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ORIGINAL PAPER

Measurement of ethanol concentration using solvent extraction and dichromate oxidation and its application to bioethanol production process

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Abstract A method for measuring the ethanol concentration in a yeast culture broth was developed using both microtubes and a 96-deepwell microplate. The strategy involved first the solvent extraction of ethanol from the yeast culture broth and measurements of the ethanol concentration using the dichromate oxidation method. Particular focus was made on selecting the extraction solvent as well as determining the measurable range of ethanol concentrations using this solvent extraction-dichromate oxidation method. This method was developed as an assay format in 2.0-ml microtubes and 1.2-ml 96-deepwell microplates, and the ethanol concentration in the batch cultures and fed-batch fermentations was measured. Tri-*n*-butyl phosphate [non-alcoholic solvent, density = 0.9727, solubility in water = 0.028% (w/v)] was used for solvent extraction when measuring the ethanol concentration from the yeast culture broth. The maximum detectable ethanol concentration was 8% (v/v) when 10 g potassium dichromate in 100 ml of 5 M sulfuric acid was used. The concentrations determined from the solvent extraction-dichromate oxidation methods were remarkably similar to those of gas chromatography in which samples were prepared from seven experiments, such as four batch cultures and three fed-batch fermentations.

Keywords Ethanol measurement · Alcoholic fermentation · Tri-*n*-butyl phosphate · Dichromate · Microtube · 96-deepwell plate

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Introduction

The ethanol concentration in aqueous solution can be determined using a variety of techniques [10], with gas chromatography being the most common method for clinical samples and alcoholic beverages. Enzymatic oxidation with alcohol dehydrogenase and chemical oxidation with acid dichromate are also used. In particular, measurements of the specific gravity of a distillate have been traditionally used to determine the ethanol concentration in the wine industry, in which distillation is carried out before the measurement. In some other cases, a solvent extraction method is used as a substitute for distillation because the distillation process is time-consuming and laborious [14, 19], and refractometry is used as an alternative for ethanol measurement [14]. In addition, the dichromate oxidation method has been examined as a substitute for measuring the

specific gravity in alcoholic beverages [11, 18]. Moreover, it has been reported that the dichromate oxidation method can be used directly to determine the ethanol concentration in blood and urine in clinical laboratories [4], as well as in culture broths of yeast *Candida shehatae* [13]. Although the well-instrumented gas chromatography produces more rapid results, a simpler, more convenient and higher throughput determination of the ethanol concentration is required in many industries and research fields, such as the selection of a strain having high productivity, the development of a bioethanol production process, process monitoring and control in alcoholic beverage production, and the determination of ethanol in clinical samples.

In this study, the strategy involved solvent extraction of ethanol from a culture broth followed by measurements of the ethanol concentration using the dichromate oxidation method. When ethanol is present in an aqueous solution, chromium ions oxidize ethanol, and these ions are reduced from the +6 oxidation state to +3, changing the color from orange to green. Dichromate oxidation method is recently used for the measurement of ethanol in a flow injection analysis [8, 11, 20]. Moreover, the international ethanol reference solution is prepared by the dichromate oxidation method [3]. Meanwhile, in the culture broth for bioethanol production, it is probable that unhydrolyzed insoluble material exists and its color is dark. The culture broth contains a variety of nutrients (monosaccharides, salts, metal ions, etc.), and by-products such as glycerol [2, 9], which can react with dichromate. Therefore, the extraction of ethanol from the culture broth is essential before the assay can be performed without interference. Interestingly, Offeman et al. [17] reported the physicochemical properties of a variety of solvents for ethanol extraction from aqueous solutions. Therefore, two non-alcoholic solvents (di-*n*-butyl phthalate (DBP) and tri-*n*-butyl phosphate (TBP)) in various solvents were investigated in this study, because alcoholic solvents can be oxidized by dichromate.

The aim of this study was to develop a simple bioethanol measurement method in both microtubes and 96-deepwell microplates. This study focused particularly on the solvent selection for the extraction of ethanol, the determination of the measurable concentration range of ethanol in the microtube and 96-deepwell microplate, and the measurement of the ethanol concentration from the culture broths of a batch and fed-batch fermentation.

Materials and methods

Dichromate reagent preparation

The dichromate reagent was prepared by dissolving potassium dichromate (Duksan Pharmaceutical Co., Republic of Korea) in 100 ml of a 5 M sulfuric acid solution. The 5, 8 and 10 g potassium dichromate-containing dichromate reagents were designated DR3, and DR5, DR8, and DR10, respectively.

Solvent extraction and dichromate oxidation in microtubes

Two solvents such as DBP [di-*n*-butyl phthalate, density = 1.0465, solubility in water = 0.0011% (w/v), Sigma] and TBP [tri-*n*-butyl phosphate, density = 0.9727, solubility in water = 0.028% (w/v), Sigma] were used to extract ethanol in an aqueous solution; 1 ml of DBP or TBP, and 1 ml of an ethanol standard solution or sample were mixed in a 2.0-ml microtube (Axygen, USA), and then vortexed vigorously using a vortex mixer (Vortex-Genie2, Scientific Industries Inc., USA), which was equipped with a microtube foam insert, for 10 min or 1 h. After phase separation, 750 µl of the solvent phase (lower for DBP and upper for TBP) was transferred to a new microtube. The dichromate reagent (750 µl) was then added, and then vortexed vigorously for 10 or 30 min. After phase separation, 200 µl of the dichromate reagent-containing lower phase was transferred to a flat-bottomed 96-well microplate (SPL Life Sciences, Republic of Korea) using a gel loading tip (QSP, USA), and the OD_{595} (optical density at 595 nm) was measured using a microplate reader (Model 680, RioRad, USA).

Solvent extraction and dichromate oxidation in 96-deepwell microplate

A deepwell microplate was used to set-up the high-throughput assay. In particular, a 1.2-ml 96-well microplate (ABgene, UK) was used. TBP was used as the extraction solvent. Five hundred microliters of TBP and the ethanol standard solution or sample were mixed in a 96-deepwell microplate, capped with a plate cap strip (ABgene, UK), and then shaken in a shaking incubator at 150 rpm for 10 min. After phase separation, 350 µl of the upper TBP phase was transferred to the new 96-deepwell microplate, and 350 µl of the dichromate reagent was added. The well was capped with a plate cap strip, and then shaken in a shaking incubator at 150 rpm for 10 min. In addition, after phase separation, 100 or 200 µl of the dichromate reagent containing the lower phase was transferred using a gel loading tip into a flat-bottomed 96-well microplate, and the OD_{595} was measured in a microplate reader.

Yeast strain, batch culture, and fed-batch culture

For the yeast culture, *Saccharomycese cerevisiae* (*S. cerevisiae*) ATCC 24858 was used, in which YPD (yeast extract 1%; peptone 2%; glucose 2 or 10%), and CD [Corn steep liquor (CSL) 2%; glucose 2%] media were used as the

batch culture media in a 250-ml Erlenmeyer flask, which was kept at 30°C stirred at 150 rpm. When necessary, the autoclaved YPD, CD, and YPG (yeast extract 1%; peptone 2%; glycerol 2%) media were used to prepare the standard ethanol samples. YPG was the medium in this study but was not used in the yeast culture. The fed-batch culture was carried out in 5-l laboratory fermenter (KoBiotech Co., Republic of Korea) with a 1.5-l working volume using a previously reported glucose feeding strategy [1, 2]. The initial batch culture medium contained 6% glucose, 2% CSL, 1.2% $(NH_4)_2SO_4$, 2.4% KH_2PO_4, 1.2% $MgSO_4{\cdot}7H_2O$. The temperature, pH, and agitation speed were controlled at 27°C, 4.0, and 200 rpm, respectively.

Gas chromatography

The ethanol concentration was also analyzed by gas chromatography (HP 6890, Agilent technologies, USA) using a flame ionization detector (FID). An HP INNOWax column (Agilent 19091N-113, film thickness; 0.25 μm, length; 30 m, inner diameter; 0.32 mm) was used. The initial temperature, maximum temperature, and rate of temperature rate in the oven were 50°C, 170°C and 10°C/min, respectively. Both the injector and FID temperatures were controlled at 250°C. Nitrogen was used as the carrier gas with a flow rate of 40 ml/min. For quantitative analysis, *n*-butanol was used as an internal standard.

Cell growth and glucose

Cell growth was monitored by measuring the optical density at 600 nm (OD_{600}) using a spectrophotometer (Spectronic, Thermo Scientific, USA). The residual glucose in the culture medium was analyzed using the dinitrosalicylic acid method [7].

Results

Dichromate oxidation for the ethanol-containing aqueous solutions

The medium ingredients were reacted directly with the dichromate reagent to determine if solvent extraction is necessary, as well as to determine how the medium ingredients affect the color development in the dichromate oxidation. When the ethanol standard solution was prepared using the yeast culture media and the medium ingredients reacted with the dichromate reagent without solvent extraction, YPD, CD media and glucose led to intense color development at an OD_{595} of approximately 0.9–1.1 (Fig. 1). In addition, CSL and YP affected the color development remarkably, in which the blank OD_{595} was approximately

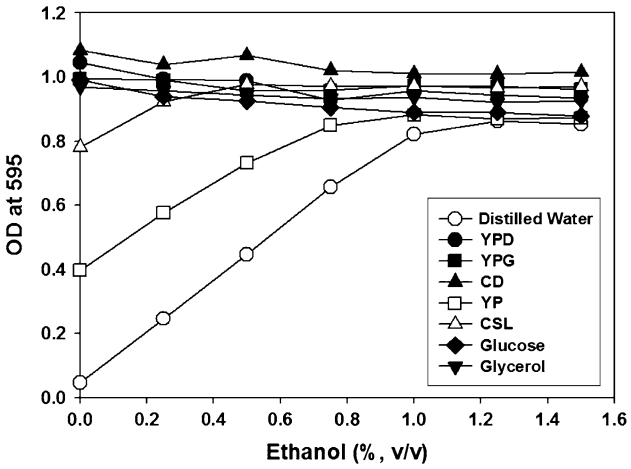

Fig. 1 Dichromate oxidation for the ethanol-containing standard solutions. *YP* indicates the solution prepared by the yeast extract and peptone. *DR3* was used as the dichromate reagent, a microtube was used for solvent extraction and dichromate oxidation, and the times for extraction and oxidation were 1 h and 30 min, respectively. Autoclaved media were used to prepare the standard ethanol sample. All the data were not subtracted from the blank

0.4 and 0.8, respectively. In particular, glycerol as a by-product of bioethanol production also led to intense color development. Only when the ethanol standard solution was prepared using distilled water was a linear standard curve made, in which a relatively low blank OD_{595} was observed.

Selection of extraction solvent and preparation of ethanol standard curve

The ethanol concentration was measured by dichromate oxidation after solvent extraction of ethanol using two non-alcoholic solvents (DBP and TBP) to determine the better of the two. First, solvent extraction was carried out in a microtube. The DBP phase was the lower phase for the DBP-water mixture, and the TBP phase was the upper phase for the TBP-water mixture. In order to determine the ethanol concentration in these solvent phases, the samples were collected and mixed with the dichromate reagent. The color development of the solvent phase of the solvent dichromate reagent mixture was investigated (Fig. 2a). TBP showed more intense color development than DBP when an ethanol standard solution was prepared in the culture medium (YPD). In addition, DBP was not clearly separated from the water phase (data not shown). Therefore, TBP was selected as the extraction solvent for subsequent experiments. As shown in Fig. 2b, the distribution coefficient of ethanol in TBP and water (K_{DE}) is 0.576 as an average value (standard deviation = 0.026, $n = 4$). That is, 57.6% ethanol in samples was extracted into TBP, and the remaining existed in water phase. Moreover, the standard ethanol curves for color development versus the ethanol concentration,

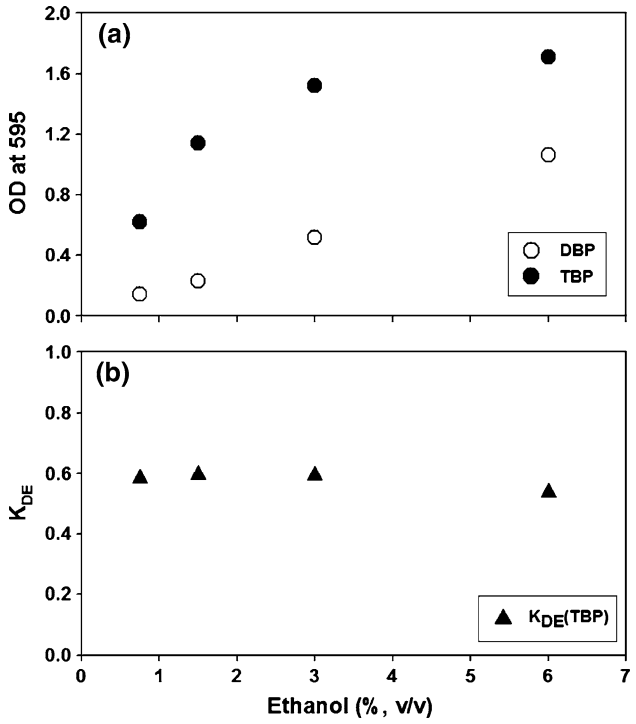

Fig. 2 **a** Dichromate oxidation of ethanol-containing DBP and TBP. DR3 was used as the dichromate reagent, a microtube was used for solvent extraction and dichromate oxidation, and the times for extraction and oxidation were 1 h and 30 min, respectively. **b** Distribution coefficient for ethanol in TBP. K_{DE}(TBP) is defined as the ratio of the weight percent of ethanol in TBP to that in the aqueous phase. Autoclaved YPD media were used to prepare the standard ethanol sample

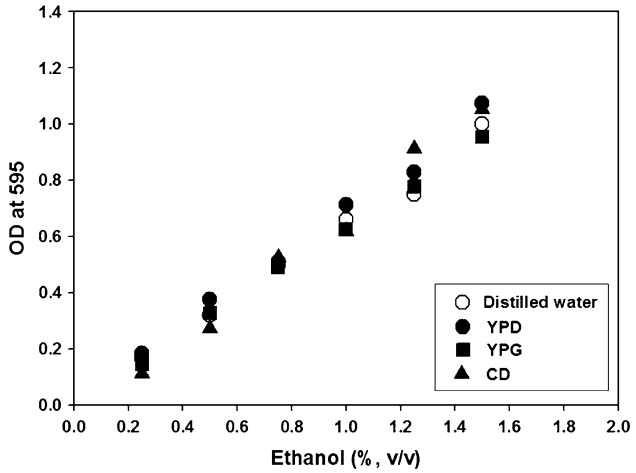

Fig. 3 Standard curves for ethanol dissolved in distilled water, YPD, YPG and CD media. TBP and DR3 were used as the extraction solvent and dichromate reagent, respectively, a microtube was used for solvent extraction and dichromate oxidation, and the times for extraction and oxidation were 1 h and 30 min, respectively. Autoclaved media were used to prepare the standard ethanol sample

in which the standard ethanol samples were prepared using the yeast culture media, showed linearity up to 1.5% (v/v) ethanol (Fig. 3).

Measurement of ethanol in the yeast culture broth

In order to confirm that this rationale is applicable for measuring the ethanol concentration in a culture broth of *S. cerevisiae*, the batch culture was carried out in YPD and CD medium, and the ethanol concentration was measured (Fig. 4). The data obtained from gas chromatography (GC) and the solvent extraction-dichromate oxidation method in a microtube were similar (Fig. 4a). Moreover, when solvent extraction and dichromate oxidation were carried out using a 96-deepwell microplate to set-up a high-throughput assay format, both solvent extraction-dichromate oxidation and GC produced similar results in the batch culture (Fig. 5).

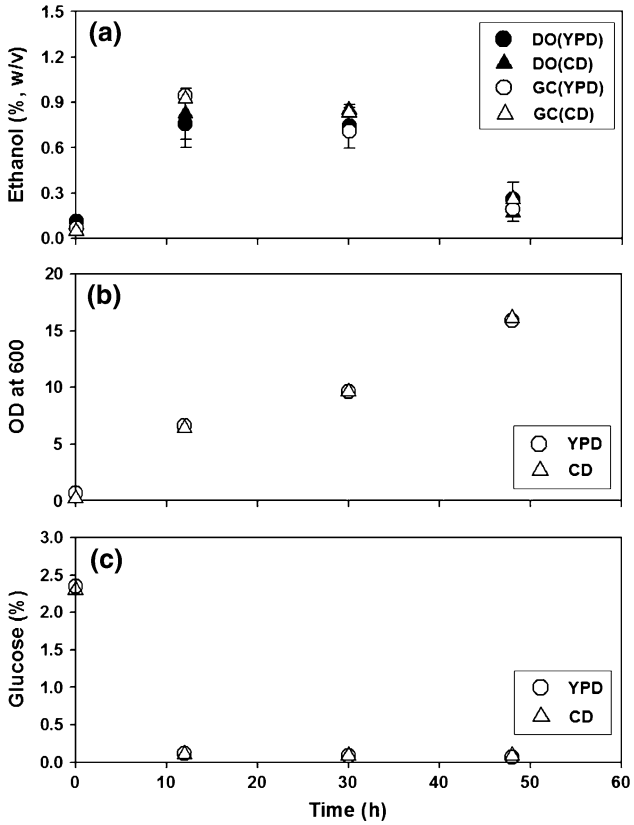

Fig. 4 Batch cultures of *S. cerevisiae* in 250-ml Erlenmeyer flask [ethanol concentration (**a**); cell growth (**b**); and glucose consumption (**c**)]. YPD and CD indicate the culture in the YPD and CD media, respectively. TBP and DR3 were used as the extraction solvent and dichromate reagent, respectively, a microtube was used for solvent extraction and dichromate oxidation, and the times for extraction and oxidation were 1 h and 30 min, respectively. DO(YPD) and DO(CD) indicate the data ($n = 3$) from the solvent extraction-dichromate oxidation method in which the sample was prepared from a culture in YPD and CD media, respectively, and GC(YPD) and GC(CD) indicate the data obtained from gas chromatography in which the sample was prepared from the culture in the YPD and CD media, respectively

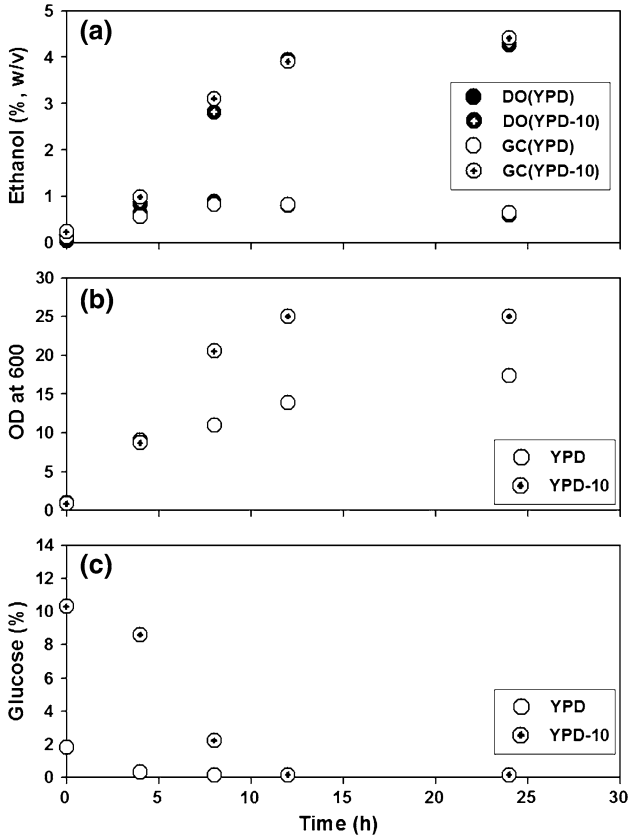

Fig. 5 Batch cultures of *S. cerevisiae* in 250-ml Erlenmeyer flask [ethanol concentration (**a**); cell growth (**b**); and glucose consumption (**c**)]. YPD and YPD-10 indicate the culture in YPD and 10% glucose-containing YPD media, respectively. TBP and DR3 were used as the extraction solvent and dichromate reagent, respectively, a 96-deepwell microplate was used for solvent extraction and dichromate oxidation, and the times for extraction and oxidation were 10 min each. Sample loading volume for measuring the OD_{595} was 200 µl. DO(YPD) and DO(YPD-10) indicates the data from the solvent-extraction-dichromate oxidation method, in which the sample was prepared from the culture in YPD and YPD-10 media, respectively, and GC(YPD) indicates the data from gas chromatography, in which the sample was prepared from the culture in YPD and YPD-10 media, respectively

Extension of the measurement range of ethanol concentration

An extension of the measurement range of the ethanol concentration is needed before the solvent extraction-dichromate oxidation method can be applied to industrial bioethanol production. As shown in Fig. 6, the linear range of the ethanol standard curve increased in proportion to the amount of dichromate in the reagent preparation. It was found that DR5, DR8 and DR10 extended the linear range to 4% (v/v), 6% (v/v), and 8% (v/v) ethanol, respectively, in both the microtube and 96-deepwell microplate cases.

Correlation between the data from solvent extraction-dichromate oxidation method and gas chromatography

The data of both solvent extraction-dichromate oxidation methods and GC from seven experiments, four batch cultures (Figs. 4, 5) and three fed-batch cultures for bioethanol production, were examined. The correlation showed that the data from the two methods were remarkably similar, even though these are not perfectly the same (Fig. 7). The linear equation for the data from the two methods from linear regression analysis was $Y = 1.05X + 0.01$ with a correlation coefficient of $r^2 = 0.978$.

Discussion

In this study, dichromate works as a reagent for color development, in which it oxidizes ethanol. With this technique for measuring the ethanol concentration, the ethanol was first extracted from the aqueous phase using the solvent, even though not all the ethanol had been extracted (Fig. 2). The ethanol in the solvent phase moved to an acidic aqueous phase, and subsequently reacted with dichromate. The ethanol concentration was measured from the increase in green color from orange according to the OD_{595}. Figure 1 shows that the 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. Their intrinsic colors can also affect the final reaction color. 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 can also apply to other ethanol production processes (bioethanol, alcoholic beverage, wine, etc.).

Many solvents have previously been used for the selective extraction of ethanol, particularly for ethanol extraction fermentation [6, 15]. Primary aliphatic alcohol (e.g. *n*-dodecanol, *n*-decanol) [12, 16] is a representative solvent for extracting ethanol from a culture broth, and benzyl alcohol [14] had been used as an alternative to distillation. In this study, however, two non-alcoholic solvents (TBP and DBP) were investigated in order to find better solvent for the extraction of ethanol from a culture broth, because the alcoholic solvent is oxidized by dichromate. In Fig. 2, the more intense color development when TBP was used as solvent was attributed to the difference in K_{DE}(TBP), 0.76 for TBP and 0.095 for DBP [8]. In addition, the interface between DBP and water was not distinctive because the difference in density between DBP and water is 0.0273 (data not shown). This is smaller than that between TBP

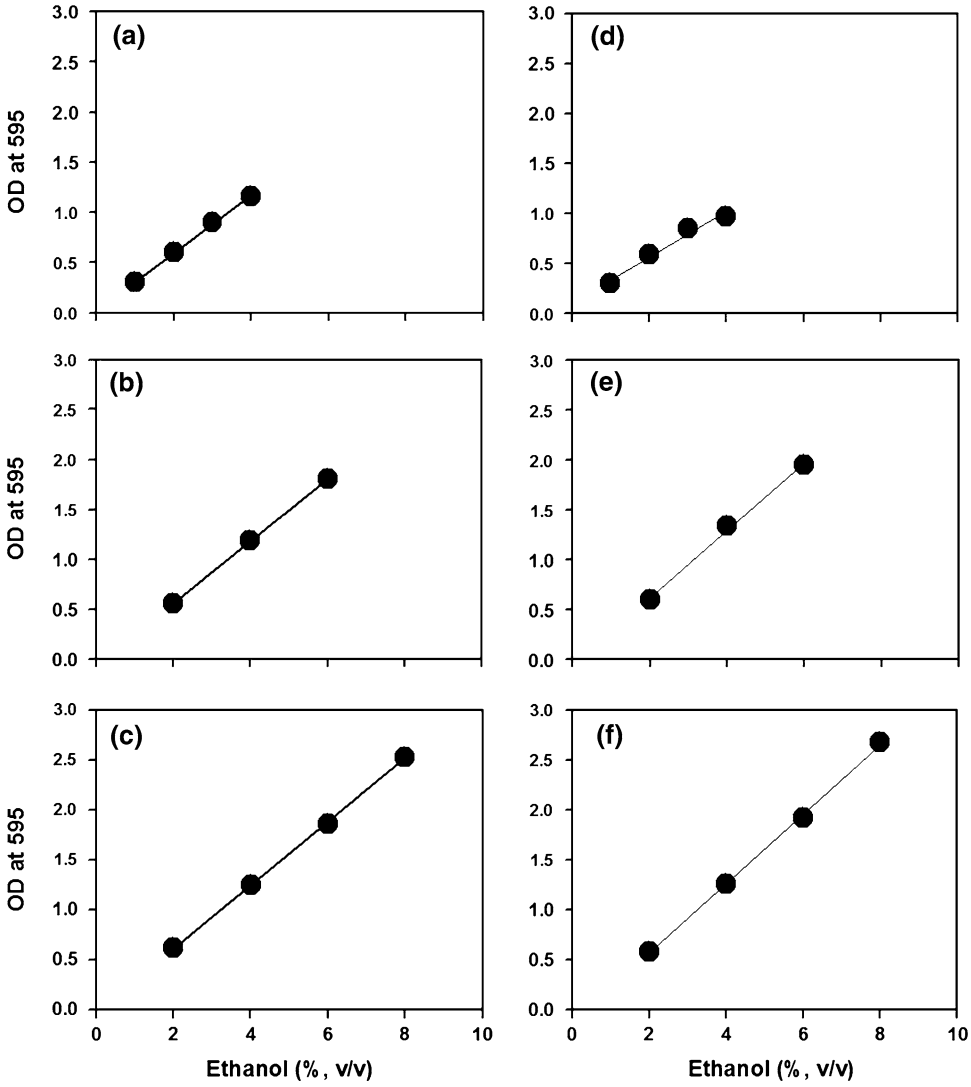

Fig. 6 Linear range of the ethanol standard curve according to the amount of dichromate used. TBP was used as the extraction solvent, microtube (**a**–**c**) and 96-deepwell microplate (**d**–**f**) were used for solvent extraction and dichromate oxidation, and the times for extraction and oxidation were 10 min each. The sample loading volume for measuring the OD_{595} was 100 µl. Autoclaved YPD medium was used to prepare the standard ethanol sample. DR5 (**a**, **d**), DR8 (**b**, **e**), and DR10 (**c**, **f**) were used as the dichromate reagents

and water. Moreover, phase separation after solvent extraction was inhibited when the culture medium was used to prepare the ethanol standard solution. However, TBP made a distinct interface between TBP and water, and showed a linear standard ethanol curve in various culture media (Fig. 3). K_{DE}(TBP) in this work was similar to those cited in a reference [17] in which K_{DE}(TBP) was reported as 0.46–0.79, including Offenman et al.'s data [K_{DE}(TBP) = 0.76). Offenman et al. [17] described K_{DE} as a variable affected by incomplete equilibration, entrainment, temperature, analytical method, solvent and water impurities, sample volatility, etc. Fig. 3 shows that TBP can extract selectively ethanol from the ethanol-containing aqueous solution, which consists of glucose, yeast extract, peptone, CSL and glycerol, and removes their effect on color development. In Figs. 1, 2 and 3 explain the rationale of the solvent extraction-dichromate oxidation method for ethanol measurements in a yeast culture medium and culture broth.

In the ethanol measurement of the yeast culture supernatant of a batch culture, it was observed that the data from the solvent extraction-dichromate oxidation method in the microtube and 96-deepwell microplate format were similar to those from gas chromatography (Figs. 4a, 5a). In addition, in 96-deepwell microplate format, the measurable range of ethanol concentrations could be extended to 8% (v/v) (Figs. 6d–f). Therefore, this ethanol assay format is practically useful for the selection of a strain having high productivity, the development of a bioethanol production process, and monitoring and control in alcoholic beverage production. In Fig. 7, some of the concentrations measured from the solvent extraction-dichromate oxidation methods were somewhat higher than those from GC, particularly in the fed-batch culture. This might be because the composition of culture medium in fed-batch culture was not consistent during the cultivation, and the initial batch culture medium was used to prepare the standard ethanol sample. In addition, the samples with more than 8% (v/v) ethanol

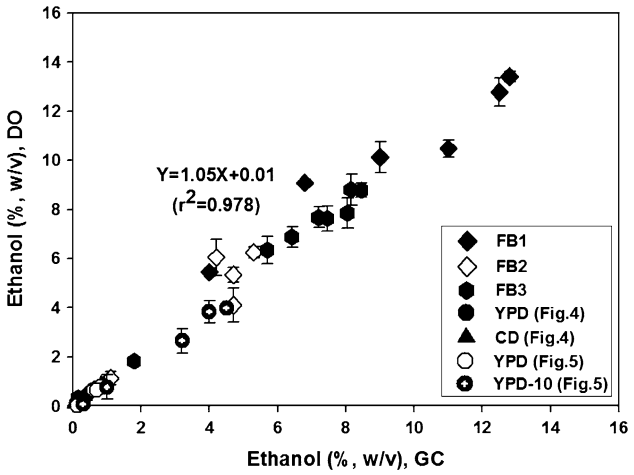

Fig. 7 Correlation between the data from the two methods for measuring the ethanol concentration. *Y*-axis [ethanol (%, v/v), DO] and *X*-axis [ethanol (%, v/v), GC] were the data obtained from the solvent extraction-dichromate oxidation method and gas chromatography, respectively. FB1, FB2, and FB3 were the data ($n = 3$) obtained from the fed-batch cultures in which the measurement was carried out in 96-deepwell microplate format

were diluted into the measurable range using the initial batch culture medium. In other words, other ingredients in the culture broth of fed-batch were not the same as those in the standard ethanol samples.

Recently, some reports show the results of ethanol determination, based on flow injection analysis and dichromate oxidation. Fletcher et al. [11] established an ethanol determination method using sequential injection analysis with an assay range up to 6% (v/v), a 100-µl sample, and an assay speed of 19 samples per hour. Based on the same principle, Vicente et al. [20] 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. [8] also introduced the flow injection analysis, up to 42.2% (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, the 96-well microplate kit for the ethanol assay has already been commercialized based on dichromate oxidation (QuantiChrom™ ethanol assay kit, Bioassay Systems, USA). However, the assay kit did not overcome the interference by glucose and glycerol. Although GC can be used for the determination of bioethanol, the dichromate oxidation method is still used after distillation of the culture broth [5].

In conclusion, we established the solvent extraction-dichromate oxidation method in both microtube and 96-deepwell microplate, in which this method overcame the interference by the medium ingredients through TBP extraction prior to dichromate oxidation, and the range of ethanol measurement was extended to 8% (v/v). In particular, the ethanol concentration in a yeast culture broth can be measured at a rate of 96 samples per 30 min using a 96-deepwell microplate. 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. In addition, it is believed that the speed and capacity of bioethanol measurements will be increased if this method can be developed as an automatically well-instrumented tool.

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