Determination of the percentage of ethanol produced by Saccharomyces cerevisiae from semi-purified glycerin

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Abstract. The percentage of ethanol produced by Saccharomyces cerevisiae yeast in semi-purified glycerin at three concentrations (2.8%, 5%, 10%) was determined. Two phases were established: an aerobic phase evaluating temperatures of 20ºC and 37ºC with an inoculation rate of 4% and 1vvm (volume/volume of medium per minute) and an anaerobic phase at a temperature of 10ºC, which were monitored for a period of 36 hours. The main kinetic parameters such as specific growth rate, doubling time and the percentage of ethanol produced by the Winnick technique were determined. The results showed that the best temperature to produce biomass of S. cerevisiae is given around 20ºC with concentrations of 1.30x10⁸ cells/ml with a maximum peak biomass production at 21 hours, which differ significantly from the other treatments evaluated (Sig.=0.000). The highest production of ethanol was obtained in the treatment of 20% glycerin and 10% of inoculum of the microorganism (p<0.05) at a temperature of 10ºC, corresponding to 176.87mg of ethanol/ml of glycerin. Therefore, the viability of this agroindustry by-product is deduced as an alternative source for the biomass and ethanol production from Saccharomyces cerevisiae.

1. Introduction
At world level, during the last few years, crude oil reserves have experienced a marginally decreasing increase, while production has varied according to demand and the production policies of the Organization of Petroleum Exporting Countries (OPEC), registering high reserve-production ratios (R/P) that in some cases were higher than 100 years, thus achieving an R/P of 85.3 years, in contrast, with the countries that make up the Organization for Economic Cooperation and Development (OECD), which contribute 6.8% of the world’s total proven reserves with a R/P (reserve/production) ratio of 13.5 years[1]. Similarly, the volume of crude oil reserves has shown a downward trend over the last ten years, with an average annual reduction of 5.7%, while production has registered a decrease of 0.5% on average over the same period. According to the oil statistics report, the remaining oil reserves in 2011 totaled 2259 million barrels. Compared to 2010, reserves increased by 201 million barrels, or 9.8 percent [2].

In this sense, it is important to be more energy efficient, for this reason, at present work is being done on obtaining two biofuels: biodiesel (based on vegetable oils) and bioethanol (through the fermentation of the by-product, glycerol), where in comparison with lignocellulosic raw materials, the production of ethanol based on glycerol is considered cheaper because it is not necessary to subject the raw material to pre-treatment. However, nowadays the increase in demand for biodiesel, there are fears that this
production will generate an overabundance of glycerol, with the consequent problem of management or elimination of this [3]. There are different possible applications for glycerin and its derivatives, which are marketed according to their quality grades; but the truth is that there are still no proposals for the use of glycerin obtained as a by-product of biodiesel, due to the fact that in general it does not meet the minimum quality standard and its refining cost leads to a product that is not viable [4].

For this reason, the research of other resource recovery strategies has been motivated, being an option the biocconversion of glycerol to high value compounds through anaerobic microbial fermentation since glycerol is not only economical and abundant but also offers the opportunity to obtain reduced chemical products such as succinate, ethanol, xylitol, propionate, hydrogen, etc. due to its higher degree of sugar reduction, with higher yields than those obtained using sugars [5], which represents a promising pathway to economic viability in the biofuels industry. Chloë et al. [6] and González et al. [7], have proposed glycerin as an alternative for the bioethanol production in replacement of corn and sugarcane, through anaerobic fermentation, such studies have shown that 99.5% purity ethanol is obtained from raw glycerin. Nwachukwu et al. [8], studied a strain of Enterobacter aerogenes, designed for the efficient conversion of glycerin into ethanol, obtaining 1.02mol ethanol/glycerin mol after 120 hours of processing. Glycerin has also become an ideal raw material for producing reduced compounds through anaerobic fermentation. Yazdani and González [9], identified the environmental conditions that allow glycerin fermentation in Escherichia coli, along with the pathways and mechanisms that mediate this process for the efficient conversion of raw glycerin into ethanol at a lower cost than the traditional processes that use sugarcane. Dharmadi et al. [5], reported glycerol fermentation from E. coli, where they analyzed pH dependence and CO2 availability. On the other hand, Ito et al. [10], showed that glycerol at a concentration of 10g/L was consumed almost completely within 84h and the main products obtained were ethanol and succinic acid with molar yields of 86% and 7%, respectively. According to these authors, E. coli is a good biocatalyst for the conversion of glycerol into ethanol and hydrogen.

However, Saccharomyces cerevisiae yeast is the most commonly used microorganism in the production of ethanol by fermentation, because it allows a better separation process after fermentation, also produces a much lower toxin content than other microorganisms and under anaerobic conditions, reduces pyruvate to ethanol with CO2 emissions, obtaining a theoretical stoichiometric yield of 0.511g ethanol and 0.489g CO2 per 1g of metabolized glucose. However, it has been observed that, at an experimental and industrial level, only between 87% and 95% of the theoretical yield for ethanol is reached, since this yeast also uses glucose in the production of other by-products and its use is preferred because it requires lower handling costs, since the presence of sucrose in some substrates does not affect ethanol yield, and the by-products of fermentation and the concentration at which these are produced do not create side effects[11]. Some research by Yu et al. [12], shows improved ethanol production using glycerol from a metabolically designed strain of Saccharomyces cerevisiae. The high rates of glycerol utilization were achieved by simultaneous overexpression of glycerol dehydrogenase (gcy) and dihydroxyacetone kinase (Dak). Overall ethanol production in the yph 499 strain (pGeyaDak, pGupCas) was 3.4 times higher than in the wild strain, with about 2.4g/L ethanol produced. Subsequent studies demonstrated the possibility of providing protection against osmotic pressure while increasing the amount of ethanol and reducing glycerol production in S. cerevisiae strains, using glycerin as a carbon source [12].

In this sense, at the regional level, there are still no studies known that show interest in the use of glycerol as a by-product obtained from the production process of the oil palm, known as one of the main economic axes according to the Norte de Santander development plan. For this reason, the purpose of this investigation was to determine the percentage of ethanol produced by Saccharomyces cerevisiae by semi-purified glycerin fermentation.

2. Materials and methods
The experimental work was carried out in the Microbiology laboratories of the Universidad de Santander (UDES), Cúcuta, Colombia. Semi-purified glycerine used for the experiments was supplied by a biodiesel production company. Table 1 specifies the composition of the substrate (glycerine).
### Table 1. Chemical composition of glycerin.

| Test               | Unit | Method   | Results |
|--------------------|------|----------|---------|
| Glycerin Content   | %    | A0CS Ea 6-94 | 80.13   |
| Moisture- Karl Fischer | %    | ASTM E203     | 10.00   |
| Methanol           | %    | EN 14110    | 1.31    |
| Ash                | %    | ASTM D482   | 5.03    |
| MONG               | %    | Std method 3-137 | 4.84  |
| Salt (NaCl)        | %    | USP 32      | 5.10    |

2.1. Reactivation of Saccharomyces cerevisiae

The dry yeast Saccharomyces cerevisiae was used, 50ml of sterile distilled water and 5g of sugar were added to a beaker; it was mixed and heated to a temperature of 37°C, then 1g of it was added, mixing constantly until homogenized. It was lowered and allowed to cool to room temperature. After 15 minutes a sample was taken and sown by exhaustion in Saboreaud Agar, incubated at 37°C for 48 hours.

2.2. Production of Saccharomyces cerevisiae at laboratory scale (aerobic phase)

The growth of Saccharomyces cerevisiae (Sc) was studied using growth curves, using yeast extract broth (CEL) as the control medium and semi-purified glycerin (GSP) at three concentrations, in a batch culture system, performing repetitive tests to determine the standard growth curve of this microorganism. The experimental design consisted of three treatments in triplicate, under two temperature conditions (20°C and 37°C), 1vvm of aeration (air volume/volume of medium a minute) and a working volume of 0.8L. In the monitoring of the first 12 hours of work, three-hour intervals were established; after this period of work, other monitoring was carried out until 24 hours were completed. The main parameter evaluated in this phase was the production of biomass by direct counting in Neubauer's chamber, these measurements allowed to determine the kinetic variables of importance among which are, specific growth rate (μ) shown at Equation (1), doubling time (td) shown at Equation (2) and biomass yield. Where, X is the biomass at the end of the aerobic phase, X₀ is the initial biomass and Δt is the measure of time in the aerobic phase.

\[ \mu = \frac{\ln(x) - \ln(x_0)}{\Delta t} \]  
\[ t_d = \frac{\ln(2)}{\mu} \]

2.3. Optimization of the culture medium and standardization of fermentation (anaerobic phase)

The fermentation or anaerobic phase was carried out on a scale of 2L for 24 hours for a total period of 36 hours at a temperature of 10°C, the optimum temperature to increase the production of this metabolite (ethanol) [13]. The experimental design was a factorial design conformed by a culture medium at 3 different concentrations, for a total of 3 treatments (2.8%, 10% and 20%), each one at concentrations of 1%, 5% and 10% of the inoculum, the same were made in triplicate, for a total of 27 assemblies. The variables evaluated were biomass concentration, pH and ethanol percentage.

2.4. Ethanol production yield by Saccharomyces cerevisiae

For the determination of ethanol, different concentrations of the inoculum (1%, 5% and 10%) were evaluated, after knowledge of the behavior of Saccharomyces cerevisiae in the substrate in the first stage of aerobic and a second stage of anaerobic processes. The determination of the ethanol content was carried out at the University de Santander campus Cucuta using the Winnick technique.

3. Results and discussion

Figure 1 shows the growth curve developed by Saccharomyces cerevisiae in the medium glycerine and yeast extract broth (CEL) at temperatures of 20°C and 37°C; showing for the glycerine medium at 20°C
the logarithmic phase between 6 and 21 hours of culture and at 21 hours the time in which the microorganisms experience their maximum growth potential with 1.3x10⁶ cel/ml, while at 37°C this microorganism reaches its maximum potential at 24 hours with concentrations of 1.2x10⁶ cel/ml.

![Average growth curve of Saccharomyces cerevisiae](image)

**Figure 1.** Average growth curve of Saccharomyces cerevisiae.

According to the kinetic data reported in Table 2, it is established that when using semi-purified glycerin for the growth of *S. cerevisiae* at 20°C, a higher specific growth rate (μ) of 0.2923h⁻¹ approx. is obtained, this value exceed the results obtained by this microorganism in the same medium at a temperature of 37°C (0.2292h⁻¹ approx.), without forgetting that in the CEL control medium the value of this same parameter (μ) is 0.0107h⁻¹ and 0.0989h⁻¹ respectively, this confirms what Álvarez and Sierra [14] and Fuentes *et al.* [15], who state that the low doubling times in the development of a microorganism clearly imply high rates of specific growth speed (μ). According to Garzón and Hernández, [16], this means that the fermentation stage (ethanol producer) can begin in a shorter time, which in the long term reduces the production costs of alcohol when it is taken to industrial scale. These results differ from those obtained by Sarmiento [17], who obtained a doubling time for *S. cerevisiae* equal to 2.83h by using solid media based on molasses at different concentrations. Studies by Villegas *et al.* [13], state that when using substrates such as cane molasses and fresh de-proteinized serum hydrolyzed at room temperature, *S. cerevisiae* can generate doubling times of 2.2h and 1.3h with concentrations of 2.2x10⁶cells/ml respectively, this data being very close to those obtained in this study from glycerin. This shows how this substrate can replace traditional carbohydrates such as sucrose, glucose and starch in some industrial fermentation processes, since due to its extensive presence in nature, *S. cerevisiae* yeast can be used as a source of carbon and energy [3].

**Table 2.** Comparative analysis of the kinetic behavior of *Saccharomyces cerevisiae* in semi-purified glycerin and CEL media.

| Treatment  | Specific growth rate (μ) | Duplication times (Td) | Maximum cell concentration |
|------------|--------------------------|------------------------|----------------------------|
| CEL 20°C.  | 0.0107h⁻¹                | 64.80h                 | 1.14x10⁶ cells/ml          |
| CEL 37°C.  | 0.0989h⁻¹                | 7.00h                  | 1.20x10⁶ cells/ml          |
| Glic. 20°C.| 0.2923h⁻¹                | 2.37h                  | 1.30x10⁶ cells/ml          |
| Glic. 37°C.| 0.2292h⁻¹                | 3.02h                  | 1.18x10⁶ cells/ml          |

Nevertheless, the kinetic coefficients obtained (Table 2), allowed to deduce that the best temperature for the production of biomass of this yeast in aerobic phase, is given with the treatment based on
glycerin at 20°C, which is corroborated when observing the minimum differences between the means of the cell concentration obtained in the CEL at 37°C (T1), the mean of the treatment with Glycerin at 37°C (T2) (Sig.=0.023) and the mean of the treatment with Glycerin at 20°C (T4)(Sig.=0.000), being these three significantly different from the CEL at 20°C (T3) (Sig.=0.224). Similarly, minimal differences are observed between CEL at 20°C (T3) and Glycerin treatment at 20°C (T4) (Sig.=0.003).

3.1. Establishment of the process conditions (culture medium and temperature) for the optimization and standardization of fermentation

Preserving the initial growing conditions (pH 6.0±0.5, T° 20°C and 1vvm) for 18 hours, S. cerevisiae shows better biomass yields in the semi-purified glycerin substrate, surpassing the CEL commercial synthetic medium, as shown in Figure 2 of the interaction of the microorganism, growing medium and its effect on the biomass reached. After this interval of time, the anaerobic phase of fermentation began, where it was kept at a temperature of 10°C because temperature is a determining factor in the growth and development of microorganisms, directly influencing the bioconversion rates of sugars into ethanol [18]. These results differ from those obtained by Villegas et al, [13] Those who found that the highest percentages of alcohol were achieved by subjecting the culture media to temperatures of 28 to 32°C, regardless of the microorganism and the culture medium evaluated, possibly due to the higher metabolic rate reached by the microorganisms, although as suggested by Garzón and Hernández [16], an increase in temperature increases the probability of obtaining greater traces of methanol and propanol in the final distilled product, this factor is not relevant in the present study because it is not intended for human consumption, but for its possible use as fuel alcohol.

![Figure 2](image)

**Figure 2.** Interaction diagram of the variables microorganism, culture medium and its effect on the biomass reached, according to Tukey (p<0.05).

3.2. Ethanol production yield of Saccharomyces cerevisiae from semi-purified glycerin

Maintaining a temperature of 10°C and pH between 5.0 and 6.0 in the anaerobic phase (Figure 3), it was established that the production of ethanol depends on the interaction between the concentration of glycerin and the concentration of the microorganism inoculum, which demonstrates that the treatment at a concentration of 20% glycerin and a concentration of 10% of inoculum of the microorganism under study (p<0.05) gives the best results in terms of ethanol concentration at a temperature of 10°C, corresponding to 176.87mg of ethanol/ml of glycerin (Table 3), these results coincide with those
reported by Peña and Arango,[19] who state that when S. cerevisiae is grown at high concentrations of sugar (less than 30-40%) increases ethanol production. These results differ from those obtained by Villegas et al. [13], who analyzed ethanol production according to the culture medium, obtaining the highest yields when using hydrolyzed deproteinized fresh serum as a substrate with significantly higher levels of ethanol production (p<0.05) in the control medium (molasses). Therefore, the viability of this agroindustry by-product is deduced as an alternative source for ethanol production.

![Figure 3](image-url)

**Figure 3.** Interaction between the concentration of glycerin, the concentration of the microorganism and the percentage of ethanol produced, evaluated according to Tukey (p<0.05).

| Glycerin concentration in the medium (%v/v) | Percentage of inoculation | Ethanol concentration (mgEthanol/mlGlycerin) |
|-----------------------------------------|----------------------------|---------------------------------------------|
| 2.8                                     | 1                          | 17.65                                       |
|                                         | 5                          | 10.00                                       |
|                                         | 10                         | 36.07                                       |
| 10                                      | 1                          | 36.06                                       |
|                                         | 5                          | 41.05                                       |
|                                         | 10                         | 40.70                                       |
|                                         | 1                          | 89.78                                       |
| 20                                      | 5                          | 109.00                                      |
|                                         | 10                         | 176.87                                      |

**Table 3.** Percentage of ethanol produced at different concentrations of glycerine and inoculum.

4. Conclusions

According to this research it is concluded that the cell concentration of the yeast is affected by the incubation temperature of the yeast, considering the treatment with Glycerin at 20°C as the optimal treatment to achieve the highest number of biomass of *Saccharomyces cerevisiae* in comparison with the treatment with Glycerin at 37°C and the Control (CEL) treatment at 20°C.

It is concluded that the concentration of the substrate and the percentage of inoculation of the microorganism influence the production of ethanol, having that the treatment at a concentration of 20% glycerin and 10% *Saccharomyces cerevisiae* inoculum (p<0.05) produced the highest concentration of ethanol at a temperature of 10°C, corresponding to 176.87mg ethanol/ml of glycerin.

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