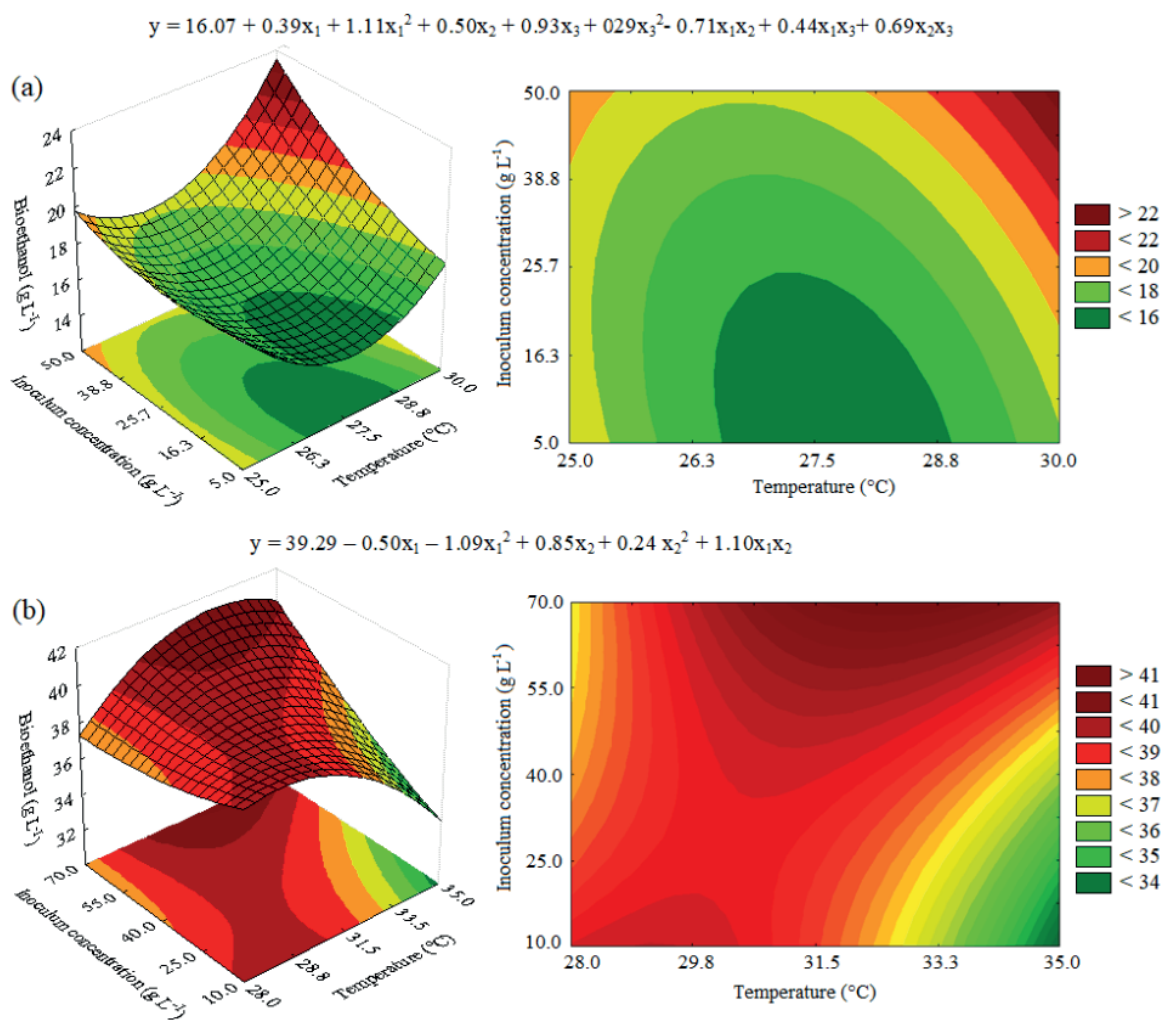


Figure 2. (a) Model, surface response and contour plot for bioethanol concentration (g L^{-1}) in function of temperature ($^{\circ}\text{C}$) and inoculum concentration (g L^{-1}) of CCRD-A - after 48 h of alcoholic fermentation; (b) Model, surface response and contour plot for bioethanol concentration (g L^{-1}) in function of temperature ($^{\circ}\text{C}$) and inoculum concentration (g L^{-1}) of CCRD-B - after 36 h of alcoholic fermentation.

Theoretically, 1.0 g of glucose should produce 0.511 g of bioethanol (Caldeirão, Tanaka, Ida, & Spinosa, 2016). However, in the CCRD-A 1.0 g of glucose resulted in a maximum of 0.29 g of bioethanol. It should be possible to obtain approximately 35 g L⁻¹ bioethanol using the DRB. However, during alcoholic fermentation, the presence of protein and lipids may reduce the performance of yeasts (Watanabe et al., 2009a) since they are unable to consume complex compounds. DRB presented significant concentrations of protein (18%) (Watanabe et al., 2019), which was probably responsible for the low yield of bioethanol observed. In order to reduce the concentration of proteins in the DRB suspension, ultrasound treatment and protease addition were evaluated.

Application of ultrasonic treatment and protease addition to increase bioethanol production from DRB

The results obtained for DRB with US+P and P addition are shown in Figure 3. The two experiments (US+P and P) presented biomass and RS kinetic similarities. For P a significant increase in the rate of conversion of substrate into bioethanol in the first 6 h was observed. After 24 h, the biomass demonstrates a higher growth rate, and the RS concentration approaches zero. These results indicate that the alcoholic fermentation process of DRB can be completed in 24 h. Significant differences between the US+P and P were not observed for these responses ($p > 0.05$ by Tukey's test). In both experiments, the substrate was consumed faster than observed in CCRD-A (24 h versus 48 h).

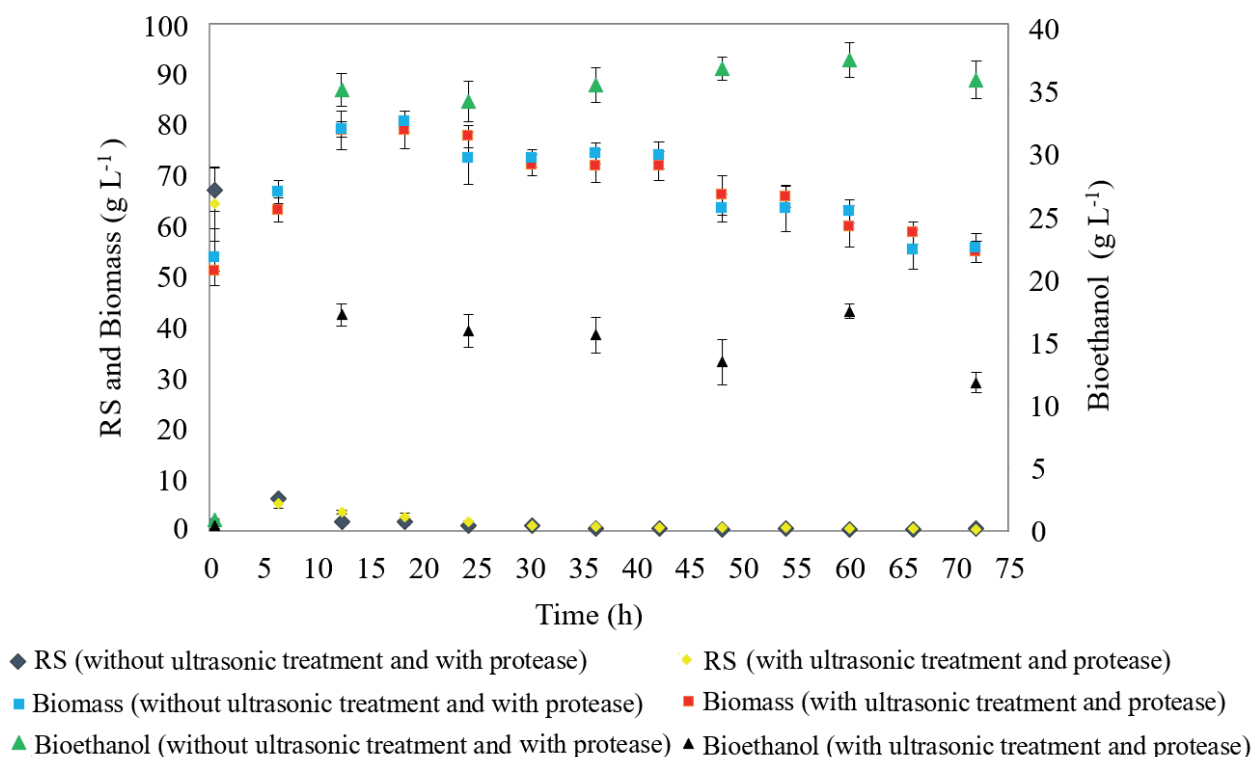


Figure 3. Kinetic profile of reducing sugar (RS), biomass and bioethanol production for experiments with ultrasonic treatment and protease addition on hydrolyzed defatted rice bran.

However, experiment P allowed high bioethanol production (36 g L^{-1}) from DRB related to US+P (11 g L^{-1}). Thus, comparing the maximum bioethanol concentration produced in P (36 g L^{-1}) with CCRD-A (20.4 g L^{-1} ; Table 1), an increase of 76% was observed. Protease addition positively affects bioethanol production, reaching a yield of 100%, similar to the 101% obtained by Singh & Singh (2007). Considering the CCRD-A variables with positive effects on increasing bioethanol concentration, the P step was included in order to improve bioethanol production. The variables temperature and inoculum concentration were adjusted, and a second CCRD was performed, named CCRD-B.

Protease addition and optimization of temperature and inoculum concentration on bioethanol production from DRB - CCRD-B

After 36 h of alcoholic fermentation, 99% of the substrate (0 h RS from 63.23 to 67.25 g L^{-1}) was consumed, resulting in final RS values between 0.34 and 0.80 g L^{-1} . At the same time, the highest concentrations of bioethanol were observed for all runs, indicating a reduction of 12 h in the fermentation time (48 h for CCRD-A and 36 h for CCRD-B). Therefore, the time of 36 h was chosen for statistical analysis of the bioethanol yield responses. The biomass values varied between 20.44 and 185.89 g L^{-1} , and the bioethanol concentration in CCRD-B runs varied from 35.80 to 40.70 g L^{-1} , and runs 4 and 8 had the highest bioethanol concentration (40.40 and 40.70 g L^{-1} , respectively) (Table 2). Comparing the higher bioethanol production of CCRD-B with those reported in the CCRD-A (20.4 g L^{-1}), an increase of 99% in bioethanol concentration was observed.

Table 3 shows the effects of the variables studied in CCRD-B. The linear and quadratic terms of temperature (x_1) had a significant negative effect on bioethanol production, probably due to the diminishing metabolic activity of the yeast. In contrast, the linear term of inoculum concentration

(x_2), and the interaction x_1 by x_2 had a significant positive effect on increasing the bioethanol concentration.

A valid quadratic model (with $R^2 = 0.95$ and $R^2\text{-adj} = 0.90$) was obtained for bioethanol production for CCRD-B (Table 3) considering all parameters. The $F_{\text{calculated}}$ for the regression was significant ($p = 0.004$), while the Lack of Fit was not significant ($p = 0.310$), indicating that the results are explained by the regression. The model was considered well-adjusted to the experimental data, and the response surface is presented in Figure 2b. It may be observed from the response surface and contour plot that the highest concentrations of bioethanol were obtained in the region of temperature between 31.5 and $33.5 \text{ }^\circ\text{C}$, and 70 g L^{-1} of inoculum, which coincides with the condition of run 8 ($31.5 \text{ }^\circ\text{C}$ and 70 g L^{-1} of inoculum).

CCRD-B yield and conversion factors

The yield and the conversion factors $Y_{P/S}$, $Y_{X/S}$, and $Y_{P/X}$ for CCRD-B runs are shown in Table 4 for CCRD-B. The yield varied between 93.26 and 113.57%; run 8 had the highest yield, and only two runs (5 and 6) had yields lower than 100%. Similarly, Das et al. (2013) reported a yield of 109% for alcoholic fermentation of hydrolyzed rice straw by an inoculum of *S. cerevisiae* and *Z. mobilis*. A yield higher than 100% was probably associated with endogenous fermentation by *S. cerevisiae*. According to Pepler (1970), yeasts used in food contain from 22% to 34% carbohydrates, and *S. cerevisiae* accumulates two storage carbohydrates, glycogen and trehalose (François & Parrou, 2001), reaching a range between 21.3% and 23.3% for the sum of mannose, trehalose, glucose, and glycogen (Plata, Koch, Wechselberger, Herwig, & Lendl, 2013).

The conversion factors $Y_{P/S}$, $Y_{X/S}$, and $Y_{P/X}$ varied between 0.48 and 0.58 g g^{-1} , 0.06 and 1.60 g g^{-1} , and 0.32 and 10.02 g g^{-1} , respectively (Table 4). For the $Y_{P/S}$ factor, runs 1 and 4 demonstrated the highest

results (0.48 and 0.58 g g⁻¹); these $Y_{p/S}$ values are greater than those cited in the literature (between 0.31 and 0.50 g g⁻¹) for the production of bioethanol from sub-products, including sugarcane bagasse, soy protein concentrate, orange peel, and corn meal (Caldeirão et al., 2016; Gutiérrez-Rivera et al., 2015; Mojović, Nikolić, Rakin, & Vukasinović, 2006; Oberoi, Vadlani, Madl, Saida, & Abeykoon, 2010).

The higher $Y_{p/S}$ in the present article should be a consequence of hydrolysis of proteins by proteases. The hydrolysis step released amino acids in the medium and reduce the yeast synthesis requirement of amino acids, resulting in a higher yield of biomass per mole of glucose consumed (Kłosowski, Mikulski, Czupryński, & Kotarska, 2010).

Table 4
Yield and conversion factors of the alcoholic fermentation of hydrolyzed defatted rice bran considering CCRD-B

Runs	Yield (%)	$Y_{p/S}$ (g g ⁻¹) ^a	$Y_{X/S}$ (g g ⁻¹) ^b	$Y_{p/X}$ (g g ⁻¹) ^c
1	110.35	0.58	0.06	10.02
2	103.03	0.53	0.11	4.50
3	103.47	0.53	0.59	0.83
4	112.02	0.57	0.78	0.67
5	95.36	0.49	0.84	0.53
6	93.26	0.48	0.60	0.78
7	106.52	0.54	0.35	1.54
8	113.57	0.51	1.60	0.32
9	106.53	0.54	0.66	0.84
10	105.71	0.54	0.63	0.86
11	108.82	0.56	0.70	0.80

^a Yield = (experimental bioethanol concentration/maximum theoretical (stoichiometric) bioethanol concentration)*100; ^b Yield factor of ethanol yield from sugar ($Y_{p/S}$); ^c Yield factor of substrate into biomass yield ($Y_{X/S}$); ^d Yield factor of ethanol yield from biomass ($Y_{p/X}$).

CCRD-B run 8 had the greatest bioethanol response (40.70 g L⁻¹) and presented the greatest transformation of substrate into biomass ($Y_{X/S}$), thereby increasing the yeast population, resulting in an increase in transformation of substrate to bioethanol. Regarding $Y_{p/X}$, run 1 presented the highest conversion of biomass to product justifying the concentration of bioethanol near 40 g L⁻¹ despite having the lower inoculum concentration (18 g L⁻¹) and temperature (29 °C).

Optimized bioethanol production by alcoholic fermentation

According to the response surface analysis (Figure 2b), the ideal point (critical values) for the variables temperature and inoculum concentration, which allowed the maximum bioethanol concentration to be calculated by the quadratic model (multiple regression), were 30.2 °C and 27.5 g L⁻¹ inoculum. At this point, the predicted bioethanol response would be 39.2 g L⁻¹ (for 36 h of fermentation). By the analysis of the results expressed in Tables 2 and

4, the run 8 conditions (CCRD-B) were selected to be tested on a major scale (benchtop bioreactor) considering that under these conditions, the highest bioethanol concentration (40.70 g L^{-1}), $Y_{X/S}$, and intermediate $Y_{P/S}$ were observed. However, in run 8 the high inoculum concentration (70 g L^{-1}) resulted in a significant increase in medium viscosity, which implies deficient homogenization, a disadvantage for industrial applications. In this way, the validation step was conducted with a 50 g L^{-1} inoculum concentration.

A similar bioethanol concentration (40.1 and 41.48 g L^{-1}) was reported for alcoholic fermentation of a hydrolyzed rice straw and corn stove (Das et al., 2013; Zhu et al., 2015). Das et al. (2013) performed alkaline and acid hydrolysis of rice straw with subsequent washing and enzymatic hydrolysis by β -glucosidase for 48 h. In addition, an associative culture of *S. cerevisiae* and *Z. mobilis* (rate 1.53) was used as the inoculum. Thus, it is apparent that the hydrolysis process carried out by these authors was slower (48 h *versus* 7 h in the present article, considering the action time of the enzymes) and more expensive to achieve the same bioethanol concentration.

In contrast, other articles reported the production of bioethanol from by-products in lower concentrations than those obtained in this article. The highest concentration of bioethanol produced from rice bran fermented by *Zymomonas mobilis* was $13.4 \pm 2.4 \text{ g L}^{-1}$ (72.47% for theoretical bioethanol yield) (Todhanakasem, Sangsutthiseree, Areerat, Young, & Thanonkeo, 2014). In the fermentation

of rice hull hydrolysates, Dagnino, Chamorro, Romano, Felissia, and Area (2013) obtained a conversion of 84% and 4.4 g L^{-1} of bioethanol. Liu et al. (2015) realized alkali-pretreated in the sugarcane bagasse supplemented with wheat bran, followed by enzymatic hydrolysis and alcoholic fermentation to obtain 5.8 g L^{-1} bioethanol (40.84% of theoretical yield).

The bioethanol production and the conversion yield of DRB by alcoholic fermentation using *S. cerevisiae* in the current work was higher than reported in the literature. Furthermore, only industrial enzymes (currently used in rice hydrolysis) without chemical treatments were applied using a short processing time, indicating the innovation and relevance of the present article in bioethanol production from DRB.

Validation of alcoholic fermentation

The greatest conversion of substrate into product occurred during the first 36 h of fermentation, reaching 98% consumption of the original substrate (71.28 g L^{-1}) (Table 5). In the same time, the maximum concentration of biomass was obtained (106.38 g L^{-1}), indicating the end of the *log* phase. After 36 h, the concentration of biomass decreased; however, the bioethanol concentration increased up to the end of the process (72 h) reaching 40.0 g L^{-1} and 109.9% yield. During yeast autolysis, carbon and nitrogen are released into the medium and can be used as substrates by live yeasts.

Table 5
Results for model validation of alcoholic fermentation of hydrolyzed defatted rice bran in a benchtop bioreactor

Time (h)	Bioethanol (g L ⁻¹) ^a	Reducing sugar (g L ⁻¹) ^a	Dry Mass (g L ⁻¹) ^a
0	9.1±0.02	71.28±4.47	51.52±1.09
12	27.6±0.04	3.45±0.33	101.14±6.11
24	27.4±0.08	1.31±2.02	102.78±1.26
36	28.1±0.02	1.17±1.86	106.38±0.03
48	36.1±0.01	0.94±0.58	91.98±0.09
60	36.7±0.02	0.50±0.46	77.26±0.18
72	40.0±0.01	0.43±0.71	74.57±0.78

^a Result expressed by mean ± standard error (n = 3).

The greater concentration of bioethanol obtained during alcoholic fermentation in CCRD-B (40.7 g L⁻¹ of run 8, at 36 h fermentation, conducted in 500 mL Erlenmeyer flasks) was similar ($p > 0.05$ by Tukey's test) to the maximum values obtained in the validation experiment carried out in the benchtop bioreactor (40.0 g L⁻¹, at 72 h of fermentation). However, the differences in total ethanol productivity observed must be considered, which probably occurred due to the scale increase and consequent homogenization trouble, since the fermentation was carried out without agitation and aeration. Moreover, the decrease in the inoculum concentration, from 70 to 50 g L⁻¹ should also have induced a decrease in bioethanol. As previously mentioned, the inoculum concentration of 70 g L⁻¹ caused a marked increase in culture medium viscosity. A more detailed study of scaling up could help increase the bioethanol productivity of the system.

Conclusion

The hydrolysis of DBR proteins by treatment with protease before amylolytic hydrolysis and alcoholic fermentation by *S. cerevisiae* allowed an increase in final bioethanol production. The temperature and inoculum concentration generate effects ($p > 0.05$) on bioethanol production, while the pH, in the range studied, had no influence on the conversion of substrate into product. The temperature of 31.5

°C and an inoculum concentration of 70 g L⁻¹ was the optimized condition for 40.70 g L⁻¹ bioethanol production by alcoholic fermentation. Validation in a benchtop bioreactor produced 40.0 g L⁻¹ of bioethanol from DRB previously hydrolyzed with protease and amylolytic enzymes. The results obtained suggest that the use of a strategy of sequential design and protease addition contributes to the improvement of bioethanol production from DRB. Additionally, the bioethanol concentration and yield obtained in this study demonstrate that the DRB alcoholic fermentation process for bioethanol production is industrially viable. Furthermore, the DRB proved to be a by-product with great potential for bioethanol production, derived from alternative sources not commonly used in human food.

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