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Production of unicelular protein and reduction of contaminating amount of vinasse

Abstract

The vinasse from ethanol distillery, which is a highly contaminating industrial waste, is produced in large quantities. Through biotechnology, it can be used for the production of protein while reducing contamination.

The *Candida utilis* strain was isolated and adapted under different concentrations of aqueous formulas of vinasse, from 10 to 50%. The experimental trials were carried out in a lab using a "batch" reactor with vinasse. The growth of the microorganism was monitored using a carbon dioxide sensor (CO_2). The parameters measured were: total nitrogen, COD (chemical demand of oxygen), pH and conductivity, at the beginning and end of each trial. Every two hours, the DO (optics density in a liquid environment) was measured with the objective of knowing its cellular concentration. For each physic- chemical variable analyzed, an ANOVA was realized to evaluate the factors of repetition, treatment and reactor, and it was chosen a significance level of 1%.

The parameters measured reported: in total nitrogen there was an enrichment of 136% in the environment composed of 50% of vinasse in aqueous formulas; the average removal of the chemical demand of oxygen was 43%; the variation of pH, which was compared at the end of the trials, was 4% less than at the beginning; and the conductivity lessened to 9%. The monitoring of yeast growth by measuring the carbon dioxide concentrations throughout the time and the environment's OD, allowed for the creation of a growth curve of the microorganism, with a fermentation period of 21 hours.

It was proved that the *Candida utilis* strain can develop in a batch reactor with vinasse, in aqueous solutions of 50%, and producing a proteic enrichment of it as well as the removal of COD. The proposed process reduces the contamination of the main industrial effluent of Tucumán.

Keywords: protein, vinasse; reducing contamination

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Introduction

Worldwide perspectives include increasing the production of alcohol, in order to be used as a fuel for vehicles. In the year 2016, Argentina increased the percentage of bioetanol in fuels to 12%. As a consequence, there was an increase in the annual volume production which reached 283 thousand m^3 of alcohol. Such production generates a liquid waste called vinasse, it is highly contaminating and it is produced in large quantities, between 12 and 16 liters for each liter of alcohol. In this context, there are 15 sugar mills and 10 alcohol distilleries in Tucuman with the subsequent environmental impact produced by their effluents. In order to contribute to the reduction of the environmental impact in the production of ethanol, it is possible to resort to bioremediation. Studies carried out by Christen et al.,[1–3] have shown the capacity of the yeast *Candida utilis* to grow in different solid and liquid environments. In these studies, it is considered that the use of *Candida utilis* for proteic growth in effluents is important due to its highly nutritional value (around 50% of the yeast is protein).

Also in his studies, Diaz, Semprúm and Gualtieri[4] have demonstrated that this yeast may grow in liquid environments composed of either vinasse, or vinasse and molasses. This was indicated in Serguera Niño et al.,[5] and Domenech López[6] who report the growth of this yeast in enriched vinasse with harmonium sulfate and dibasic harmonium sulfate in different concentrations. In these cases, it is worth noticing not only the obtention of unicellular protein, but also the diminution of the contaminating load of the vinasse. Taking into account these experiences, the objective of this paper is to study the propagation of the yeast *Candida utilis* in a batch reactor of vinasse, and monitoring both the biotechnological process of protein enrichment as well as the reduction of the contaminating load of the effluent.

Materials and methodology

In this study it was used the strain of the yeast *Candida utilis* coming from the ceparo of PROIMI- CONICET. Aqueous solutions were prepared with vinasse in the following concentrations: 10%, 20%, 30%, 40%, and 50% which were sterilized in chamberland autoclave for 15 minutes in 1 atmosphere of pressure in 121°C.

The yeast was activated in liquid medium Papa Dextrosa (PD), incubated in thermostatic cover with agitation at 34°C for 24 hours. After that, 0,5 ml were taken of this medium, and they were inoculated in a jar with vinasse in aqueous solution at 10%. This medium was incubated for 24 h in equal conditions, and 0,5 ml were transferred to the vinasse medium composed of aqueous solution at 20%. This procedure was repeated up to the point where the vinasse in aqueous solution at 50% was reached. Such solution was adopted as the concentration point to use in the experiment in the batch reactor, since there was no variability in the data when increasing the concentration of vinasse. As batch reactors, it was adapted an Erlenmeyer of 1 l with a cover of rubber with two perforations to allow the entrance and exit of sterile air through a microbiological filter, between the bomb and the reactor. So as to obtain a homogeneous air distribution, and facilitating the transference of oxygen to the cells, a malleable hose diffuser of 45 cm of length was used, which allowed us to adapt its form to the reactor. Temperature control and additional agitation were not realized. The Optical Density (OD) in the reactor was measured at

600 nm every two hours for 48hs, so as to calculate the growth curve of the yeast in the reactor.

At the beginning and at the end of each experiment, the following variables were measured: CDO (chemical demand of oxygen) through colorimetric method; total nitrogen through Kjeldhal method; pH with pH-metro Orion 420, and conductivity with a conductimeter Adwa AD32.

In total, six experiments in triplicate were realized, and the data obtained was processed statistically. For each analyzed variable, it was carried out an ANOVA (Analysis of Variance), assessing the factors of repetition, and the reactor with a significance level of 1%. In order to control the exhaustion of the vinasse in the reactor, the concept of cell breathing was used. All living cells carry out cell breathing to obtain the necessary energy for their functions. A product of cell breathing is CO_2, as the yeasts reproduce, they need greater quantities of nutrients, and they consume more oxygen and produce larger amounts of carbon dioxide. Taking CO_2 as an indicator of the physiological status of the yeasts, it was used the sensor Cozir wide range model GC-0016 to monitor the yeast's growth. This sensor uses the technique of spectroscopic infrared radiation to obtain the concentration of CO_2 in a gas sample. It is a non-dispersive sensor with a LED light of stretched band, and a photodiode detector (PD). The combination of LED/PD in infrared medium allows for the selection of the wave longitude necessary to avoid crosses sensitivity without an optical filter. The data acquired with the sensor were presented on screen by the GasLab software that allowed the calibration of the sensor, obtaining the value of the instantaneous concentration, programing an interval of mediation and the period of data collection. The experiments lasted 50 hours and a period of mediation was programed every ten minutes.

Results and discussion

Judging by the data obtained, it was observed that:

Variation of pH

At the end of the experiments, the pH in the vinasse was on average 4% lower than at the beginning, reaching in every case values close to 4,4. This indicates that the growth of the yeast produced acids like metabolic waste which caused the diminution of pH in the medium. This fact agrees with what was reported by Soto Meza[7] who informs a lowering in the pH liquid medium of 4 to 3,6 after their treatment with *Candida utilis*. The statistical analysis of the data showed a significant difference between the reactors ($p<0,01$). The factor of repetition (number of repetitions) is not quite significant ($p=0,17$), that is there are not noticeable differences between the first, the second and last measure.

Variation of chemical demand of oxygen (COD)

The average removal of the chemical demand of the oxygen (CDO) was of 43% It was noticed a variability between the first 2 experiments and the last 4, which may be due to the fact that the experiments were carried out at room temperature without using mechanisms of control and the proper temperature for the growth of the yeast is between 28-34° C, i.e. temperatures close to the proper ones caused a higher removal of COD. It is worth noticing that Domenech López[6] obtained a removal of COD between 30 and 50% of vinasse in aqueous solutions at 50% with bagasse of sugar cane which are similar to those found in this study. The statistical analysis of the data shows the existence of significant differences between reactors ($p<0,01$).

Enrichment of protein in vinasse

On average, enrichments of 136% in the liquid medium were formed of vinasse in aqueous solutions at 50%, values which are superior to the 100% reported by Serguera Niño et al.,[5] in a liquid medium composed of: urea (2,3 g), $(NH_4)_2HPO_4$, NH_4SO_4 (11,5 g), sugar cane honey B (200mg), and measured with distilled water up to 1 l, which is a medium much more nutritional than the one used in our experiments for the growth of the yeast. The results obtained through ANOVA show the existence of significant differences for the reactor ($p<0,01$).

Variation in conductivity

The conductivity of the medium conformed by vinasse in aqueous solutions at 50% diminished to 9%, ie. there was a reduction of ions in the solution. It is well-known that the ions of potassium are more abundant in the vinasse.[8] This is due to the fact that the soil in Tucuman possesses large amounts of this element. In future investigations, we propose knowing the percentage of removal of this ion, since its diminution will make it easier for the final disposition of the vinasse as a ferti-risk, avoiding the salinization of the soil were it is poured. The results obtained for the ANOVA were that there is a significant difference between reactor ($p<0,01$).

Growth curve

Using the data obtained from the measures of OD, which were realized during the experiments, it was designed a curve of growth of the yeast *Candida utilis* in vinasse in aqueous solutions at 50%, Figure 1.

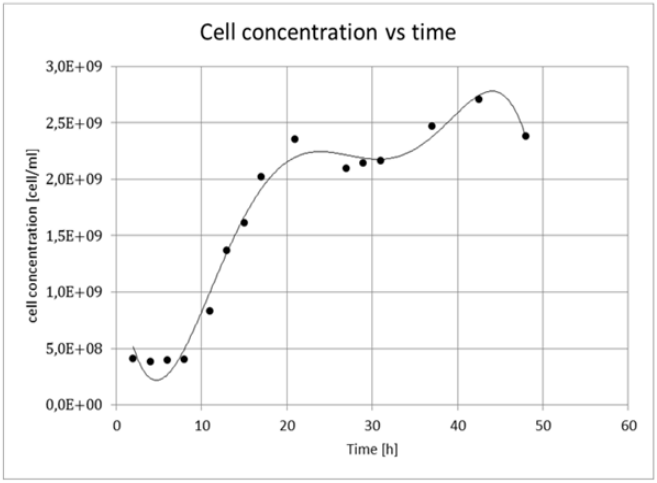

Figure 1 Growth curve of the yeast *Candida utilis* in vinasse in aqueous solution at 50%.

As it can be observed in Figure 1, the graphic presents three well-defined phases, lag phase with an average duration of 8 hours, a phase of logarithmic or exponential growth which ends, on average, in 21 hours after the initiation of the fermentation, and a stationary phase which in the experiments lasted up to the end of each of them (48h). The concentration of cells reached was of $2,5.10^9$, which is of the same order of magnitude obtained by Serguera Niño et al.,[5] in an optimal medium for the growth of the yeast, not a waste, and using the same concentration of inoculum 10^8 cell/ml and the same relation of inoculation 1/10. Moreover, Serguera Niño *et al*. (2001) reports final concentrations of 9.10^8 cell/ml in an aqueous medium composed of 70% vinasse and 30% sugar cane honey. Diaz, Semprúm and Gualtieri (2003) used enriched vinasse with 1g/l of harmonium sulfate, 1g/l of urea and extract of malt. They obtained cell concentrations of $2,2.10^8$ cell/ml. Thus, in the medium composed of vinasse in aqueous solutions at 50%, it reached a final concentration of major cells than the ones reported in the bibliography consulted.

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Monitoring with sensors of CO_2

Figure 2 shows the three experiments where the growth of the yeast was monitored, measuring the concentrations of carbon dioxide through time. The experiments lasted 50 hours, and the sensor took a sample of the concentration of carbon dioxide every 10 minutes. In all experiments, the sensor reached saturation point, ie it obtained a concentration of 1000000 PPM, or a concentration of 100% of CO_2, which is the same, in the exit gases of the fermentor. It seems difficult to believe that such a concentration was accomplished with a lab reactor, knowing that the air never stopped being introduced to it. Taking into account Saucedo by Castañeda et al.,[9] he also argues that the concentrations of CO_2 are hard to find at higher percentages than 10% (that is 100000 PPM) in aerobic fermentations. The sensor's point of saturation may be due to the gas sample coming from the sensing camera through a diffuse membrane. This method makes the sampling process slower than the concentration of CO_2 when the production rate is at the maximum and very accelerated. In this way, the sampled gas does not leave entirely the sampling chamber when a new sample is measured. As a consequence, the concentration that the sensor reads is an accumulated concentration, and not the instantaneous one during that period.

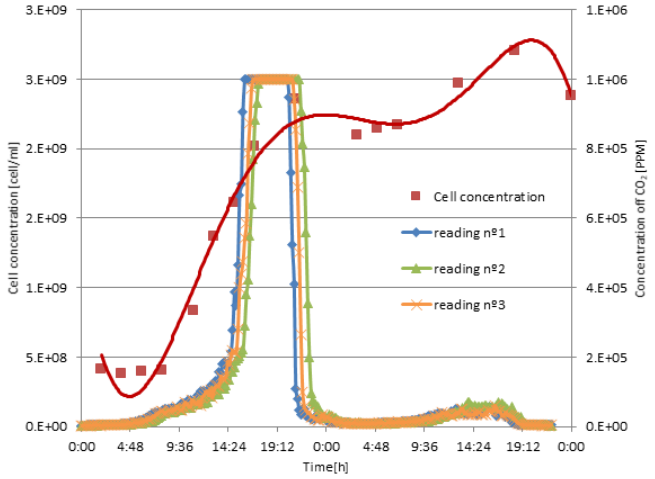

Figure 2 Curve of growth measuring OD vs Curve of concentration of CO_2 obtained with the sensor of CO_2.

Saucedo Castaña et al.,[9] warns that, even when the infrared sensor that measures the CO_2 concentration of the exit gases in an aerobic fermentation reaches saturation, it does not imply that the extraction of important information of the cell's physiological status is not allowed while they are being cultivated in the reactor. Although the CO_2 curve does not truly reflect its concentration in every instance of measurement, it is possible to observe and quantify the time that the yeast grows in each phase of growth: lag phase, exponential phase, stationary phase and cell's death. The lag phase lasts 4 hours on average, leading to the exponential phase taking place from the 4° to 8° hour; it can be clearly observed the change in the concavity of the curve.

In the 8° hour, approximately, the exponential phase begins, it lasts 20 hours on average. It is worth mentioning that, owed to the saturation of the sensor, it is not possible to appreciate the moment of deceleration in which the graphic changes its concavity to initiate the stationary phase. From there on, the stationary phase begins, the concentration of gas falls drastically since the cells stop reproducing and diminish their metabolism.

After 30 hours of the start of the experiments, the cells make a reconvertion of their metabolism so that they can consume some available nutrients in the medium. This is reflected in a slight increase in the concentration of CO_2. After that, it can be observed that the concentration decreases and stays in that way during the rest of the experiment, this period corresponds to the stage of cellular death. Comparing the two curves, the one obtained with the sensor of carbon dioxide and the other curve of growth measuring OD, it can be clearly observed that the information that one shows correlated with what the other curve demonstrates, as it can be seen in Figure 2. The monitoring of the yeast growth, which measured the concentration of carbon dioxide in time, and the OD in the medium, showed that the fermentation period lasted 21 hours.

Conclusion

The yeast *Candida utilis* proved to be able to grow in a batch reactor of vinasse, in aqueous solutions at 50%, producing a protein enrichment on its own and removal of CDO. It was possible to monitor the growth of the yeast with a sensor of carbon dioxide by spectroscopy of infrared radiation, since it gives information of the physiological status of the yeast without altering it. The proposed process diminishes the contaminating load of the principal effluent of Tucuman.

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None.

Conflict of interest

The authors declare there is no conflict of interest.

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