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RESEARCH ARTICLE

SPECTROPHOTOMETRIC METHOD FOR DETERMINATION OF BIOETHANOL PRODUCTION AMOUNT.

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Abstract

The continuous increase in global energy demand and need necessitates the availability of new energy sources. The expansion of production of bioethanol will reduce our dependence on petroleum and natural gas, and contribute to the decrease of environmental pollution by reducing emission of greenhouse gases. In the course of our studies for bioethanol production we needed a practical and inexpensive method for the extraction of ethanol from fermentation media and determination of the amount of bioethanol produced. We herein report a spectrophotometric method for bioethanol measurement which depends on the dichromate oxidation of the ethanol. Furthermore, we applied this method successfully to the determination of amount of bioethanol produced by fermentation in *Saccharomyces cerevisiae* cells.

Introduction:-

Today, as the risk of extinction of the traditionally known and widely used energy resources is increasing; the strategic importance of petroleum and natural gas, which are the most fundamental sources of energy, has increased; and these energy resources have become indispensable inputs of the economy. It is important to diversify energy resources especially for countries with high dependence on external energy supply, and to engage as much domestic resources as possible. Bioethanol may be a solution at a certain level for the consumption of petroleum, as well as reduction of greenhouse gases, and environmental pollution problems. Bioethanol in general is obtained by fermentation of plants containing sugar and starch and its production positively support the ecology by providing diversity in agricultural production. Bioethanol manufacture is important for creating sustainable agricultural structure and supporting rural development.

In the course of our studies for bioethanol production we needed a practical and inexpensive method for the extraction of ethanol from fermentation media and determination of the amount of bioethanol produced. Spectrophotometric methods based on the oxidation of ethanol with dichromate were advantageous over determination of ethanol concentration by gas chromatography due to lower cost of the previous method (Seo et al., 2009; Miah et al., 2017). We thus focused on finding suitable solvents and conditions for the efficient extraction of ethanol from aqueous fermentation media that is compatible with the dichromate oxidation conditions applied before the spectrophotometric measurement. We herein report our optimized protocol for the extraction of ethanol and measurement of its concentration and successful application of this methodology to the bioethanol production conditions.

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Materials and methods:-

Extraction of ethanol from aqueous solution and determination of degree of ethanol recovery

800 µl of extractant diluted in hexane is mixed with 800 µl of aqueous solution of ethanol (10 % v/v) in a 2.0-ml microtube and vigorously vortexed. After phase separation 500 µl of hexane layer was transferred to a new tube microtube. The % ethanol extracted to the hexane phase was determined by GC (Shimadzu, 8610A) equipped with an FID, under the following chromatographic conditions: temperature of the FID, 220ºC; temperature of the injector, 220°C; the oven temperature was set to 40°C (for 2 min), followed by an increase of 5ºC/min until 200ºC; carrier gas, helium with a flow of 1.5 mL/min.

Determination of bioethanol amount produced by fermentation in *S. cerevisiae* cells

*S. cerevisiae*YPH499 strain was cultured overnight at 25˚C in YPD broth. It was then diluted in YPD to give a final density of 1×10^6CFU/mL and incubated shaking with 150 rpm; bioethanol formation was monitored after 24h.At the end of fermentation, 0.58 g NaCl is added to 10 ml of yeast culture and stirred for five minutes. 1 ml of yeast culture is centrifuged and the amount of bioethanol produced was determined as follows: 800 µl of tri-n-butyl phosphate (TBP) is mixed with 800 µl of yeast culture supernatant in a 2.0-ml microtube and vigorously vortexed. After phase separation 500 µl of TBP layer (upper layer) was transferred to a new microtube.500 µl of dichromate reagent (prepared by dissolving 10 g potassium dichromate in 100 ml of 5 M sulfuric acid solution) was added and vortexed vigorously. After phase separation this time lower aqueous phase is separated and diluted twice with water. Optical density of the sample was measured at 595 nm with a UV spectrophotometer.

Results and Discussion:-

Due to rapid growth in population and industrialization, worldwide ethanol demand is increasing continuously. Throughout the course of our efforts towards more efficient production of bioethanol by fermentation in *S. cerevisiae* cells, we faced with a requirement for an inexpensive and practical method for the determination of amount of bioethanol produced. The method in need had to be compatible with the fermentation conditions and quick enough to allow monitoring of tens of reaction conditions run in parallel. Thus extraction of ethanol from the aqueous fermentation medium by an extractant followed by a dichromate oxidation based spectrophotometric measurement seemed a feasible methodology for our purposes.

We started our studies by searching for a suitable extractant to extract ethanol from aqueous solutions (Munson and King, 1984; Egan et al., 1988; Solimo et al., 1989; Arenson et al., 1990; Koullas et al., 1999; Offeman et al., 2005; 2006). The solvent had to be compatible with the dichromate oxidation process that will follow extraction, thus alcohols were not an option. We tested three extractants, namely tri-n-octylamine, tri-n-butyl phosphate and di-n-butyl phthalate, for their capacity as an extractant for ethanol. In this first screen extractants were mixed with an inert solvent, hexane as a diluent for ease of comparison and to overcome high viscosity of the extractants, especially oftri-n-octylamine. The percent ethanol recovered from the aqueous solution to the hexane phase is determined by GC and presented in Table 1. The most efficient of the three extractants was tri-n-butyl phosphate, up to 52 % of ethanol could be extracted.

Table 1:-Amount of ethanol recovered by extraction. 10 % ethanol solution was mixed with same amount of hexane containing mentioned extractants.

#	Extractant	Concentration of extractant(v/v)	Ethanol recovery (%)
1	Tri-n-octylamine	1:1	38
		1:2	23
		1:4	19
2	Tri-n-butyl phosphate	1:1	52
		1:2	34
		1:4	25
3	Di-n-butyl phthalate	1:1	27
		1:2	18
		1:4	16

Addition of salt to the aqueous solutions is a commonly applied strategy to partition other solutes out of the water phase in water-solvent extractions (Malinowski, and Dagulis, 1994). Thus we tested whether addition of various monovalent and divalent salts could increase the amount of ethanol extracted by tri-n-butyl phosphate. Addition of salts in general increased percent ethanol extracted to the solvent phase up to 73 % and the most efficient of the salts tested was NaCl (Table 2).

Table 2:-Amount of ethanol recovered by extraction in the presence of salt.

#	Salt	Concentration of salt (M)	Ethanol recovery (%)
1	none	-	54
2	NaCl	1	73
		0.5	68
3	KCl	1	62
		0.5	54
4	$CaCl_2$	1	55
		0.5	54
5	NaOAc	1	57
		0.5	56

At this point, whether the amount of ethanol recovered is linearly dependent on the initial ethanol concentration in aqueous solution remained unanswered. This question was key for us to be able to apply this method to our bioethanol production studies. We thus determined the amount of ethanol recovery starting with various concentrations of aqueous ethanol solutions. To our delight, the amount of extracted ethanol was increasing linearly with increased initial concentration (Fig 1).The experiment was repeated by measuring the ethanol recovery with spectrophotometer followingthe dichromate oxidation and this linear graph could be used as a calibration line for our later studies (Fig 2).

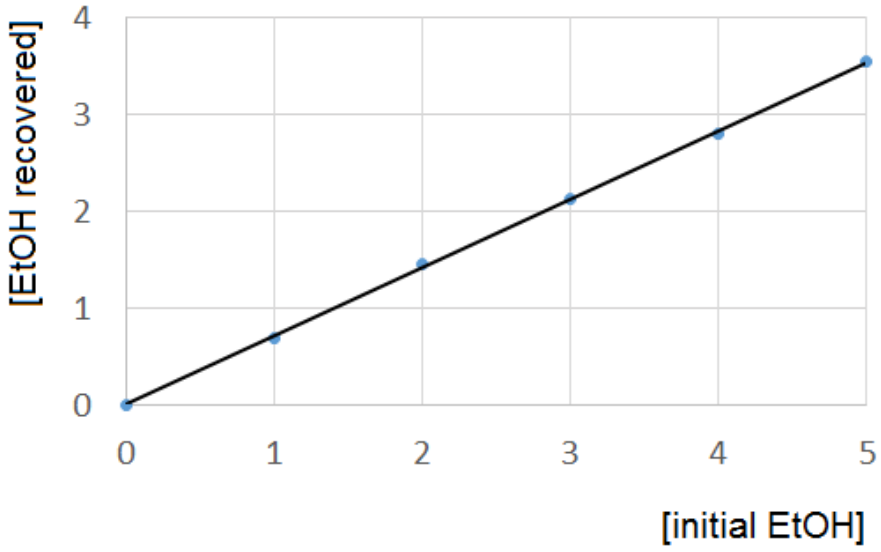

Fig 1:-Amount of ethanol extracted from its aqueous solutions with TBP is linear with initial ethanol concentration.

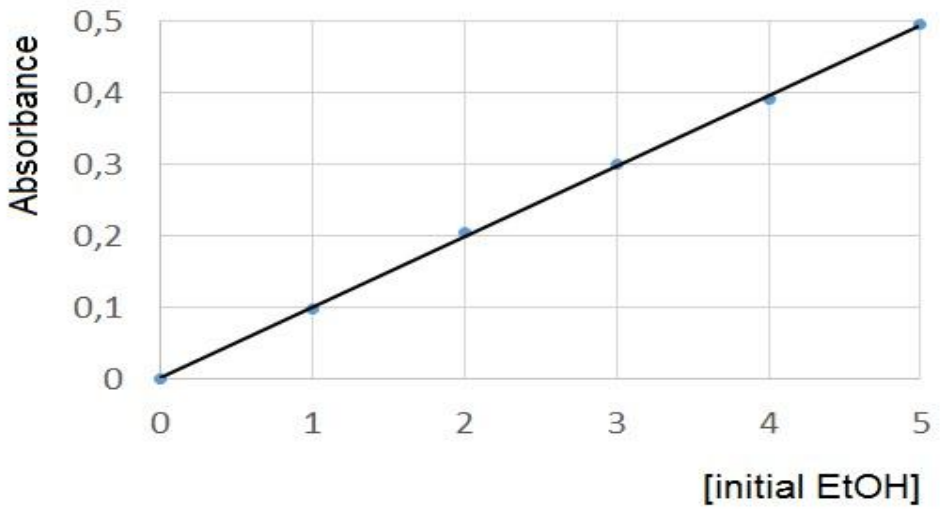

Fig 2:-Absorbance for ethanol recovered by TBP extraction for various initial ethanol concentrations.

At this point we applied our method to the determination of fermented bioethanol in yeast cells. *S. cerevisiae* culture was grown for 24 h, at 25 ºC, shaking with 150 rpm speed in YPD media. At the end of 24 h, bioethanol produced was extracted in TBP solvent and its amount was determined spectrophotometrically after dichromate oxidation. The absorbance measured at 595 nm was 0.178; and thus reading from the calibration line of Fig 2, about 1.8 % of bioethanol was produced in this study.

In conclusion, we developed a practical method for the extraction and measurement of ethanol from aqueous solutions and this protocol could be successfully applied to the monitoring of bioethanol production in YPD media by yeast.

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