A simple rapid gas-chromatography flame-ionization-detector (GC-FID) method for the determination of ethanol from fermentation processes

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In the recent years, we have been facing an exponential increase in energy demand. To date, fossil fuels are the fuel of choice, but rise in costs, depletion of reservoirs and growing awareness to the environmental effects, have elevated the appeal of renewable energy sources. Among the most attractive substitutes, especially in the context of fuel for transportation, is bioethanol – the ethanol produced by microbial fermentation of feedstock. However, research in this growing field is hampered, especially in developing countries, by the cost of current ethanol quantification methods. Here we presented a simple, rapid, low-cost method for ethanol quantification based on organic extraction followed by gas-chromatography flame-ionization-detector (GC-FID) chromatography. This method offers wide range detection (1 to 30% v/v) and was demonstrated as suitable for fermentation process.

Key words: Bioethanol, yeast, fermentation, ethanol quantification, gas chromatography.

INTRODUCTION

Over the last century, in correlation with the growth in world population and industrialization, energy consump- tion has increased dramatically (Sun and Cheng, 2002). To date, fossil fuels are the fuel of choice, but rise in oil costs, depletion of reservoirs and the growing awareness of the environmental effects, have elevated the appeal of renewable energy sources. One of the most attractive substitutes for the fossil energy sources is bioethanol- the ethanol produced by fermentation of feed-stock. The latter is conveniently classified into three main types of raw materials: sugars, starches and cellulose (Lin and Tanaka, 2006). Both starches and cellulose must first be hydrolyzed by acids or enzymes and related microorgan- isms to fermentable sugars, which consequently produce ethanol. Although the production of bioethanol offers many benefits, either when used directly or by blending with petroleum (Balat, 2009), more research is needed in the aspects like feedstock preparation, fermentation technology modification, etc., to make bioethanol more economically viable (Chandel et al., 2007). To date, research efforts in this field are limited, mainly by the high cost and/or complexity of existing methods for determination of ethanol concen-tration in fermentation process. Common methods include such strategies as chemical (Williams and Darwin, 1950) and enzymatic (Redetzki and Dees, 1976) assays, high performance liquid chromatography (HPLC) coupled to a flame ionization detector (FID) (Yarita et al., 2002), headspace gas-chromatography (Li et al., 2009), silicate membrane extraction (Nomura et al., 2002) and others. Never- theless, all these methods require the use of high-cost equipment and/or high-cost consumables.

This situation as mentioned and the growing interest in alternative feed for bioethanol production (Reddy et al., 2010, 2011) prompted us to develop a cheap and rapid approach for ethanol quantification in aqueous media during fermentation steps as part of the conversion of biomass to ethanol. The suggested method requires a

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Abbreviations: GC-FID, Gas-chromatography flame-ionization-detector; EtOH, ethanol; nBuOH, n-butanol; EtOAc, ethyl acetate.
sample of a small volume and consists of organic extraction, followed by direct use of gas chromatography with a flame ionization detector (GC-FID). The feasibility of such approach is obvious since there is no need for the headspace system, distillation, expensive reagents and sophisticated equipment.

MATERIALS AND METHODS

Chemicals

*n*-Butanol (BuOH) and ethanol (analytical grade, 99.5%) were purchased from Sigma-Aldrich (Israel) and used thereafter without any pretreatment. Ethyl acetate (analytical grade, 99.5%) was purchased from Frutarom (Israel). Glucose, KH₂PO₄, NH₄SO₄ and MgSO₄·7H₂O were from Sigma (all chemically pure or higher grade).

Standard solutions and calibration curves

Standard solutions of *n*-butanol were prepared in ethyl acetate (EtOAc) and injected without further treatment. Standard solutions of ethanol (EtOH) were prepared in distilled water containing 1% v/v of *n*-butanol as an internal standard, extracted and injected. Peak area ratios of the ethanol vs. *n*-butanol were calculated and plotted against ethanol concentration (% v/v) to afford a calibration curve which served for ethanol quantification in the fermentation samples.

Fermentation experiments

Fermentation experiments were done using commercial ale yeasts strain Hefeweize IV (White Labs, USA, cat# WLP380). Yeast were grown aerobically in modified yeast broth (30% w/v glucose, 0.1% yeast extract, KH₂PO₄ 0.1% w/v, NH₄SO₄ 0.1% w/v, MgSO₄·7H₂O 0.05% w/v; medium titrated to pH 4.5 after sterilization [120°C, 20 min] with solution of 1 M HCl). Cultures were shaken in an orbital shaker (200 rpm) at 30°C for three days. After three days the Erlenmeyer was sealed with a rubber septum and the yeast allowed to reach an anaerobic conditions. A thin needle (28G) was inserted through the septum to enable the release of pressure. Broth samples for ethanol analysis were taken as described below.

Extraction procedure

Broth samples of 600 μL containing yeast cells were collected at the designated times through the rubber septum by a syringe equipped with an 18G needle. The broth was transferred to an Eppendorf tube and the tube was centrifuged (8,000 g for 2 min at R.T.) to sediment yeast cells. Afterward, 500 μL of the clear supernatant were transferred to a new tube without disturbing the cell pellet, and then 5 μL of *n*-butanol (as internal standard) were added and the tube was vortexed for 30 s at maximum speed. Next, 1 ml of ethyl acetate was added, followed by 5 min of vortexing at maximum speed. Finally, the tubes were centrifuged to facilitate phase separation (5,000 g; 2 min at R.T.), and the organic phase (upper) was subjected to GC analysis.

GC analysis

Analysis of ethanol and butanol was conducted using SRI GC model 8610C, equipped with a 60 m column (Restec MXT-1, Id 0.53 mm, 5 μM), on-column injector and FID conditions: 250°C; H₂, 25 PSI, equivalent to 25 ml/min; air, 2 PSI, equivalent to 100 ml/min; gain set to ‘medium’. The GC was also equipped with an internal air compressor and hydrogen generator. N₂ was used as carrier gas with pressure control (24 PSI constant; equivalent to 27 ml/min). The GC was connected to a computer running Peak Simple software version 2.8. Oven temperature (and hence column and injector temperature) was initially set at 50°C and then elevated at the rate of 7°C/min to 100°C, thus giving a total run time of 7 min. Furthermore, 2 μL sample was injected manually at time 0, using a 5 μl Hamilton syringe and temperature cycle was started. Syringe was thoroughly washed with ethyl acetate between injections to avoid cross-contamination. Each injection was repeated three times, ethanol routinely came out at retention time equivalent to 65°C.

RESULTS AND DISCUSSION

Ethanolic fermentation process happen in aqueous broth containing sugars and other compounds. Direct injection of such broth into a GC is possible (Wang et al., 2003) but is undesirable due to possibility of damage to the equipment. This has led to the development of numerous sophisticated approaches to ethanol determination in fermentation broth. Most of these methods require expensive equipment and/or reagents. Here we have utilized a simple pre-injection organic extraction step to facilitate the safer use of GC for determination of ethanol in fermentation process.

Organic extraction of analytes from water for GC analysis requires careful choice of both the organic extractants (Pienta et al., 1996) and the internal standard (Hewavitharana, 2009). The criteria for the extraction solvent should include moderate to low volatility, low solubility in water and high partition coefficients for the analyte and the internal standard. All three should have distinct retention times to allow for a reliable quantification. In this study we chose the use of ethyl acetate as an extractant due to its low water solubility (8.3 g/L at 20°C) and good partition coefficient for ethanol (K<sub>EtOAc/Water</sub> = 0.91 at 25°C for ethanol; (Pienta et al., 1996)). *n*-Butanol was chosen as an internal standard. As demonstrated in Figure 1, there is no overlap in ethyl acetate, ethanol and *n*-butanol retention times.

To compensate for the imprecision in injection volumes and for differences in extraction efficiency, the use of internal standard is indispensable, in particular in case of manual injection (Hewavitharana, 2009). *n*-Butanol was the reagent of choice due to its low toxicity (it is completely metabolized in a pathway similar to ethanol), good miscibility in ethyl acetate and retention time different from that of ethanol and ethyl acetate. The concentration of the internal standard needs to be high enough to be detected easily, but not to reach a saturation level that will hinder its quantification. Therefore we tested the dose-response curve of *n*-butanol solutions in ethyl acetate. The results are presented in Figure 2, demonstrating a good linear relation (R² = 0.985) between *n*-butanol concentration and detector response in the range of 0.2 to 2% v/v. On the basis of the obtained results, 1% of *n*-butanol was used as an internal standard in all further experiments.
We also determined the applicability of the extraction–GC-FID method for determination of ethanol in aqueous solution. Figure 3 demonstrates a good linear relation ($R^2 = 0.993$) between the ratio of ethanol and butanol peak area ($R^2 = 0.993$) and the ethanol concentration in the range of 0 to 30% v/v, suggesting that the described method can be applicable in a wide range of concentrations. Limit of detection (LOD) and limit of...
quantification (LOQ) were determined by extraction of 0.1% ethanol (1% butanol) standard and repeated injection (N = 10). Peak ratio was converted to ethanol concentration using the calibration curve (Figure 3) and LOD was calculated as 0.08% (3 times SD) and LOQ was calculated as 0.26% (10 times SD). These values could be expended to lower concentrations by setting the detector gain to the 'high' mode (0.02 and 0.08% respectively) but under these conditions linearity dropped dramatically around 0.5% ethanol (Figure 3 - insert).

Finally, the proposed method was tested for its 'real-life' applicability for ethanol quantification from fermentation process. Commercial ale yeast fermenting a modified broth was used. The results presented in Figure 4 show a classical fermentation pattern with an increase in ethanol concentration starting after two days (probably after achieving anaerobiosis) and maximum of 3.5% ethanol after 10 days (and probably before). These results demonstrated not only that our tandem method of extraction-direct GC analysis is indeed well suitable for analysis of fermentation process, but also that broth or yeast generated contaminants do pose an issue to the analysis (as they are not seen in the first days).

In conclusion, we have described a low-cost, rapid and sensitive method for determination of ethanol, ranging from 0.25 to 30% ethanol and suitable for analysis of ethanol production during fermentation processes. Although the method was demonstrated for *Streptomyces cerevisiae* only, we strongly believe that its scope can be easily extended to other fermenting strains and conditions, as well as to alcohol quantification in alcoholic beverages. Its low-cost, simplicity and robustness may make it a more economically and viable substitute for the current methods.
Figure 4. Fermentation by ale yeast - % v/v EtOH vs. time. EtOH, Ethanol.

REFERENCES


