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Alternative non-chromatographic method for alcohols determination in *Clostridium acetobutylicum* fermentations

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ABSTRACT

An economic, simple, quantitative, and non-chromatographic method for the determination of alcohols using microdiffusion principle has been adapted and validated for acetone-butanol-ethanol (ABE) fermentation samples. This method, based on alcohols oxidation using potassium dichromate in acid medium, and detection by spectrophotometry, was evaluated varying, both, temperature (35 °C, 45 °C, and 55 °C) and reaction time (0 to 125 min). With a sample analysis time of 90 min at 45 °C, a limit of detection (LOD), and a limit of quantification (LOQ) of 0.10, and 0.40 g/L, respectively. The proposed method has been successfully applied to determine butanol and ethanol concentrations in ABE fermentation samples with the advantage that multiple samples can be analyzed simultaneously. The measurements obtained with the proposed method were in good agreement with those obtained with the Gas Chromatography Method (GCM). This proposed method is useful for routine analysis of alcohols and screening samples in laboratories and industries.

1. Introduction

Butanol is a potential second generation biofuel, being a better alternative to gasoline or diesel regarding combustion characteristics, engine performance, and exhaust emissions (Jin et al., 2011). The interest in producing butanol by fermentation has been increasing as a renewable fuel alternative from petroleum (Lee et al., 2008; Singh and Singh, 2011). There are many butanol-producing microorganisms, the mayor producers are *Clostridium acetobutylicum*, *Clostridium beijerinckii*, *Clostridium saccharoperbutylacetonicum* and *Clostridium sacharobutylicum*, being *Clostridium acetobutylicum* the most studied for scientific research (Lehmann and Lütke-Eversloh, 2011), thus has been considered the study model. *Clostridium acetobutylicum* produces hydrogen, carbon dioxide, acetate and butyrate in the acidogenic phase, and acetone, butanol and ethanol in the solventogenic phase; this two-staged process is called ABE fermentation with solvents concentrations up to 2% (w/v) using a wild strain (Jones and Woods, 1986). During a fermentation, several parameters can be monitored and controlled, such as biomass, lipids or ethanol with in-situ sterilizable probes, however this probes are costly, and online measurement of other fermentation products is not always feasible. The determination of products, as acids and alcohols, in ABE fermentations by *Clostridium acetobutylicum*, has been reported using Gas Chromatography and High Performance Liquid Chromatography (HPLC) methods (Buday et al., 1990; Tsuey et al., 2006; Lin et al., 2014). Nonetheless, these methods have some disadvantages such as expensiveness, and only one sample can be processed per runtime.

There are some alcohol detection kits that rely on enzymatic reactions and subsequent spectrophotometric detection with a dye (Fu, 2009; Ehrlich et al., 2012), most commonly used for food and tissue samples, since they are practical and fast to use, but are non-specific since they can detect all the linear short-chained alcohols (e.g. methanol, propanol, butanol) if the analyzed sample contains them. There is also a method for alcohol detection based on a chemical reaction, it is called microdiffusion and was described by Conway for analysis of volatile compounds in samples with a complex matrix (Conway and Malley, 1942).

The microdiffusion method is based on the oxidation of the volatile compounds present in a sample, the reaction occurs in a closed chamber with two separate compartments: one with the sample containing the volatile compounds; the other with an oxidizing agent. It also requires an incubation time with high temperatures to increase the volatilization rate of the compounds and reach the compartment with the oxidizing

Abbreviations: GCM, Gas Chromatography Method; MAM, Microdiffusion Adapted Method; ABE, acetone-butanol-ethanol; GMM, Gu modified medium; CV, coefficient of variation; LOQ, limit of quantification; LOD, limit of detection; std, standard; CE, correlation error.

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agent. If the method is applied to oxidize linear short-chained alcohols the oxidizing agent used is a solution of $K_2Cr_2O_7$ dissolved in H_2SO_4. When the reaction takes place, the chromium (VII) present is reduced to chromium (III), in this reduction a color change occurs, from clear orange, to yellow, until it reaches clear blue, depending on the quantity of linear short-chained alcohols present. This method has been used to determine linear short-chained alcohols like: low ethanol concentration in fruit juice (Gros, 2011), and in biological samples (Culik, 1986; Fakruddin, 2013). It has also been used to determine other volatile compounds, such as cyanide and azide in beverages (Rubio et al., 1987; Tsuge et al., 2001).

The aim of the present work is the adaptation and validation of the microdiffusion method as an economic, simple, rapid, and quantitative method to determine the concentration of alcohols (butanol and ethanol) in ABE fermentation samples. The proposed method is also compared with a traditional Gas Chromatography Method. To the best of our knowledge, this is the first report which describes a semi-quantitative test for butanol in ABE fermentation samples using an alternative non-chromatographic method which provides measurement of multiple samples in the same run.

2. Experimental

2.1. Chemicals

All chemicals used were of analytical grade: Acetone (99.5%), ethanol (99.5%), and acetic acid (99.7%) from Tecsiquim (Toluca, Mexico); butyric acid (99%), and n-butanol (99.8%) from Baker analyzed (Berlin, Germany); Na_2CO_3 (99.5%), and H_2SO_4 was obtained from Merck (Berlin, Germany); $K_2Cr_2O_7$ from Mallinckront (Dublin, Ireland). Distilled and deionized water were used throughout.

2.2. Standard solutions and calibration curves

Two stock solutions of: 1) acetone, n-butanol, ethanol, acetic acid and butyric acid (4, 10, 1, 4 and 10 g/L, respectively); 2) n-butanol (10 g/L); were prepared in deionized and degassed water. Calibration curves solutions were obtained with the two stock solutions previously described with appropriate dilutions. The standard solutions of solvents and acids were eight, diluting stock solution 1, the acetone concentrations were 0.4, 0.8, 1.6, 2, 2.4, 2.8, 3.2 and 4 g/L; the n-butanol concentrations were 1, 2, 3, 4, 5, 6, 7, 8 and 10 g/L; the ethanol concentrations were 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 and 1 g/L; the acetic acid concentrations were 0.4, 0.8, 1.6, 2, 2.4, 2.8, 3.2 and 4 g/L; the butyric acid concentrations were 1, 2, 3, 4, 5, 6, 7, 8 and 10 g/L. Ten standard solutions of butanol, with the concentrations 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 g/L, were prepared diluting stock solution 2.

2.3. Microorganism and fermentation

Clostridium acetobutylicum 824 (ATCC, www.atcc.org) wild type was obtained from the National Collection of Microbial Cultures at CINVESTAV. The strain was conserved in Gu modified medium (GMM), asparagine was removed from the formulation (Gu et al., 2009), that contained per liter: 50 g glucose, 0.75 g KH_2PO_4, 0.75 g K_2HPO_4, 0.4 g $MgSO_4$, 0.01 g $MnSO_4$, 1 g NaCl, 5 g of yeast extract, 2 g $(NH_4)_2SO_4$, and 0.01 g $FeSO_4$. *C. acetobutylicum* was inoculated in a 100 mL serum bottle with 20 mL of GMM, purged with nitrogen gas for 20 min and incubated at 37 °C for 36 h without agitation, 20% (v/v) of a glycerol solution (80% w/v) was added into the serum bottle. The mixed suspension was dispensed (1 mL) in 1.5 mL cryovials that were immediately flash frozen with dry ice and stored at −70 °C.

C. acetobutylicum (1 mL stored vial) was grown in 20 mL of GMM in a 100 mL serum bottle, purged with nitrogen gas for 20 min and incubated at 37 °C for 36 h, without agitation, before being transferred into the fermentation medium as inoculum. The batch control experiment (ABE fermentation) was carried out using a 0.75 L bioreactor with magnetic agitation at 200 rpm, the initial medium volume was 0.4 L of GMM previously sterilized at 121 °C for 15 min. Nitrogen gas was used to purge the oxygen from the culture medium before inoculation and during the experiments. The initial pH value was 6.5 and pH control was carried out only to keep the pH value above 4.5 by automatically adding 2 N NaOH solution as buffer. The temperature was maintained at 37 °C the whole experiment. Throughout the fermentation, 5 mL samples were collected regularly to determine optical density, glucose concentration, and solvents production. Cell density was measured at 600 nm (OD_{600nm}) with appropriate dilution using a Jenway 7315 spectrophotometer. The fermentation samples were centrifuged (Sorvall MC 12) at 12,000 rpm at 4 °C for 15 min to separate cells, and the clear liquid was analyzed for ABE fermentation products by Gas Chromatography Method (CGM), and glucose concentration by the method described by Miller (Miller, 1959).

2.4. Gas Chromatography Method for alcohols determination

A Gas Chromatograph (Perkin Elmer, Auto System), equipped with a flame ionization detector (FID) and a ZB-FFAP column (30 m × 0.25 mm × 0.25 μm), was used to determine the ABE fermentation products concentrations. The separation conditions were: the column temperature was held at 70 °C for 5 min after injection, then increased at a rate of 15 °C/min until 148 °C was reached, this temperature was held for 3 min, then increased at a rate of 30 °C/min until 200 °C was reached, held 8 min, and increased again at a rate of 10 °C/min until 240 °C was reached, this temperature was held 15 min. The injector and detector temperature were 220 °C and 250 °C, nitrogen was the carrier gas with a pressure of 9 psi. Samples were centrifuged (Sorvall MC 12) at 12,000 rpm at 4 °C for 15 min and filtered with 0.2 μm membrane filters. The injection volume was of 2 μL and each sample was injected three times.

2.5. Alcohols determination by microdiffusion assay

For the Microdiffusion assay two reagents were prepared: 30.7 g Na_2CO_3 dissolved in 100 mL distilled water (Reagent 1) and 0.145 g $K_2Cr_2O_7$ dissolved in 100 mL of 10 N H_2SO_4 (Reagent 2, stored in the dark). The assay required two concentric tubes (placed one inside the other): The outer tube (7 × 1.5 cm) contained 0.5 mL of Reagent 1, and, either, 0.1 mL standard solution for the calibration curve or 0.05 mL of ABE fermentation sample; 0.1 mL of distilled water was used as blank. The inner tube (4.5 × 0.9 cm) contained 1 mL of Reagent 2; once readied the device, it was covered with a rubber stopper (Fig. 1). An incubation period is needed for the reaction to occur and after the incubation period has passed, the small tube is removed and 1 mL of distilled water was added to measure the absorbance at 450 nm ($OD_{450\ nm}$) in the spectrophotometer.

The mass of the butanol standard solutions was calculated with the following equation:

$$mass[\mathrm{mg}] = std\ solution\ concentration\left[\frac{\mathrm{mg}}{\mathrm{mL}}\right] * volume\ of\ std\ solution\ used\ [\mathrm{mL}]. \quad (1)$$

A plot was constructed using the calculated mass of the standard solutions vs the absorbance ($OD_{450\ nm}$) measurements of the standard solutions, to obtain the linear equation by means of a least-squares method using Excel (Microsoft, USA), the criterion for acceptance was a regression's value ≥0.99 (r^2). The linear equation correlated the absorbance measurement with the mass of the sample measured, presented in the slope-intercept form: $y = mx + b$, where y = absorbance (OD_{450nm}), m = slope, x = butanol mass, and b = the y-intercept. To obtain the concentration of the sample, the calculated mass was divided by the sample volume.