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Optimization of a low-cost defined medium for alcoholic fermentation – a case study for potential application in bioethanol production from industrial wastewaters

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In bioethanol production processes, the media composition has an impact on product concentration, yields and the overall process economics. The main purpose of this research was to develop a low-cost mineral-based supplement for successful alcoholic fermentation in an attempt to provide an economically feasible alternative to produce bioethanol from novel sources, for example, sugary industrial wastewaters. Statistical experimental designs were used to select essential nutrients for yeast fermentation, and its optimal concentrations were estimated by Response Surface Methodology. Fermentations were performed on synthetic media inoculated with 2.0 g L^{-1} of yeast, and the evolution of biomass, sugar, ethanol, CO_2 and glycerol were monitored over time. A mix of salts [10.6 g L^{-1} $(NH_4)_2HPO_4$; 6.4 g L^{-1} $MgSO_4{\cdot}7H_2O$ and 7.5 mg L^{-1} $ZnSO_4{\cdot}7H_2O$] was found to be optimal. It led to the complete fermentation of the sugars in less than 12 h with an average ethanol yield of 0.42 $g_{ethanol}/g_{sugar}$. A general C-balance indicated that no carbonaceous compounds different from biomass, ethanol, CO_2 or glycerol were produced in significant amounts in the fermentation process. Similar results were obtained when soft drink wastewaters were tested to evaluate the potential industrial application of this supplement. The ethanol yields were very close to those obtained when yeast extract was used as the supplement, but the optimized mineral-based medium is six times cheaper, which favorably impacts the process economics and makes this supplement more attractive from an industrial viewpoint.

Introduction

Currently, bioethanol is one of the most important renewable fuels. It is added to gasoline to reduce the negative environmental impact generated by the worldwide use of fossil fuels [1]. Several energy crops, including sugarcane, corn and jatropha, are used as raw materials for bioethanol production [2,3]. The high worldwide bioethanol demand exerts enormous pressure on primary production capacity. Thus, it is imperative that new renewable sources are identified for the production of this 'green' fuel [4]. As such, several alternatives have received increased focus, such as lignocellulosic biomass [5,6], regional agricultural discards [7] and wastewaters of the soft drink industry [8]. Although lignocellulosic residues represent an attractive renewable source for bioethanol production, the technology is not sufficiently developed, and the large quantity of wastewaters produced by the fermentation process poses a problem for large-scale production. Sugar-sweetened beverage wastewaters are generated in large quantities in proportion to the high production of these beverages (e.g., 6000 million L/year in Argentina), and some of them exhibit a high sugar content of approximately 60–180 g L^{-1}. In addition to using renewable raw materials, these alternative processes are environmentally friendly, and when compared to the bioethanol production from energy crops, they neither demand natural resources nor compete for land that could be used for food production.

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In biotechnology-based industrial processes, the composition of media is of critical importance due to its impact on product concentration and yield as well as on the overall process economics. To produce bioethanol at a laboratory scale, media was generally supplemented with yeast extract to provide specific nutrients such as organic nitrogen (in the form of amino acids and dipeptides), trace elements and vitamins. However, it is interesting to explore other sources of these additional nutrients in order to diminish production costs. For instance, mineral salts are generally used in industry to supplement the fermentation media and provide acceptable yields.

The main purpose of this research was to develop a low-cost mineral-based medium to replace yeast extract as the supplement for successful alcoholic fermentation. In this context, selected soft drink wastewaters were chosen as a test media to evaluate the potential industrial application of this supplement.

The use of a statistical approach has gained traction for medium optimization and for understanding the interactions among various factors using a minimum number of experiments. The combination of Full Factorial Designs (FFD) and Response Surface Methodology (RSM) is a commonly used method to assess the optimal media compositions and fermentation conditions, and it is an efficient statistical technique for the optimization of multiple variables [9]. This method has been successfully applied to optimize the composition of different media for alcoholic fermentations [10–13].

In this study, a statistically designed approach was used to optimize a low-cost mineral-based supplement for alcoholic fermentations mediated by *Saccharomyces cerevisiae*. The minerals that significantly improved the ethanol production were selected according to a series of FFD whereas the optimal concentration of key factors and parameter analysis were performed using a Central Composite Design (CCD) and RSM. Furthermore, the optimized mineral supplement was compared with the yeast extract in fermentation assays performed on a synthetic medium and on selected wastewaters. The concentrations of biomass, sugars, glycerol and ethanol, as well as the carbon dioxide production, were monitored over time in these experiences.

Material and methods

Strain, media and fermentations

The commercial yeast strain *S. cerevisiae* var. Windsor (Lallemand Brewing Co., Felixstowe, UK) was used throughout the screening and optimization experimental designs. Stock cultures were maintained on YPD (yeast extract 5 g L^{-1}, peptone 5 g L^{-1} and D-glucose 20 g L^{-1}) agar plates at 4°C. The culture was transferred to fresh medium monthly.

Fermentation assays were performed in triplicate using 500-mL glass flasks (300-mL working volume) operated in batch mode under anaerobic conditions and at a constant temperature of 30°C. Once inoculated with biomass, the reactors were closed until the end of the experiments. The gasses outlet passes through a water trap and the sampling was always outward, so that no air entry during the experiences. Despite that experiments began under a microaerobic atmosphere due to the oxygen present in the headspace of the reactor and in the initial fermentation medium, it is quickly displaced by the CO_2 produced during fermentation. Therefore, it can be assumed that the assays were carried out under anaerobic conditions. The pH was initially adjusted to 4.50 ± 0.10 and an orbital shaking (100 rpm) was maintained along the experiments to avoid the biomass precipitation. The initial concentration of yeast in each assay was 2.00 ± 0.10 g L^{-1}. The samples were collected immediately after inoculation ($t = 0$) and every 1.5 h until the end of the experiments.

Analytical procedures

During the fermentation assays, samples (1 mL) were taken in duplicate and immediately centrifuged for 5 min at 1200 × *g*. The pellet (yeasts) was washed five times with distilled water and resuspended to the starting volume prior to biomass determination. The initial supernatants were transferred to sterile 1.5 mL tubes and stored at −20°C until the corresponding determination.

The biomass concentration was indirectly determined by turbidity measurements at 600 nm using a VIS spectrophotometer (DR/2010, HACH, USA). These measurements were correlated to biomass concentration using a calibration curve built according to the standard technique for determination of Volatile Suspended Solids (VSS). To build the calibration curve, the yeasts were grown on YPD medium at 30°C for 12–18 h and were then harvested by centrifugation for 5 minutes at 1200 × *g*, washed five times using distilled water. Several dilutions on distilled water were made by triplicate. An aliquot of each diluted sample was used for measure of turbidity (at 600 nm) using distilled water as blank. Another aliquot of the well-mixed sample (50-mL) was filtered in vacuum through a weighed standard Whatman GFC glass fiber filter (47 mm diameter and 1.2 μm nominal pore size, Biopore, Buenos Aires, Argentina) and the residue retained on the filter was dried to a constant weight at 103–105°C. The increase in weight of the filter represents the total suspended solids (TSS). The next step was the combustion of the filter at 500°C for 15 minutes and the weight lost after combustion represents the weight of Volatile Suspended Solids (VSS) in the sample [14].

The total sugar content was determined using the phenol-sulfuric acid colorimetric method [15], and the reducing sugar content was measured using the Miller colorimetric method [16]. The sugar concentration was calculated indirectly using a standard curve constructed from different concentrations of D-glucose (Merck, NJ, USA).

The ethanol concentration was determined using a device based on a SnO_2 sensor (TGS Figaro 2620; Figaro Engineering Inc., Osaka, Japan) as described in a previous work [8]. The CO_2 production was measured online using a mass flowmeter with a transducer (Matheson, East Rutherford, NJ, USA) and the total CO_2 production was estimated by integration. Glycerol was measured using an enzymatic kit (SB Lab., Santa Fe, Argentina), whereas ammonium, magnesium, zinc and inorganic phosphorus were determined by colorimetric methods (Wiener Lab., Rosario, Argentina).

Identification of the most important nutrient components

A statistical approach was performed to screen the following salts at the initial concentration recommended in the literature: $(NH_4)_2SO_4$ 10 g L^{-1}, $(NH_4)_2HPO_4$ 10 g L^{-1}, NH_4Cl 8 g L^{-1}, K_2SO_4 13.2 g L^{-1}, K_2HPO_4 13.2 g L^{-1}, KCl 11.3 g L^{-1}, $MgSO_4{\cdot}7H_2O$ 5 g L^{-1}, $MgCl_2{\cdot}6H_2O$ 4.2 g L^{-1}, $ZnSO_4{\cdot}7H_2O$ 10 mg L^{-1}, $ZnCl_2$ 4.8 mg L^{-1}, $CaSO_4{\cdot}2H_2O$ 2.2 g L^{-1}, $CaCl_2$ 1.4 g L^{-1}, $FeSO_4{\cdot}7H_2O$ 5 mg L^{-1}, $FeCl_3{\cdot}6H_2O$ 4.8 mg L^{-1}, $CuSO_4{\cdot}5H_2O$ 10 mg L^{-1}, $CuCl_2$ 5.3 mg L^{-1}, $CoSO_4{\cdot}7H_2O$ 5 mg L^{-1}, $CoCl_2$ 2.2 mg L^{-1}, $MnSO_4{\cdot}H_2O$