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Inexpensive Procedure for Measurement of Ethanol: Application to Bioethanol Production Process

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Abstract

We have developed a spectrophotometric method for measurement of ethanol concentration in any unknown sample using a solvent tri-*n*-butyl phosphate [TBP, non-alcoholic solvent, density = 0.975 to 0.976, solubility in water = 0.028% (w/v)]. Solvent TBP separates ethanol from the non-hydrolyzed substrates that could interrupt the desired result by reacting with dichromate reagent. Oxidation of ethanol with dichromate reagent imparts blue green-colour to the solvent, which is easily detected by Spectrophotomer at 595 nm. Our established method showed similar results performed by relatively expensive Gas Chromatography (GC) method. In our present study we put forth a cheap alternate method for determining ethanol concentration in any aqueous solution.

Keywords

Bioethanol, Yeasts, *Saccharomyces cerevisiae*

1. Introduction

A variety of techniques can be used to determine ethanol concentration in aqueous solution [1]. Among them, gas chromatography (GC) is the most common method for determination of ethanol from alcoholic beverages. Distillation is carried out before the measurement of ethanol concentration [2]. However, this process is expensive, time-consuming and laborious [3] [4]. Solvent extraction and dichromate oxidation method is a simpler, more convenient for ethanol measurement in many industries and research fields, such as the selec-

tion of a strain having high ethanol productivity, the development of a bioethanol production process etc. [5]. During fermentation, culture broth contains a variety of complex polysaccharides like starch, cellulose etc. In saccharification, enzymatic degradation of complex sugars produces additional components as byproducts. These could react directly or indirectly with dichromate reagent, thus may interfere with reaction color [6] [7]. Therefore, the extraction of ethanol from the culture broth is essential before the assay is performed. In this study, we have developed a method for bioethanol estimation from any aqueous solution by solvent extraction process, which was compared with the results performed by the GC method. Our result clearly demonstrates that inexpensive solvent extraction method can easily be applied for estimation of ethanol concentration in any aqueous solution.

2. Materials and Methods

Sample Preparation

Microbial cells growth was carried out in Yeast extract Peptone Dextrose (YPD) liquid medium. At first, one loopful fresh microbial colony was inoculated from a fresh YPD plate into the test tube containing 3 ml of YPD broth and incubated at 30˚C for 24 hours in a shaking water bath (100 rpm). Freshly grown cultures were inoculated 100-fold dilution into the conical flask containing 30 ml of YPD medium, incubated further at either 30˚C or 37˚C for 72 hours. The fermented sample was centrifuged at 14,000 rpm for 10 minutes. After centrifugation, the supernatant was collected, and filtered using syringe filter (0.22 - 0.45 µm). Then the bioethanol concentration was measured by following two methods.

Bioethanol Estimation

Bioethanol concentration was estimated either by solvent extraction and dichromate oxidation method or by Gas Chromatography (GC) method discuss below. The dichromate reagent was prepared by dissolving fresh 10 gm potassium dichromate in 100 ml of 5 M sulfuric acid solution [5]. Tri-*n*-butyl phosphate [TBP, density = 0.975 to 0.976, solubility in water = 0.028% (w/v), Sigma] was used to extract ethanol in an aqueous solution. In detail, 1 ml of TBP and 1 ml of standard solutions; 0% to 5% (v/v) or samples were mixed in a 2 ml microtube and then vortex vigorously until the reaction mixture was separated into two phases (upper phase-very clear, transparent and lower phase-turbid). From the upper phase, the 500 µl solution was transferred to a new 2 ml microtube then 500 µl dichromate reagent was added in a tube and vortex vigorously again for 10 minutes. The reaction mixture is separated into two phases (lower phase-blue green color). Dichromate reagent-containing lower phase (100 µl) was transferred in to the test tube and diluted up to 10-times with both standard and experimental samples. Optical density (OD) for standard ethanol solution and samples were measured at 595 nm in a UV spectrophotometer (Thermofisher). Bioethanol content of unknown sample was estimated from the ethanol standard curve as shown below.

Standard ethanol solutions with water were prepared from 0.0% - 1.0% (v/v) and 1 - 2 µl was injected into the injection port of the GC and then subjected to quantitative analysis of ethanol on a GC apparatus (SRI Model 8610A GC 5890 GC, Shimadzu, Japan). Packed column head pressure: 30 psi, column: carbo wax 20 M, 1/8" OD, length = 6 feet, oven temperature: 115˚C, carrier gas flow rate: 30 - 40 ml/min. Retention time, peak area and height of the injected standard ethanol solution was measured and recorded. That was used to determine the concentration of unknown experimental samples. In order to determine the reproducibility for injection method, standard solutions were injected at least three times. After the standards were completed, we replicate injections of our unknown samples same as standard solutions. The peak areas were measured and retention times were adjusted for the ethanol peaks in each chromatogram. A standard ethanol curve was prepared by plotting the % of standard ethanol solution on the X-axis and the peak areas on to the Y-axis. Ethanol concentrations from unknown samples were estimated from the ethanol standard curve.

3. Result and Discussion

Bioethanol is alternative fuel of fossil fuel, which is well known to keep environment clean. We are looking for some thermotolerant microbes from the natural fermented sources of Bangladesh for fuel ethanol production. The main aim of this study is to develop cost effective eco-friendly method to measure bioethanol content from our experimental samples. Our developed method was designed based on method published previously [5]. Ethanol was first extracted from the aqueous phase using the solvent dichromate, which works as a reagent for color development from orange to green, in which it oxidizes ethanol. Gas Chromatography method is relatively expensive compared with solvent extraction and dichromate oxidation method and our main target was to screen bioethanol-producing microbes from the large population. Solvent extraction in this ethanol measurement is a crucial step because glucose, yeast extract, peptone, CSL, and glycerol can cause a change in color through a reaction with dichromate and their intrinsic colors can also affect the final reaction color [7]. In other words, without the ethanol extraction step, the measured ethanol concentration in the culture medium may be tainted by a direct reaction between the dichromate reagent and the other components in the culture medium. Therefore, this solvent extraction process might be applied to other ethanol production processes like bioethanol, alcoholic beverage, wine, etc. Many solvents have previously been used for the selective extraction of ethanol, particularly for ethanol extraction from fermentation reaction [8]. It has been reported that the dichromate oxidation method can be used directly to determine the ethanol concentration in culture broths of yeast like *Candida shehatae*, *Saccharomyces cerevisiae*, *Pichia kudriavzevii and Candida tropicalis* [9] [10]. When ethanol is present in an aqueous solution, chromium ions oxidize ethanol, and these ions are reduced from the +6 oxidation state to +3 and changing the color from

orange to blue-green. Dichromate oxidation method is recently used for the measurement of ethanol in a flow injection analysis [6] [7]. Moreover aliphatic alcohol like *n*-dodecanol or *n*-decanol is a representative solvent for extracting ethanol from a culture broth and benzyl alcohol had been used as an alternative to distillation [11] [12]. In this study, non-alcoholic solvent (TBP) was used for extracting ethanol from a culture broth, because the alcoholic solvent is oxidized by dichromate solution more efficiently than others [5]. Three yeast strains were used to check their bioethanol activity in two different methods and bioethanol estimation were compared and found more or less similar result are shown in the Figure 1 and Table 1.

It was reported that the high content of ethanol even 8% (v/v) could be estimated by Solvent Extraction and dichromate oxidation method using both microtubes and a 96-deepwell microplate [5]. Therefore, this ethanol assay format is practically useful for the selection of a strain having high productivity, the development of a bioethanol production process, monitoring and control in alcoholic beverage production. Fletcher *et al.* [13] established an ethanol estimation

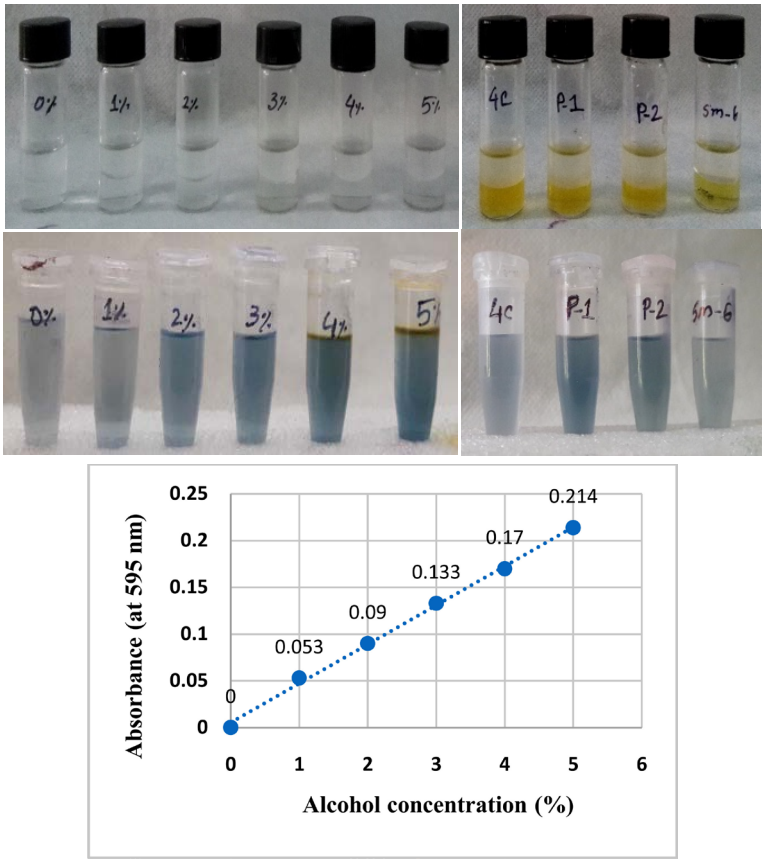

Figure 1. Solvent extraction procedure for bioethanol measurement in any solution. Here, top left and bottom panel represent the solvent extraction and dichromate oxidation steps of standard alcohol solution, respectively, and right top and bottom showed experimental samples marked by strain number on eppendrof tube. Bottom graph represents the standard curve of known alcohol solution, which was used for measurement bioethanol concentration of experimental or unknown samples.

Table 1. Comparison of bioethanol production level estimated by two distinct methods.

Strain	Bioethanol yeald % (v/v)			
	30˚C		37˚C	
	GC	SE	GC	SE
4C	5.00	5.35	3.00	2.80
P-1	2.75	3.00	2.12	2.50
Sm-6	3.00	3.50	4.50	4.80

Here, GC = Gas Chromatography and SE = Solvent Extraction. Experiments were carried out at least 3-times and the mean values are shown. The strain 4C, P-1 and Sm-6 represent *Saccharomyces cerevisiae*, *Pichia kudriavzevii* and *Trichosporon coremiiforme*.

method using sequential injection analysis with an assay range up to 6% (v/v). Based on the principle, Vicente *et al.* [6] carried out the determination of ethanol concentration, up to 50% (v/v) in alcoholic beverages, and at a rate of 30 samples per hour. In addition, Choengchan *et al.* [14] also introduced the flow injection analysis, up to 42% (v/v) ethanol in whiskey. However, these analysis systems require a very complicated instrumentation due to the automated set-up for sequential operation and online monitoring. Therefore, these systems are not easily available in a laboratory for ethanol determination. Meanwhile, UV visible spectrophotometer for the ethanol assay has simple instrumentation and operation where computer monitoring is not required. We have established the solvent extraction dichromate oxidation method in UV visible spectrophotometer in which this method overcame the interference by the medium ingredients through TBP extraction prior to dichromate oxidation. Moreover, our established method was inexpensive and less time consuming compared with other methods [2] [5] [6] [14].

4. Conclusion

Although this method cannot determine precisely the bioethanol concentration in the culture broth, it might be sufficient for this assay system to screen a strain having high bioethanol productivity, and monitor the bioethanol production process. The proposed bioethanol concentration measurement method in this work is comparatively cheap and easy to perform, thus can easily be adopted by others for bioethanol production assays as well as in potent strain sorting.

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