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Produção de biomassa de levedura com potencial para controle biológico: estratégias de processo para aumentar o rendimento

Yeast biomass production with potential for biological control: process strategies for increasing yield

Producción de biomasa de levadura con potencial para el control biológico: estrategias de proceso para aumentar el rendimiento

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Resumo

A qualidade dos produtos vegetais está diretamente ligada às técnicas empregadas no campo, a fim de garantir produtos seguros e saudáveis à saúde. Nesse contexto, o uso de leveduras com potencial para controle biológico provou ser uma alternativa promissora para garantir a segurança desses alimentos. Os processos de fermentação têm sido utilizados para promover o desenvolvimento de muitos produtos, incluindo a produção de biomassa de levedura. O objetivo deste trabalho foi verificar a influência da taxa de aeração e do processo de batelada alimentada na produção de biomassa de levedura. Uma estirpe de levedura com potencial de controle biológico, pertencente à coleção de culturas do Semi-Árido da Embrapa, foi submetida a cultivos em batelada simples e alimentada com diferentes taxas de aeração (3, 4, 6 e 8 L.ar/min) e concentração de fonte de carbono no meio de alimentação (200, 400 e 600 g/L). A maior biomassa (6,99 g/L) após 24 horas de fermentação foi observada no experimento que utilizou a taxa de aeração de 8 L.ar/min. Em relação à concentração da fonte

de carbono no meio de alimentação, verificou-se que a concentração de 200 g/L favoreceu uma maior biomassa total (11,21 g/L) e reduziu a produção de etanol (0,65 g/L), já a concentração de 600 g/L favoreceu uma menor produção de biomassa (7,90 g/L) e uma maior produção de etanol (9,26 g/L). Dessa forma, constatou-se que a taxa de aeração e o processo de batelada alimentada favorecem a estratégia de fermentação, pois contribuem para a produção de biomassa de levedura e o rendimento geral do processo.

Palavras-chave: biocontrole; biomassa microbiana; fermentação; bioprocesso industrial.

Abstract

The quality of vegetable products is directly linked to the techniques used in the field, in order to ensure safe and healthy products to health. In this context, the use of yeasts with potential for biological control proved to be a promising alternative to assure the safety of these foods. Fermentation processes have been used to promote the development of many products, including the production of yeast biomass. The objective of this work was to verify the influence of the aeration rate and the fed batch process in the production of yeast biomass. A yeast strain with biological control potential, belonging to Embrapa's Semi-Arid crop collection, was subjected to simple batch cultivation and fed with different aeration rates (3, 4, 6 and 8 L.ar/min) and concentration of carbon source in the feed medium (200, 400 and 600 g/L). The highest biomass (6.99 g/L) after 24 hours of fermentation was observed in the experiment that used an aeration rate of 8 L.ar/min. Regarding the concentration of the carbon source in the feed medium, it was found that the concentration of 200 g/L favored a greater total biomass (11.21 g/L) and reduced the production of ethanol (0.65 g/L), while the concentration of 600 g/L favored less biomass production (7.90 g/L) and higher ethanol production (9.26 g/L). Thus, it was found that the aeration rate and the fed batch process favor the fermentation strategy, as they contribute to the production of yeast biomass and the overall yield of the process.

Key-words: biocontrol; microbial biomass; fermentation; industrial bioprocess.

Resumen

La calidad de los productos vegetales está directamente relacionada con las técnicas utilizadas en el campo, para garantizar productos sanos y seguros para la salud. En este contexto, el uso de levaduras con potencial para el control biológico demostró ser una alternativa prometedora para garantizar la seguridad de estos alimentos. Los procesos de fermentación se han utilizado para promover el desarrollo de muchos productos, incluida la producción de biomasa de

levadura. El objetivo de este trabajo fue verificar la influencia de la velocidad de aireación y el proceso por lotes alimentado en la producción de biomasa de levadura. Una cepa de levadura con potencial de control biológico, perteneciente a la colección de cultivos semiáridos de Embrapa, se sometió a un cultivo por lotes simple y se alimentó con diferentes tasas de aireación (3, 4, 6 y 8 L.ar/min) y concentración de fuente de carbono en el medio de alimentación (200, 400 y 600 g / L). La mayor biomasa (6,99 g / L) después de 24 horas de fermentación se observó en el experimento que utilizó una tasa de aireación de 8 L.ar/min. Con respecto a la concentración de la fuente de carbono en el medio de alimentación, se encontró que la concentración de 200 g / L favoreció una mayor biomasa total (11.21 g / L) y redujo la producción de etanol (0.65 g / L), mientras que la concentración de 600 g / L favoreció una menor producción de biomasa (7.90 g / L) y una mayor producción de etanol (9.26 g / L). Por lo tanto, se descubrió que la velocidad de aireación y el proceso por lotes alimentado favorecen la estrategia de fermentación, ya que contribuyen a la producción de biomasa de levadura y al rendimiento general del proceso.

Palabras clave: biocontrol; biomasa microbiana;fermentación; bioprocesos industriales

1. Introduction

Colletotrichum gloeosporioides e Lasiodiplodia theobromoe they are microorganisms that cause postharvest mango diseases, such as anthracnose and pendulum rot. These pathogens are mainly responsible for phytosanitary problems and large losses in the economic sector.

The quality and safety of plant-derived products are directly related to the techniques used in the field which contribute to obtain harmless and safe food and environmental protection. In this context, several alternatives are being used objectifying to minimize the damage caused by pathogens and pests that attack the crops, with emphasis on biological control.

Among the several microorganisms that can be used for biological control, yeasts stand out because they are non-toxigenic organisms. do not produce allergenic spores and antibiotic metabolites. These characteristics potentiate their antagonistic effect against some species of pathogenic fungi and minimize the environmental impact of its utilization (Coelho et al., 2003; Gouvea, 2007; Bendo et al, 2009).

The fermentation processes have been used to assist the development of many products. including the production of yeast biomass. New and economically viable processes, presenting reproducibility and reliability, require a better monitoring. However, progress in

this area needs a better understanding of microbial physiology, microorganism-environment interactions and the ability to manipulate metabolic fluxes (Neves, 2003).

Oxygen transfer is considered the most important factor affecting the fermentation process, since cellular growth and multiplication of the microorganism need energy supply. The use of bioreactors with aeration and agitation promotes the transfer of oxygen from the gas phase to the liquid phase, allowing dissolved oxygen to reach the cells and to be consumed in the reaction, which contributes to the increase in cell concentration.

Frequently, discontinuous processes can result in low yields and/or productivity, because the substrate added at once at the beginning of the fermentation exerts inhibitory effects, repression or redirect the cell metabolism to different products. High concentrations of sugars and higher oxygen supply can lead to a repressor effect called *Crabtree*, where enzymes of the microbial respiration are inhibited and the production of ethanol increases, in a process where alcoholic fermentation occurs under aerobic conditions (Rodrigues et al., 2006).

According to Win et al. (1996), the *Crabtree effect* can be controlled through cultivation under fed batch processes, since the essential nutrients can be supplied (fed) as required by the microorganism during cultivation, which favors a higher biomass production.

The fed batch process is characterized as a technique in which one or more nutrients are added to the bioreactor during the cultivation and the products remain there until the end of the fermentation. In some cases, the nutrients are gradually fed at a constant or variable rate. According to Kim et al. (2007), the fed batch processes help the increase of the cell mass and productivity due to the control of the cultivation conditions such as pH, culture medium and feeding rate of the substrate.

The aim of this work was to evaluate the effect of the aeration rate and the fed batch process in the production of yeast biomass with potential for biological control.

2. Methodology

Microorganism

The microorganism used in this study belongs to the collection of Embrapa Semi-Arid (Petrolina-Pernambuco. Brazil) and was isolated from the natural microflora of tropical fruits.

Activation of the microorganism

The culture maintained in Agar potato dextrose (Difco) was previously activated in test tubes containing 5 mL of Sabouraud broth supplemented with 1 % yeast extract (Difco) at 30 °C for 24h. After this period, an aliquot containing 1 % (v/v) of inoculum was transferred to 250 mL flasks containing 100 mL of the culture medium and incubated at 30 ± 1°C, 150 rpm on orbital shaker for 24 hours.

Fermentative Process

The Brand New Brunswick Scientific Co fermenter, model Bioflo/CelliGen® 115 with 3 liters vats was used. At this stage, two flat 6-blade turbines were used, also known "Rushton" turbines, aiming to improve the agitation of the reaction medium. The pH was monitored throughout the fermentation process, using a combination pH electrode (Metler-Toledo; 405-DPAS-SC-K8S/225)

The culture medium was prepared directly in the vat and pH was adjusted according to the manufacturer specifications (Difco, 2009). Inverted sugar was used as carbon source in this phase at concentration of 20 g/L. Commercial soybean oil was added to the medium at the end of preparation at 0.30% concentration as a foam prevention agent and then the bioreactor containing the medium was autoclaved at 121°C for 30 minutes.

The fermentation was performed under simple batch and fed-batch, after adding 1% (v/v) of previously activated inoculum. The processes were conducted at 30 ±1 °C, 150 rpm for 24 hours for batch process and 40 hours for the fed batch process.

In this step, the influence of the aeration rates (3, 4, 6 and 8 $L._{ar/min}$) and feeding of system (culture medium containing 10, 20 and 30 times more the carbon source and 2.5 times more nitrogen source) were evaluated. The feeding was carried out continuously using nutrient solution in concentrations of 200, 400 and 600 g/L, at a feeding rate of 10 mL/hour until the end of the fermentation.

Analytical Determinations

At each time interval established for sampling, the following parameters were determined: 1) Cell Quantification: biomass produced was determined by spectrophotometer (Varian. Model Cary 50 Conc) at 600 nm with the aid of a previously established dry weight standard curve; 2) quantification of total reducing groups (TRGs) was determined by a colorimetric method using 3,5 dinitrosalicylic acid (DNS) according to Miller (1959); 3) control of pH: the pH was potentiometrically monitored in pH meter (Hanna Instruments.

model HI 2221); 4) determination of ethanol: performed by high performance liquid chromatography (Varian ProStar model 355 RI detector).

4. Results and discussion

The highest biomass production (6.99 g/L) after 24 hours of fermentation was observed in the experiment that used the aeration rate of 8 $L._{ar/min.}$ In this experiment, it was also observed that the sugar consumption was relevant, favoring the biomass yield ($Y_{x/s}$) of 0.31 (Table 1).

Table 1. Results for the production of yeast biomass in experiments using invert sugar (20 g/L) as the carbon source and aeration rates of 3, 4, 6 and 8 L $_{air/min,}$ respectively.

Time (h)	Biomass (g/L)	Sugar consumed (g/L)	pH	Yx/s
0	0.16±1.44*	23.15±10.08	6.26±0.24	0.00
4	0.63±1.24	24.14±9.95	6.01±0.21	0.47
8	2.36±0.73	14.37±6.41	5.63±0.24	0.25
16	4.08±0.62	5.26±2.30	5.86±0.17	0.19
24	2.83±1.39	2.00±15.19	6.10±0.25	0.12
Time (h)	**Biomass (g/L)**	**Sugar consumed (g/L)**	**pH**	**Yx/s**
0	0.05±2.89	23.49±8.88	6.45±0.58	0.00
4	0.12±2.81	23.86±7.95	6.17±0.46	0.21
8	1.89±2.25	6.41±1.02	5.47±0.21	0.11
16	7.37±1.12	6.26±1.04	5.09±0.04	0.42
24	5.14±2.56	4.17±10.80	5.00±0.75	0.26
Time (h)	**Biomass (g/L)**	**Sugar consumed (g/L)**	**pH**	**Yx/s**
0	0.01±2.87	25.78±9.91	6.50±0.72	0.00
4	0.06±2.78	22.16±8.54	6.28±0.59	0.01
8	1.68±2.21	13.78±5.57	4.90±0.02	0.14
16	5.63±0.62	2.10±0.14	4.94±0.01	0.24
24	6.87±3.41	1.82±13.09	4.95±0.57	0.29
Time (h)	**Biomass (g/L)**	**Sugar consumed (g/L)**	**pH**	**Yx/s**
0	0.04±2.89	28.00±10.72	6.09±0.58	0.00
4	0.20±2.69	27.41±9.66	5.69±0.42	0.27
8	2.94±1.73	13.22±4.54	4.90±0.10	0.20
16	6.08±0.46	2.47±1.47	4.68±0.01	0.24
24	6.99±0.00	5.41±0.00	4.67±0.00	0.31

***Standard deviation**

The growth of aerobic microorganisms is affected by oxygen availability, causing a higher or lower efficiency of the microbial culture. Besides the available oxygen, another factor favoring microbial growth is stirring, as causes a better aeration of the medium and dispersion of nutrients.

According to Aiba et al. (1973), aeration and agitation in a fermentation process aim to supply oxygen to the microorganisms and promote agitation of the fermentation broth, which contributes to a dispersion and uniform growth of the microorganisms in the culture medium.

Kim et al. (2007), evaluated the effect of different aeration rates (2, 4 and 6 $L_{air/min}$) in a fermentation process and found that increased aeration rate resulted in a significant production of yeast biomass ranging from 36.4 to 39.3 g/L.

The aerobic growth of yeasts and fungi present a typical biomass yield ranging from 0.4 to 0.8 g/g (Bayle et al., 1986). In the present study the yields observed were inferior to the mentioned, indicating that the carbon source was not only used for biomass production, but also for production of energy, primary and secondary products and for maintenance of the system (Taccari et al., 2012).

Regarding pH values, a decrease (6.5 to 4.6) was observed during the fermentation process. Comparing the consumption of sugars and biomass production, the inverse situation was observed: the highest values of sugars consumption and production of biomass occurred in the lowest pH values. According to Oliveira (2006), the pH decrease can be caused by the formation of products due to a high concentration of protons and CO_2 in the medium that occurs during the aerobic metabolism of the carbon source.

The lag phase was four hours long in all the experiments, followed by a relatively short exponential phase. Although initially the biomass production was not extensive, the cell growth of the exponential phase was continuous until the entire carbon source was consumed, when the stationary phase started, from 16 hours of fermentation. Sugars consumption was significant in both experiments, showing a significant increase of biomass, suggesting the conversion of substrate into biomass.

Products derived from nutrient consumption can be formed during an aerobic fermentation, characterizing an oxidative metabolism which can be easily converted into a reductive metabolism, which favors the production of ethanol and other compounds. In fermentation processes for biomass production, ethanol is not an interesting product, because the entire substrate should be converted into biomass.

Thus, the ethanol produced during this experiment can be attributed to a suppression of the metabolism by the high concentrations of the carbon source in the medium, characterizing the *Crabtree effect.* This effect reduces respiratory capacity (oxidation) and causes a change in the metabolism, resulting in the formation of ethanol, even in the presence of oxygen (Reis, 2009).

The highest amounts of ethanol (18.97; 17.61 and 18.31 g/L) were produced in the exponential phase of microbial growth, with aeration rates of 3, 4 and 6 $L_{.ar/min}$, respectively. Studies show that under decreasing aerobic conditions, yeasts increase ethanol production and decrease biomass production (Yamane at al., 1997; Johnson et al., 1979; Moriel, 2004).

The results showed that sugar was not completely used by the microorganism in the exponential phase, which may have caused an accumulation of this substrate in the medium favoring the repression of the respiratory activity.

According to Reed and Peppler (1973), repression of respiratory activity occurs when the carbon source is present in concentrations from 0.2 % w/v. and this is one of the reasons to use fed-batch fermentations.

Although the feeding of the system is one alternative to reduce the amount of ethanol produced, it is known that increased aeration rate can directly affect the decrease of the ethanol produced in the medium, favoring an increase of the biomass produced. In the tests performed, after 24 hours of fermentation the aeration rate of 8 $L_{.ar/min}$ led to a reduced production of ethanol (0.59 g/L) compared to other experiments.

Studies have shown that high concentrations of oxygen are required to suppress the *Crabtree effect*, since high aeration rates prevent the ethanol formation and favor the biomass production (Moriel, 2004; Yamane et al., 1997; Fang et al., 1996).

According to Agrano (1996), the use of aeration rate 27 $L._{ar/min}$ in 16 hours fermentation in a process for the production of yeast biomass, favored a high biomass (18 g/L) and a low ethanol (3.01 g/L) production. Ghaly and Ei-Tawell (1995), evaluated the effects of aeration rate on the growth of yeast and reported that the increase of aeration rate from 150 to 480 $mL._{ar/min}$ increased the biomass production and reduced the ethanol formation.

As previously mentioned, the ethanol formation can decrease the biomass yield, being necessary to control the sugar feeding rate in the fermenter in order to minimize the ethanol production of a fed batch process. Thus, the sugar concentration is considered the main parameter for the effective cell mass production of yeast (Miskiewicz et al., 2000).

In the present study, the effect of the fed batch process was observed from 16 hours of fermentation. This period was chosen because in the discontinuous process was observed that the microbial growth showed a decrease after that stage due to the scarcity of the substrate, so migrating to the stationary phase.

It should be noticed that in the fed batch process was used the aeration rate (8 $L._{ar/min}$) because it promoted a greater biomass and a lower ethanol production in the batch process.

Furthermore, the concentration of sugars in the culture medium was maintained around 20 g/L during the feeding period in order to avoid the decrease in biomass yield.

The three experiments used different concentrations (200, 400 and 600 g/L) of carbon source and all showed a continuous increase in the biomass when more substrate was added to the medium by feeding the system. After 40 hours of fermentation the medium with 200 g/L of carbon source showed a greater total biomass (11.21 g/L) and reduced ethanol production (0.65 g/L), while the medium containing the carbon source at 600g/L presented a lower production of biomass (7.90 g/L) and higher ethanol production (9.26 g/L) (Figures 1 a 3; Table 2).

Figure 1 - Results of batch process fed with feed rate of 200 g/L.

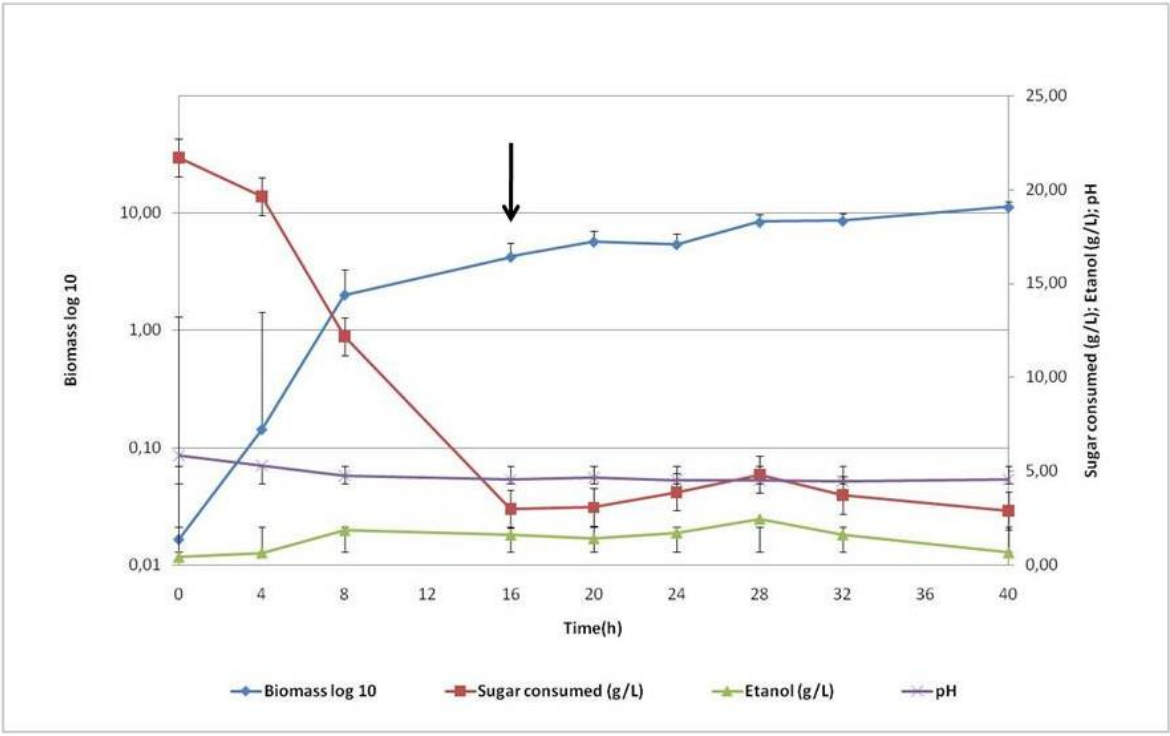

Figure 2 - Results of batch process fed with feed rate of 400 g/L.

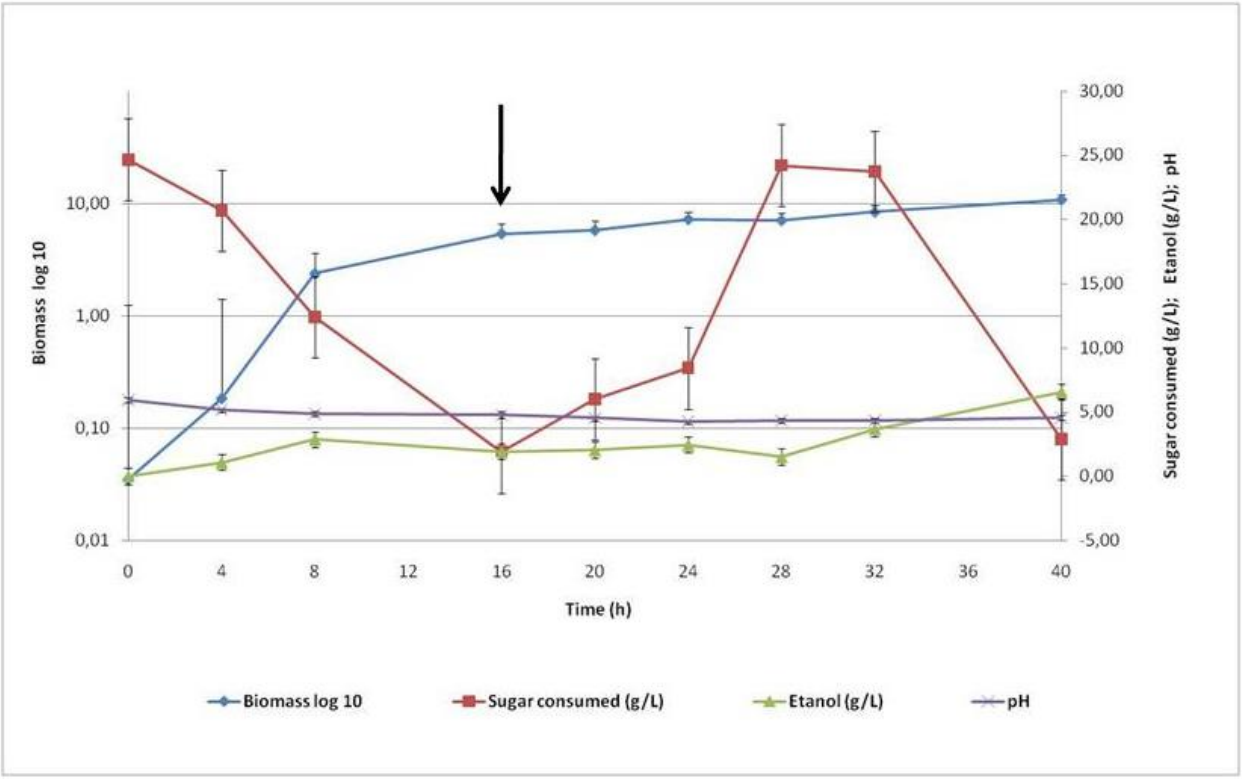

Figure 3 - Results of batch process fed with feed rate of 600 g/L.

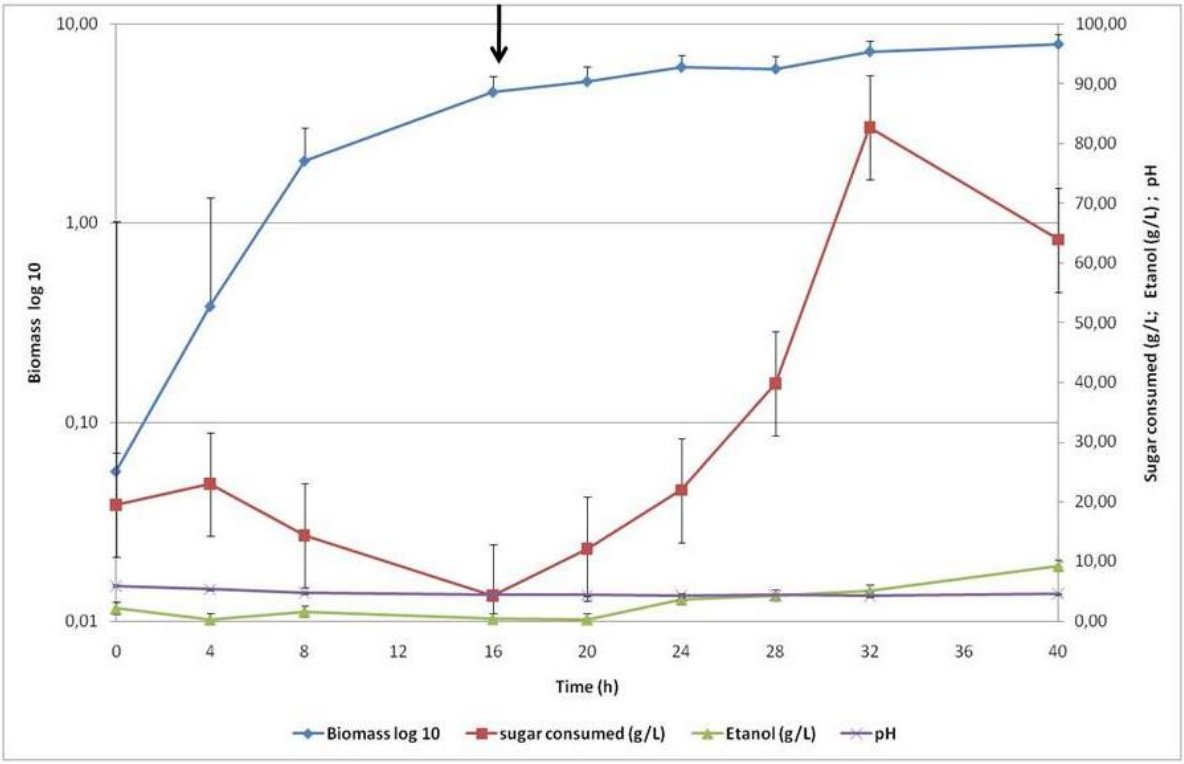

Table 2. Results obtained in the fed batch process with different concentrations of the carbon source in the feed medium.

Medium for feeding a carbon source at a concentration of 200 g/L					
Tempo (h)	**Biomassa (g/L)**	**Açúcar consumido (g/L)**	**pH**	**Etanol (g/L)**	**Yx/s**
0	0.02±0.00*	21.69±1.77	5.84±0.07	0.42	0.00
4	0.14±0.01	19.62±0.06	5.30±0.05	0.64	0.01
8	2.00±0.30	12.15±1.25	4.74±0.02	1.83	0.14
16	4.20±2.04	2.98±0.81	4.53±0.02	1.60	0.17
20	5.67±0.64	3.07±0.20	4.65±0.12	1.40	0.02
24	5.39±1.00	3.89±0.46	4.49±0.05	1.71	0.08
28	8.33±0.62	4.82±0.66	4.51±0.04	2.44	0.12
32	8.54±1.10	3.73±0.59	4.45±0.05	1.61	0.20
40	11.21±0.21	2.89±1.02	4.55±0.12	0.65	0.59
Medium for feeding a carbon source at a concentration of 400 g/L					
Tempo (h)	**Biomassa (g/L)**	**Açúcar consumido (g/L)**	**pH**	**Etanol (g/L)**	**Yx/s**
0	0.03±0.02	24.66±2.84	5.90±0.07	0.00	0.00
4	0.18±0.02	20.69±0.59	5.11±0.24	1.08	0.11
8	2.36±0.28	12.39±2.34	4.85±0.03	2.86	0.19
16	5.35±0.22	1.86±0.06	4.72±0.02	1.91	0.23
20	5.73±0.60	6.01±0.80	4.50±0.02	2.03	0.09
24	7.17±0.31	8.41±0.03	4.22±0.00	2.44	0.02
28	7.02±1.72	24.23±17.13	4.31±0.20	1.50	0.13
32	8.34±0.82	23.71±14.83	4.30±0.2	3.68	0.18
40	10.71±1.51	2.87±0.88	4.53±2.27	6.57	0.49
Medium for feeding a carbon source at a concentration of 600 g/L					
Tempo (h)	**Biomassa (g/L)**	**Açúcar consumido (g/L)**	**pH**	**Etanol (g/L)**	**Yx/s**
0	0.06±0.02	19.45±1.27	5.90±0.06	2.17	0.00
4	0.38±0.30	22.90±1.37	5.32±0.16	0.30	0.01
8	2.05±0.44	14.32±2.86	4.76±0.13	1.65	0.02
16	4.54±0.62	4.19±2.35	4.38±0.02	0.39	0.01
20	5.13±0.64	12.08±1.16	4.30±0.04	0.37	0.38
24	6.06±0.00	21.91±14.26	4.22±0.00	3.71	0.08
28	5.94±1.85	39.75±1.27	4.35±0.08	4.33	0.16
32	7.26±2.03	82.69±2.14	4.25±0.00	5.16	0.11
40	7.90±0.83	63.88±11.53	4.53±0.00	9.26	0.16

***Standart deviation**

In the experiment with the carbon source at a concentration of 200 g/L the yield was 0.59 ($Y_{x/s}$). This high yield may be occurred because the carbon source was completely used by the microorganism to produce biomass.

Atasoy et al. (2013), optimized the operating conditions of the fermentation process for yeast production and observed that the amount of biomass produced in the fed batch fermentation increased while ethanol formation was minimized by the regulation of substrate feeding and air.

When the carbon source was at 600 g/L, the high concentration of this nutrient could have caused growth inhibition due accumulation of substrate and ethanol in the medium. Thus, the *Crabtree effect* was again observed, confirmed by the concentration of residual sugar at the end of the experiment (63.88 g/L).

When the concentration of the carbon source in the feeding medium exceeds the critical value, even under aerobic conditions, a decrease in the biomass yield occurs, since the formation of ATP in the fermentation is much lower than that of the respiratory chain (Campos et al., 2013).

A deficiency in oxygen transfer possibly occurs in a fed batch system for yeast biomass production, which leads to an anaerobic condition and promotes the production of ethanol (Daramola et al., 2008).

Comparing the two methods used in this study (batch and fed batch fermentation) for the production of yeast biomass, it was observed that the fed batch process presented better results (Table 3). Biomass and the biomass yield increased 1.6 and 1.9 times, respectively. Biomass yields showed no significant differences in both processes. The increase in fermentation time and the feeding of the system increased the yeast biomass production.

Table 3. Comparative results between the process batch and fed batch

	Biomass (g/L)	**Etanol (g/L)**	**Yx/s**	**Px ($g.L^{-1}h^{-1}$)**
Process batch (24h)	6.99	0.59	0.31	0.29
Process fed batch (40h)	11.21	0.65	0.59	0.28

Yx/s: biomass yield

Px(g/L/h): biomass productivity

Kim et al. (2007), compared the batch and fed-batch process for producing yeast biomass and observed an increase in the production of biomass from 39.3 g/L to 95.7 g/L. respectively.

Chang et al. (2013), observed an increase in the yeast biomass from 4.1 to 8.3 g/L in a comparative study of the batch and fed-batch processes from a non conventional carbon source (hydrolyzed corn).

4. Conclusions

These results demonstrate the importance of the aeration rate and the fed batch fermentation process to improve the fermentation strategy in order to significantly increase the production of yeast biomass and the overall yield of the process.

However, high concentrations of the carbon source and low concentrations of aeration rates can be considered limiting factors in the process, since they favor low cell yield and significant ethanol production.

New studies must be carried out in order to establish the best parameters to produce yeast biomass with potential for use in biological control.

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