CIÊNCIA e NATURA

Universidade Federal de Santa Maria
Ci. e Nat., Santa Maria v.42, e52, 2020
DOI:10.5902/2179460X35612
ISSN 2179-460X
Received 12/11/18 Accepted: 14/02/20 Published:13/08/20

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Chemistry

Glycerol and ethanol accumulation profile of the yeast FT-858 on saccharine substrates

O perfil do acúmulo de glicerol e etanol da levedura FT-858 em substratos sacarinos

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ABSTRACT

The production of Brazilian ethanol has aroused worldwide interest, since it is made from a source of renewable biomass, sugarcane and yeast metabolism. Thus, the objective of this study is to compare the characteristics of sugarcane and sorghum biomass and to evaluate the ethanol and glycerol production of yeast FT-858 in different culture conditions. An exploratory qualitative bibliographical study was performed regarding the technological characteristics of saccharine substrates. A pre-inoculum using sterile liquid medium YPD 2% was prepared, in which 0.10 g of FT-858 yeast was inoculated and incubated at 30°C for 10h at 250rpm. Cells were collected by centrifugation and inoculated in the fermentation medium based on sugarcane and sorghum at the concentration of 22 °Brix. The process was carried out by cellular recycling e inoculated into the substrate at 30 and 40 °C at 250rpm. Aliquots of 4 ml were collected for intracellular glycerol analysis via enzymatic kit and ethanol concentration by gas chromatography. It can be observed that both substrates have a similar composition in relation to sugars, although the sugarcane juice has a higher sucrose. The accumulation of intracellular glycerol was more significant in the cane juice at both temperatures and the ethanol concentration decreased throughout the recycle.

Keywords: Metabolism; Biomass; Cell recycling

RESUMO

O processo fermentativo para a produção de etanol brasileiro vem despertando interesse mundial, pois é realizado a partir da cana-de-açúcar, uma biomassa renovável de fermentação direta e, por meio do metabolismo das leveduras. Assim, este estudo visa comparar as características das biomassas cana-de-açucar e sorgo sacarino e avaliar o acúmulo de glycerol e a produção de etanol da levedura FT-858. Inicialmente, foi realizado um estudo bibliográfico qualitativo-exploratório acerca das características tecnológicas dos substratos sacarinos e, para a parte laboratorial um pré-inoculo com o meio líquido YPD 2%, esterilizado, no qual foi inoculada 0,10 g da levedura

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liofilizada FT-858 e incubada a 30 °C por 10h a 250rpm, que foi prontamente centrifugação e a biomassa inoculada no meio fermentativo a base de cana e de sorgo na concentração de 22 °Brix, que foi conduzido por reciclo celular a 30 e 40 °C a 250rpm e alíquotas de 4ml foram retiradas para as análises de glicerol intracelular utilizando o kit enzimático de triglicerídios e a concentração de etanol por cromatografia gasosa. Os substratos sacarinos apresentaram composição semelhante de açúcares, contudo, a sacarose foi superior ao encontrado no sorgo. O acúmulo de glicerol intracelular foi maior em caldo de cana em ambas as temperaturas e a concentração de etanol sofreu queda ao longo dos reciclos fermentativos. O acúmulo de glicerol e etanol foi inversamente proporcional, independente do substrato da sacarina. No entanto, os fatores de estresse associados causaram alterações no perfil de produção do metabólito da levedura, o que influenciou um maior acúmulo de glicerol. Assim, o sorgo sacarina tem grande potencial para ser utilizado na produção de etanol, uma vez que pode ser processado na mesma instalação industrial.
Palavras-chave: Metabolismo; Biomassa; Reciclagem de células

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1 INTRODUCTION

Bioethanol production in Brazil has aroused worldwide interest, since the product from this process comes from renewable sources, the ethanol (RAMOS et al., 2013). This biofuel is derived from sugarcane by a process that uses yeast for conversion and has competitive prices and low environmental impacts (DELLA-BIANCA and GOMBERT, 2013). However, other biomasses can be employed in this process, depending exclusively on the amount of total soluble solids contained in the raw material, which can be obtained from various raw materials such as saccharins with direct fermentation and the cellulosic and starches (BAZILIO; ALVES and WANDER, 2008),

A good example is the saccharine sorghum that has similar characteristics to sugarcane, which according to the Empresa Brasileira de Pesquisa Agropecuária – Milho e Sorgo (EMBRAPA, 2012) which contains fermentable sugars. In

addition, according to Almodares and Hadi (2009), saccharine sorghum can be directly metabolized by yeasts like sugarcane. This process has as its main condition the quality of raw materials and the choice of yeast, considering that this microorganism is of utmost importance for the substrate bioconversion, thus the process conditions guide the efficiency of this organism metabolism.

Yeasts of the species *Saccharomyces cerevisiae* are microorganisms widely used in Brazilian distilleries for fuel ethanol production representing a successful example of biotransformation, which results in a biofuel with quality, competitive price and low environmental impact (BRANDUARDI; SMERALDI and PORRO, 2008). Therefore, it can be emphasized that the production and amount of other compounds generated in the process result from the stress level to which these organisms are subjected (BASSO et al., 2008). One of the effects of this stress is the production of secondary metabolites, such as glycerol (LIU, WANG, and ZHOU, 2008), which can remain intracellular or be excreted to the extracellular medium (DOS SANTOS et al., 2018).

Intracellular metabolites are a direct consequence of the concentration and properties of enzymatic processes, whose quantity and activity can be directly related with the content of a particular metabolite. In addition, depending on the substrate, chemical compounds may be formed. Sucrose is an example of this, as it can transform into a series of processes that can be corrected or rearranged and form compounds that can be economically exploited (SANTOS et al., 2018), as shown in Figure 1.

Figure 1- Products formed from sucrose

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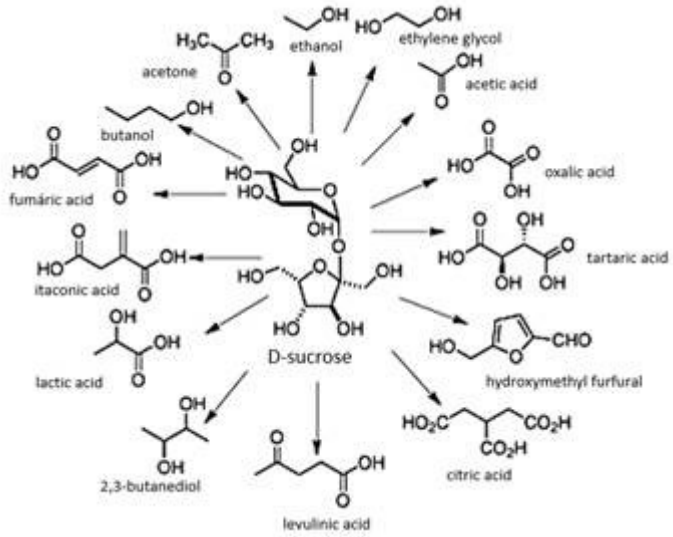
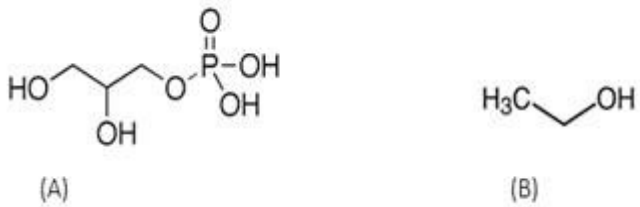


Adapted from Ferreira, Da Rocha and Da Silva (2009)

In the intracellular medium, numerous molecules are produced and display complex functions in different regulatory processes, such as regulation of transcription and transduction, regulation of protein-protein interactions and metabolic regulation of proteins by metabolites (VILLAS-BÔAS et al., 2004; MASHEGO et al., 2007). Figure 2 shows the molecular structure of ethanol and glycerol.

Figure 2- Molecular structure of ethanol (A) and glycerol (B).



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Glycerol is one of the most important metabolites produced by yeast during fermentation (GOMBERT and MARIS, 2015). In this sense, the production of this compound induces the yeast to activate cellular adaptation mechanisms to maintain its integrity in the fermentation medium under different stressing conditions (PEREIRA et al., 2011). According to Da Silva Santos et al. (2018), studies regarding the physiological response of yeasts in relation to metabolites production can influence the choice of yeast for the fermentation process. Thus, this study aims to assess the technological

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KEYWORDS

Amazon Arduino Biodiversity Contamination Educação Ensino Ensino de Matemática Environmental licensing Extension GeoGebra Interdisciplinaridade Programming Remote sensing Survival analysis Sustainability Série histórica Temperature Thermal comfort Turbulence WRF Water pollution

characteristics of the biomasses with potential for ethanol production, as well as evaluate the fermentative parameters and metabolites production of yeast FT-858.

2 MATERIAL AND METHODS

2.1 Technological characteristics of saccharine substrates

The assessment of saccharine substrates was performed by an exploratory qualitative bibliographical study comparing the technological characteristics of saccharine substrates. The study was performed in the laboratory of Biotecnologia, Bioquímica e Biotransformação of Centro de Estudos em Recursos Naturais – CERNA of the Universidade Estadual de Mato Grosso do Sul - UEMS/Dourados-MS.

2.2 Collection and preparation of substrates

The sugarcane broth was collected from the process of Bunge industry, the sorghum broth was collected and processed by Embrapa and both were transported to the laboratory in sterile bottles. This material was filtered in cotton and filter paper in order to remove most of the impurities. The Brix was concentrated by evaporation and monitored using a refractometer. The pH was adjusted to 5.0 using a benchtop pH meter.

2.3 Microorganism

The yeast used in this study was the *Saccharomyces cerevisiae*: FT-858 obtained from Fermentec.

2.4 Pre-inoculum and fermentative condition

The pre-inoculum was prepared using liquid cultivation medium YPD 2%, containing 1.0% (p v^{-1}) yeast extract; 1.0% (p v^{-1}) peptone; 2.0% (p v^{-1}) glucose, autoclaved at 120°C for 20 minutes, in which 0.10 g of lyophilized yeast FT-858 was inoculated and incubated at 30 °C for 10 hours at 250rpm. After this period, cells were collected by centrifugation (800g, 20 min), re-suspended three consecutive times in sterile saline solution (0.85%), resulting in a final concentration of 10 mg mL^{-1} of wet mass that was used for the fermentative experiments. The fermentative experiment was performed in

Erlenmeyer flasks of 125 mL, containing 50mL of sterile sugarcane and sorghum broth, at the concentration of 22 °Brix, incubated at temperatures of 30 and 40 °C at 250rpm. Cellular recycling consisted in recovering the cells by centrifugation and re-inoculating them into the fermentation medium with the same initial characteristics, every 10 hours of fermentation, composing a total of five recycles. From each recycle, aliquots of 4 mL were collected for analyses of intracellular glycerol and ethanol concentration.

2.5 Analytical methods

Cell viability was determined by methylene blue staining (LEE SS, ROBINSON FM, WANG, 1981).

To quantify intracellular glycerol, a cell lysis was performed with the obtained biomass, in which 2 ml of lysis buffer containing (Tris / HCL 0.01m with pH 7.2) and 10g of glass beads were added to a Falcon tubes. The process consisted of 10-minute vortexing steps interspersed for 2 minutes in an ice bath and an additional 10 minutes in an ultrasound vat. The process was accompanied by a microscope to verify the efficiency of the process. The glycerol concentration was determined from the triglyceride analysis enzyme kit (Laborlab®), and consisted of adding 10 µL of sample and 1000 µL of the enzyme reactive to a test tube which after incubated in a water bath at 37 °C for 10 minutes adapted by Sousa Jr et al. (2014).

Ethanol was analyzed on a CG 3900 gas chromatograph equipped with a flame ionization detector (Varian), using a 30 m–long fused silica capillary column (ZB-5). The chromatographic conditions adopted were 1 µL injection volume, 1:20 displacement ratio, 90 °C oven temperature, and 240 °C detector injector temperature. The samples were filtered with a 0.22 µm ultrafilter (BATISTOTE et al., 2010).

2.6 Statistical analyses

The results were analyzed using the Excel 2016 software and expressed as means followed by standard deviations, then the graphs were plotted using the Origin 8 software. All experiments were performed in triplicate.

3 RESULTS AND DISCUSSION

The analysis of technological characteristics of substrates showed that both the sugarcane and the saccharine sorghum broth have a similar composition regarding sugars. However, sugarcane broth featured sucrose content from 14 to 22%, greater than that found in sorghum, from 8 to 13%. The percentage of monosaccharides present in the sorghum broth was higher, 0.5 - 2% fructose and 0.5 - 1.5% glucose and the mean concentration of soluble solids (°Brix) was higher in the sugarcane broth, from 18 to 25 (Table 1).

The biomasses, sugarcane and sorghum, have carbohydrates of direct fermentation in their composition, according to Barcelos (2012), which can be metabolized by yeasts *Saccharomyces cerevisiae* and converted into ethanol (ALMODARES and HADI, 2009). Studies developed by Masson et al. (2015), comparing saccharine sorghum broth with sugarcane broth, found higher Brix values in the sugarcane broth, 21.2%. Serna-Saldívar et al. (2012) reported in their studies values of total soluble solids of 20% for saccharine sorghum broth.

Saccharin substrates have important technological characteristics such as high levels of fermentable sugars and high productivity and have been shown to be an important renewable source to be kept in the energy matrix for ethanol production (ALVES and PAIXÃO, 2018). Given the environmental issues, it is necessary to increase biofuel production to meet the consumption of society and the different industrial segments (MUELLER et al., 2019).

Table 1 – The main chemical compounds present in the biomasses of direct fermentation direct fermentation

Substrates	**Parameters**		
	°Brix	Sucrose (%)	Glucose (%)
Sugarcane	18 – 25	14 – 22	0.2 – 1
Saccharin sorghum	15 – 19	8 – 13	0.5 – 2

Source: Adapted from EMBRAPA – Milho e Sorgo, 2012

FT-858 yeast showed the best cell growth rates in the first

recycle at 30 °C when grown on the substrate based on saccharin sorghum broth, with 77% of viable cells at 18 °Brix and 80% at 22 °Brix. The viable cell rate of this yeast when grown in sugarcane juice at 30 °C was 74 and 76% at 18 and 22 °Brix concentrations respectively. Yeast showed a drop in viability rate throughout the recycle, this loss of viability was observed mainly at 40 °C (Table 2). Noticeably, the yeast showed higher percentage of viable cells when grown on sorghum broth.

In studies on the physiology of industrial yeast strains performed by Moreira et al. (2015), growing strains on substrate based on sugarcane broth at 12, 15, 24 and 30 °Brix and temperature of 30 °C. The authors observed that the best rates were obtained at the time of 8 hours of fermentation at 15 °Brix, with cell viability of 96% and ethanol concentration of 8.5% (v v^{-1}), concluding that the temperature of 30 °C provided the best results. This physiological response corroborates the studies of Batistote et al. (2010), which highlights that fermentative processes for ethanol production can happen in a temperature range from 30 to 35 °C depending on the region where industries are located.

In the works developed by Santos et al. (2018), studying the physiological response of yeast FT-858 grown in sugarcane juice and saccharin sorghum, observed that this strain presented the best results at 30 °C for both viability and biomass in both substrates. Changes in the fermentative environment such as nutrient availability, pH, temperature, contamination may cause loss of fermentative efficiency and cell viability, and may infer the cellular mechanisms of yeast (AMORIM et al., 2011). Studies carried out on saccharin substrates under different cultivation conditions showed that different industrial yeast strains presented different concentrations of ethanol. Our results also showed that under different fermentative niche conditions, yeast has a different physiological response to substrate-to-product conversion.

Table 2- Assessment of fermentative parameters of FT-858 yeast on saccharine substrates under different growing conditions

			Viability (%)
	Sugarcane		
Substrate	18 °Brix	22 °Brix	18
Fermentation Recycling			Temperature of 30
1	74.00 ± 0.01b	76.02± 0.08ab	77.01
2	50.01 ± 0.14e	53.00 ± 0.05de	58.02
3	35.02 ± 0.01f	37.01 ± 0.00f	33.0(
			Temperature of 40
1	55.00 ± 0.07de	56.01 ± 0.08d	57.01
2	37.02 ± 0.12f	35.01 ± 0.09f	37.0:
3	26.03 ± 0.05g	25.04 ± 0.04g	27.0(

Values (means ± standard deviation) followed by the same letter are not statistically different from each

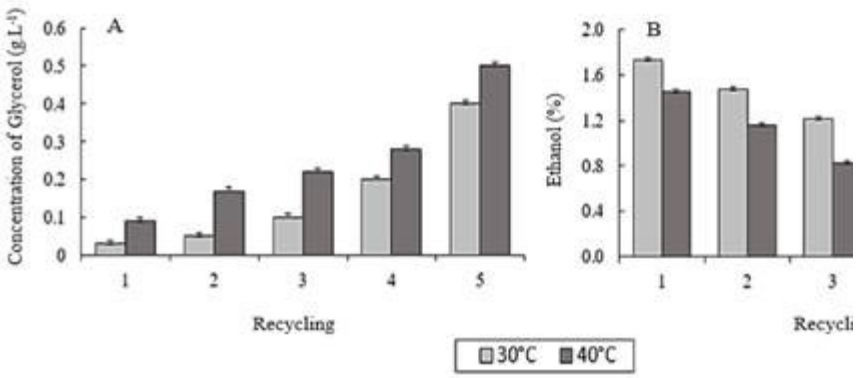
other (P <0.05) by the Tukey test at 5% significance.

The analysis of intracellular glycerol and ethanol quantification showed that there was an inversion in the metabolites production along recycling. At the temperature of 30 °C, glycerol accumulation of the fifth recycle in relation to the first was 0.37 g L^{-1} on sugarcane broth. At 40 °C, the accumulation of this metabolite was 0.40 g L^{-1} (Figure 3A). Noticeably, the ethanol concentration declined along recycling at both temperatures (Figure 3B). It can be observed that glycerol accumulation occurred throughout the recycles as well as at the highest temperature. Possibly the synergisms of stress factors have favored a higher production of this metabolite, leading the yeast to a physiological response in relation to stress factors present in the fermentative niche.

The yeasts *Saccharomyces cerevisiae* have the ability to reverse their metabolic pathway and produce, for example, glycerol which can both be excreted to the medium and be retained in its interior as a mean of protection against stressing conditions to which they are subjected (HUBMANN et al., 2011). According to Wei et al. (2013), yeast cells can control the internal glycerol content by channels located in the plasma membrane with the aim of promoting a balance between the intracellular and extracellular medium. This mechanism is directly related with cell survival to adapt to stress (TULHA et al., 2010).

Figure 3 - Profile of intracellular glycerol accumulation (A) and ethanol concentration (B) of the yeast FT-858 on

substrate based on sugarcane broth performing cellular recycling at the concentration of 22 °Brix



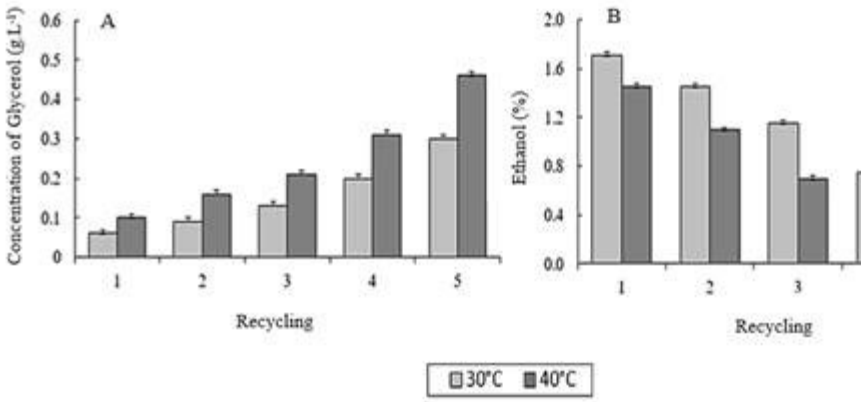
The yeast grown on saccharine sorghum broth at the temperature of 30 °C showed similar glycerol accumulation along recycling to that observed on sugarcane broth. However, this accumulation was lower at both temperatures, 0.24 g L^{-1} at 30 °C and 0.37 g L^{-1} at 40 °C (Figure 4A). A decline in ethanol concentration was also observed on this substrate along recycling at the temperatures studied (Figure 4B).

Studies by Dos Santos et al. (2018), which evaluated the accumulation of glycerol in Catanduva-1 yeast grown in sugarcane juice at concentrations of (18, 22 and 25 °Brix) under different cultivation conditions. The authors report that a higher glycerol accumulation of 0.50 g L^{-1} occurred at 40 °C at the highest Brix concentration. It can be observed that the associated stress factors can trigger physiological mechanisms that lead the yeast to produce more glycerol over ethanol. Given that glycerol is a cell protection related compound.

The by-products of fermentation such as glycerol are formed in response to the stress level of the medium, presenting, therefore, an important role for control of osmotic pressure in the microorganism in relation to fermentation medium (BASSO et al., 2008). For Pérez-Torrado et al. (2016), the main cause of accumulation of this metabolite is temperature oscillation. The growing conditions, cellular recycling and high temperature, affected the yeast's metabolites biosynthesis pathway, since there was glycerol production to the detriment of ethanol concentration. This might happen because the ethanol concentration is related to glycerol synthesis in *Saccharomyces cerevisiae* and when there is a high rate of intracellular glycerol,

the yeast inhibits the ethanol production according to Pagliardini et al. (2013).

Figure 4 - Profile of intracellular glycerol accumulation (A) and ethanol concentration (B) of the yeast FT-858 on substrates based on saccharine sorghum performing cellular recycling at the concentration of 22 °Brix



CONCLUSIONS

The sucrose substrates analyzed are similar to the composition of fermentable sugars, which favored an efficient fermentative performance of yeast FT-858.

Glycerol and ethanol accumulation were inversely proportional independent of saccharin substrate. However, the associated stress factors caused alterations in the yeast metabolite production profile which influenced a higher glycerol accumulation. Thus, saccharin sorghum has great potential to be used in ethanol production, since it can be processed in the same industrial facility.

ACKNOWLEDGMENT

Fundação de Apoio ao Desenvolvimento do Ensino, Ciência e Tecnologia do Estado de Mato Grosso do Sul (FUNDECT), Financiadora de Inovação e Pesquisas (FINEP), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (311975/2018-6 CALC); Programa Institucional de Bolsas aos Alunos de Pós-Graduação (PIBAP) da Universidade Estadual de Mato Grosso do Sul (UEMS); Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil

(CAPES) – funding code 001.

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