Single cell protein production by *Candida robusta* isolated from sugar cane (*Saccharum sp.*) for animal feed

Produção de Biomassa por *Candida robusta* isolada da Cana-de-açúcar (Saccharum sp.) para alimentação animal

DOI:10.34117/bjdv8n8-081

Recebimento dos originais: 21/06/2022
Aceitação para publicação: 29/07/2022

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ABSTRACT
The protein obtained from the microorganisms is not only cheap but can be used as additive to provide a balanced nutrition for many animals feeding. The aim of this work was the single cell protein (SCP) production by *Candida robusta* URM5293 using sugarcane bagasse as substrate. The yeast *C. robusta* URM5293 was isolated from root sugarcane and was identified based on morphological and biochemical characteristics. Biomass production was done into Erlenmayers flasks (250 mL) containing culture medium supplemented of sugarcane bagasse hydrolyzate. Fermentations were carried varying culture conditions through the study of four different variables: temperature (25, 30, and 35°C), agitation intensity (110, 140 or 170 rpm), pH (6.0, 7.0, and 8.0) and production time (72, 96, and 120h), according to a 2^{4-1} fractional factorial design. Results demonstrated that this yeast was able to ensure the highest level of biomass (141 g/L) when cultivated at 25°C, pH 6.0, 170 rpm of agitation intensity after 72h of cultivation using sugarcane bagasse. The present results demonstrate the potential of sugarcane bagasse hemicellulosic hydrolyzate as a substrate for the production of microbial protein by *C. robusta* URM5293.

Keywords: single cell protein, *Candida robusta*, sugarcane bagasse, fractional factorial design.

RESUMO
A proteína de origem microbiana tem alto valor agregado e pode ser utilizada como aditivo para fornecer uma nutrição balanceada para diversas espécies animais. O objetivo deste trabalho foi a produção de biomassa por Candida robusta URM5293 utilizando bagaço de cana-de-açúcar como substrato. A levedura C. robusta URM 5293 foi isolada da raiz da cana-de-açúcar e identificada com base nas características morfológicas e bioquímicas. A produção da biomassa foi realizada em frascos Erlenmayers (250 mL) contendo meio de cultura suplementado com hidrolisado de bagaço de cana-de-açúcar. As fermentações foram realizadas variando as condições de cultivo através do estudo de quatro variáveis diferentes: temperatura (25, 30 e 35°C), intensidade de agitação (110, 140 ou 170 rpm), pH (6,0, 7,0 e 8,0) e tempo de produção (72, 96 e 120h), de acordo com um planejamento fatorial fracionário 2^{4-1}. Os resultados demonstraram que esta levedura foi capaz de garantir o maior nível de biomassa (141 g/L) quando cultivada a 25°C, pH 6,0, 170 rpm de intensidade de agitação após 72h de cultivo utilizando bagaço de cana. Os presentes resultados demonstram o potencial do hidrolisado hemicelulósico do bagaço de cana-de-açúcar como substrato para a produção de proteína microbiana por *C. robusta* URM5293.

Palavras-chave: proteína unicelular, *Candida robusta*, bagaço da cana-de-açúcar, planejamento fatorial fracionário.

1 INTRODUCTION

Since the early 1950s intense efforts have been made to explore new alternate protein sources as food supplements, primarily in anticipation of a repeatedly predicted insufficient future protein supply. For these, i.e. yeasts, fungi, bacteria and microalgae, the name single cell protein (SCP) was coined to describe the protein production from biomass, originating by different microbial sources (Becker, 2007). The production of SCP from various microorganisms, particularly from fungi and bacteria has received considerable attention (Mahasneh, 1997).

Recently, more and more yeast species are isolated from nature and proven superior to conventional protein producer in laboratory or field studies (Arnold et al., 2000; Nigam, 2000; Urano et al., 2002; Yang et al., 2003), which offers more choices for the microbial protein process. For example, a yeast species, *Candida langeronii*, is isolated under selective conditions as an alternative microorganism of *C. utilis* because of the inability of the latter to utilize L-arabinose at temperatures above 42ºC in the absence of vitamins (Nigam, 2000). In comparison with a strain of *C. utilis*, the isolate, *Galactomyces geotrichum* T2B, gives consistently higher biomass yields from silage effluent along with excellent nutrient removal (Arnold et al., 2000). The co-culture of two yeast isolates, *C. halophila* and *Rhotorula glutinis*, could effectively remove organic

pollutants, above 85%, from fermentative wastewater even when ammonium–nitrogen concentration reaches as high as about 19g/L (Yang et al., 2003).

The protein obtained from the microorganisms is not only cheap but also may provide balanced nutrition. It is also a potential supplemental protein source for feeding poultry, livestock and humans (Singh et al., 1991; Pacheco et al., 1997). *C. utilis* has been frequently used in biomass production because of its ability to utilize a variety of carbon sources and to support high protein yield. It has been used for production of several industrial products both for human and animal consumption (Zayed and Mostafa, 1992; Kondo et al., 1997; Pacheco et al., 1997; Otero et al., 1998). Several agro-industrial wastes have been used to produce SCP for rumen and poultry feed (Haddadin et al., 1999; Paul et al., 2002).

The food value and usefulness of SCP from any source is based on its composition. Nutrients, vitamins, nitrogen, carbohydrates, fats, cell wall components, nucleic acids, protein concentration and amino acid profile, should be analyzed before the product is used for food or as feed supplementation. Yeast contains thiamine, riboflavin, biotin, niacin, pantothenic acid, pyridoxine, choline, streptogenin, glutathione, folic acid and *p*-amino benzoic acid (Frazier and Westhoff, 1988; Ravindra, 2000).

A variety of substrates have been utilized to cultivate bacteria, fungi and algae. Technically, SCP is the manufacture of cell mass using microorganisms by culturing on abundantly available agricultural and industrial wastes. The production of microbial biomass is done either by a submerged or solid state fermentation process. After fermentation, biomass is harvested and may be subjected to downstream processing steps like washing, cell disruption, protein extraction and purification (Faust, 1987).

Lignocellulosic wastes from different sources have varying composition of hemicellulose, cellulose and lignin. Some sources of lignocellulosic material are wood from angiosperms and gymnosperms, grasses, leaves, wastes from paper manufacture, sugarcane bagasse, wheat straw, wheat bran, rice bran, groundnut shell and other agricultural wastes (Tanaka and Matsuno, 1985; Gupte and Madamwar, 1997). Based on the dominant component in the waste used, specific fungi can be utilized for biomass production. The biomass thus produced can be harvested and used as SCP.

One of the largest ligno-cellulosic agro-industrial by-products is sugarcane bagasse (or bagasse as it is generally called) a fibrous residue of cane stalks left over after the crushing and extraction of the juice from the sugar cane (Pandey et al., 2000).

One of the significant applications of bagasse has been for the production of protein-enriched cattle feed (SCP) and enzymes. The new awareness of the importance of utilizing renewable resources such as bagasse for value addition has led to the development of several processes for the production of protein-enriched cattle feed (Pandey et al., 2000). Brazil is the greatest sugar cane producer in the world. However, technologies concerning the utilization of the by-product's utilization of sugar cane processing still needs to be developed and optimized through experimental factorial design (Roberto et. al., 1995).

The fermentation process is significantly influenced by various physical as well as chemical parameters. The first step in process optimization is screening of the important factors, followed by estimation of optimal levels of these factors. The conventional approach is to investigate one factor at a time, while keeping the others constant. This approach is tedious, time consuming and does not take into consideration the interactions among the factors (Stowe and Mayer, 1966). A statistical approach provides an efficient alternative, which is economical and allows the study of interactions among the factors, and at the same time predicts the optimum values of the variables.

The influence of pH, temperature, agitation intensity and time on the SCP production was studied using a 2^{4-1} fractional two-level factorial design by *Candida robusta* URM5293 isolated from root sugar cane using sugar cane bagasse as substrate, selecting the biomass concentration as the response.

2 MATERIALS AND METHODS

2.1 MICROORGANISM

The yeast *C. robusta* URM5293 used in this work was isolated from sugarcane (*Saccharum* sp.), was supplied by the Pernambucana Company of Farming Reasearch (IPA), located in Recife, Pernambuco, Brazil. The sugarcane root was removed from the stem and washed with water. After this, the root was washed with NaClO 15% (v/v). Soon after, the samples were crushed. The liquid obtained of maceration was diluted with 5 mL of sterilized water and inoculated using Drigalsky spatial (1 mL) in Petri dish containing Sabourand agar medium.

2.2 YEAST IDENTIFICATION

The identification was made through observation of macroscopic and microscopic characteristics, reproduction type and physiological characterization according to the literature Barnett et al., (2000); Hoog et al., (2000) and Domsch et al., (1993).

2.3 SUGARCANE BAGASSE HYDROLYSIS

Alkaline hydrolysis was performed at 121ºC for 30 minutes after mixing an over-dried chopped bagasse with NaOH 4%. After heating, the solid residue was separated by filtration. The hydrolyzate bagasse was washed with deionized water to withdraw the NaOH and remained in heater for 24 hours at 80ºC to withdraw the excess of water (Aguiar and Menezes, 2000).

2.4 SINGLE CELL PRODUCTION IN SUGARCANE BAGASSE

Fermentations were carried out in Erlenmeyer flasks (250 mL) containing the production medium with the following composition: sugarcane bagasse hydrolyzate, 1.5 g/L; added 15 mL of synthetic medium (KH_2PO_4 2.0 g/L; peptone (2,0 g/L); yeast extract (2,0 g/L), $(NH_4)_2HPO_4$ (2,0 g/L), $MgSO_4.7H_2O$ (1,0 g/L). After inoculating cells up to a concentration of 10^6 cells/ml in Erlenmeyers containing the production medium, fermentations were performed in an orbital shaker at different agitation intensity, pH, temperature, and time production according to the 2^{4-1} fractional factorial design (Table 1). Samples were collected every 12 h and assayed for biomass concentration.

Table 1. Levels of variables used in the 2^{4-1} fractional factorial design.

Variables	Level		
	Lower (-1)	Central (0)	Higher (+1)
(x_1) Agitation intensity (rpm)	110	140	170
(x_2) Temperature (°C)	25	30	35
(x_3) pH	6.0	7.0	8.0
x_4- Production Time (h)	72	96	120

2.5 BIOMASS DETERMINATION

The absorbance was measured in 660 nm by a spectrophotometer. The broth medium was collected and separated by filtration, being placed in the heater at 80°C by 12 hours overnight and the day after were weighted. The biomass density in the erlenmayers flasks was determined by using a calibration curve to correlate optical

density (O.D.) with dry weight (g/L). Maximum specific growth rate (μ_{max}) was determined from the growth curve in the exponential phase obtained by next equation:

$$\mu Max = \frac{1nX - 1nX_0}{t - t_0} \quad (1)$$

according to Pirt (1975). The "X" represents the final biomass, "X_0" represents the initial biomass, "t" final time and "t_0" initial time.

2.6 EXPERIMENTAL DESIGN

Batch fermentations were carried out according to a 2^{4-1} fractional factorial design, which is a one-third fraction of the full two-level factorial design. It was used to evaluate the relative influence of several variables on the biomass production. The experimental design was composed of 8 runs and 4 repetitions in the central point, needed to calculate the pure error (Table 1). We selected four variables (agitation intensity, temperature, pH, and production time) and biomass concentration as the response variable after 120 h of microorganism growth, i.e. the time at which the highest biomass concentration was obtained. The following linear regression model was employed to predict the response:

$$Y = b_0 + \sum b_i x_i + \sum b_{ij} x_i x_j \quad (2)$$

where Y is the variable response, b_0 is the interception coefficient, b_i are the linear coefficients, b_{ij} are the interaction coefficients and X_i and X_j are the coded values of the independent variables.

The goodness of fit of the model was evaluated by the determination coefficient (R^2) and the analysis of variance (ANOVA) at a significance level of $p \leq 0.05$. The first-order model equation was determined by the Fischer's test. All statistical and graphical analyses were carried out using the "Statistic 6.0" software (StatSoft, Tulsa, OK).

2.7 REAGENTS

All chemicals used in this work were of analytical grade and were obtained from Merck (Darmstadt, Germany) and Sigma (St. Louis, MO).

3 RESULTS

In our research isolated, from on petiole the leaf only bacteria; only yeast in the stem; and of root were isolated bacteria, fungi and yeasts. The microorganisms of interest for the study were the yeasts isolated from stem and root. The yeasts two endophytes were isolated and purified in culture medium ágar Sabourand and used for biomass production stage. Both yeasts were identified as *Candida robusta* URM5293, to be the asexual state of *Saccharomyces ceresiviae*, in which there is no production of ascospores. The species were incorporated to the Mycology Department's Micoteca URM, at Federal University of Pernambuco, Recife-PE.

3.1 INFLUENCE OF THE INDEPENDENT VARIABLES SIGNIFICANTLY AFFECTING BIOMASS PRODUCTION

Four variables were evaluated: agitation intensity, temperature, pH, and production time supposed to affect the biomass production were selected for the 2^{4-1} fractional factorial design. Table 2 shows the four independent variables and their values at the different levels of the fractional factorial design experiments and the corresponding response. The corresponding first-order model equation fitted to the data obtained from the factorial design experiment has the formula:

$$Y = 120.2 + 1.6\, x_1 + 4.1\, x_2 - 1.4\, x_3 + 8.1\, x_4 - 6.6\, x_1x_2 - 1.1\, x_1x_3 + 9.4\, x_1x_4$$

where x_1, x_2, x_3, and x_4 are the independent variables: agitation intensity, temperature, pH, and production time, respectively; x_1x_2, x_1x_3, and x_1x_4 are the interaction of the independent variables.

Table 2. Level combinations of the four independent variables used in the 2^{4-1} fractional factorial design and related values of response.[a]

Run	Agitation Intensity (rpm)	Temperature (°C)	pH	Production Time (h)	[a]Biomass Production (g/L)
5	110	25	8	120	103
4	170	35	6	72	101
3	110	35	6	120	125
1	110	25	6	72	106
12 (C)	140	30	7	96	123
10 (C)	140	30	7	96	130
2	**170**	**25**	**6**	**120**	**141**
8	170	35	8	120	131
9 (C)	140	30	7	96	127
7	110	35	8	72	127

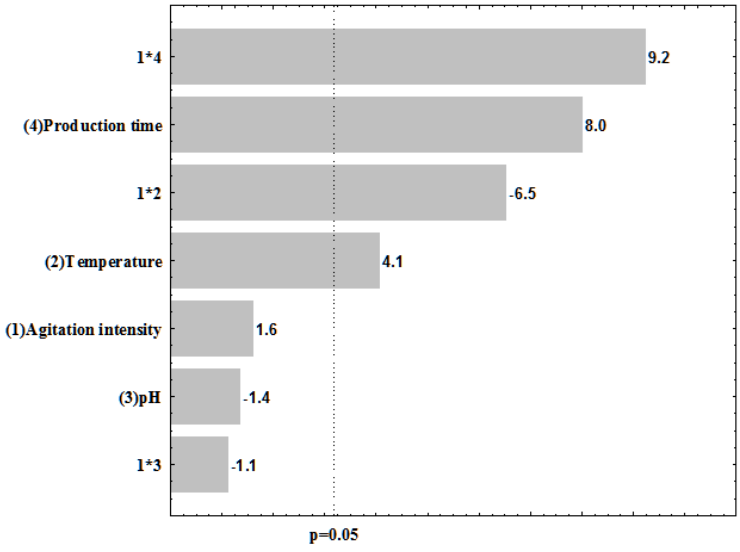
6	170	25	8	72	101
11 (C)	140	30	7	96	127

Some significant findings of the statistical analyses are evident in Figure 1, where the Pareto chart shows the estimated effects of the independent variables (agitation intensity, temperature, pH, and, production time) and of their interactions on the biomass production, according to a decreasing order of magnitude. Whereas the length of each bar is proportional to the standardized effect, bars extending beyond the vertical line correspond to the effects statistically significant at a confidence level of 95%. It should be noted that only the temperature and production time main effects (2 and 4) and its interaction with agitation intensity (1*2 and 1*4) were significant. From Eq. (2), we could also see that production time (x_4) was the most significant variable due to its coefficient effect being heavily pronounced, followed by the interactions.

The agitation intensity and pH main coefficient and its interaction were not statistically significant ($p > 0.05$). Although, these terms were kept in the model to minimize the error determination.

In particular, the negative sign of the agitation intensity and temperature interation (1*2) means that biomass production was favored by a decrease in this variable that achieved maximum values (141 g/L) at 170 rpm and 25°C. Moreover, the positive effect also of the interaction among production time and agitation intensity means that the simultaneous increase in the levels of both independent variables led to an improvement in biomass production.

Figure 1. Pareto chart for the effects of variables time production (4), pH (3), temperature (2) and agitation intensity (1) on SCP production.



3.2 CULTURE UNDER THE BEST CONDITIONS

The best result was obtained in run 2 (Table 2) performed at 170 rpm, 25°C, pH 6.0. The biomass production was shown to be dependent on the culture time. In fact, the maximum biomass concentration (141 g/L) was obtained after 120 h of growth. Resuming, according to this behavior, too long fermentation times should be avoided in an industrial process to get satisfactory performance.

4 DISCUSSION

As a rule, production time is very important effect on the activity of some enzyme and some enzymes included in nonribosomal peptide synthetases (NRPSs) were Mg2+-depentent (Koop and Marahiel, 2007). So Mg2+ in present study was speculated to play an important role in the process of antifungal active substances synthesis, and specific biochemical mechanism needs further research.

When there is a situation of scarce information, firstly the attitude should be carry out a selection and to rule out the factors what it is not important, in order to avoid the loss of time and to reduce the costs. The use of fractional factorial design is a possibility to get this purpose. The fractional designs are extremely economical because it can be used to analyse about ten factors at once time (Barros Neto et al., 2002). The biggest advantage of statistical analysis comes from the study of interactions between the multiple experimental variables (Swalley et al., 2006). The current work used fractional factorial design as scientific tool for the study of the available factors in the process with speed and reduction of costs, basic characteristics in the biotechnological development.

Pessoa Jr. et al., (1996) studied the cultivation of *C. tropicalis* IZ 1824 in hydrolyzate sugar cane bagasse for production of microbian protein (SCP) and it was found values of 9.6 g/L for the SCP production for 48h with orbital agitation (200 rpm) at 30ºC. *C. robusta* produced a quantity bigger of SCP (141 g/L) after a time of 120h at 25ºC.

Chanda and Chakrabarti (1996) analysed the SCP production by *Saccharomyces cerevisiae* isolated from the extract that was obtained through maceration of four plants: *Brassica campestris* L., *B. oleracea*, *B. nigra* e *Raphanus sativus*, obtaining the next values respectively (g/L): 9.4; 6.1; 8.9; 10. Regarding biomass production, the present work demonstrated that *C. robusta* produced approximately twelve times more of SCP.

El-Nawwi and El-Kader (1996) analyzed the protein production by *Aspergillus terreus* grown on alkali-treated bagasse under various culture conditions. Optimum

biomass protein content was in the range of 21-28% (w/w) and protein recovery was in the range of 11-14.5 g 100 g^{-1} bagasse with an alkali-treated bagasse substrate concentration of 1.5% (w/v), pH 4.5, 35ºC, 1:5 (v/v) culture broth and 4% (v/v) inoculum and continuous shaking for seven days.

Rosa-Magri et al., (2012) evaluates the role of the yeast *Torulaspora globosa*, isolated from the sugar cane rhizosphere, in the solubilization of potassium from alkaline ultramafic rock powder and results showed that as much as 38% of the total potassium in the rock was released in the medium with the yeast during a 15-day period of incubation.

Johnson (2012) in your mini-review cite non-*Saccharomyces* ascomycetous yeasts, including species of *Candida*, of importance in biotechnology with ability of oleaginous yeasts to accumulate high quantities of lipids offering the commercial potential for production of lipids or "single-cell oils" and production of SCP from a variety of substrates including hydrocarbons, as in our search for production by single cell protein utilized sugarcane bagasse.

5 CONCLUSION

The present results demonstrate the potential of sugar cane bagasse hemicellulosic hydrolyzate as a substrate for the production of microbial protein by *C. robusta*. The application of factorial experimental designs for selection of culture condition for SCP production by *C. robusta* enables the rapid identification of key factors such as pH, agitation intensity, temperature, production time and interactions between them, which together are necessary for a production optimization of biomass by this microorganism. The present study on SCP production from *C. robusta* clearly indicates the potential of this organism in the field of industrial SCP production.

ACKNOWLEDGEMENTS

The authors acknowledges the financial supported provide by CAPES (Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brasilia, Brazil) and CNPq (Coordenacão de Aperfeiçoamento de Pessoal de Nível Superior, Brasilia, Brazil). Sincere thanks are due to LIKA (Laboratory of Immunopathology Keizo Asami, Federal University of Pernambuco, Recife – PE, Brazil).

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