International Journal of Biological Sciences and Applications
2014; 1(3): 46-54
Published online July 20, 2014 (http://www.aascit.org/journal/ijbsa)

Keywords
Electrochemical Cell,
Ethanol,
Lignocellulosic Biomass,
Voltage

Received: June 23, 2014
Revised: July 15, 2014
Accepted: July 16, 2014

Improvement of ethanol production by electrochemical redox combination of yeast cells

Jarina Joshi[1, *], Rejeena Shrestha[1], Rojlina Manandhar[1], Krishna Manandhar[1], Lakshmaiah Sreerama[2], Tribikram Bhattarai[1]

[1]Central Department of Biotechnology, Tribhuvan University, Nepal
[2]Department of Chemistry and Physics, St. Cloud State University, USA

Email address
jarinarjoshi@gmail.com (J. Joshi)

Citation
Jarina Joshi, Rejeena Shrestha, Rojlina Manandhar, Krishna Manandhar, Lakshmaiah Sreerama, Tribikram Bhattarai. Improvement of Ethanol Production by Electrochemical Redox Combination of Yeast Cells. *International Journal of Biological Sciences and Applications.* Vol. 1, No. 3, 2014, pp.46-54.

Abstract
Electrolytic cell are used for enhancement in production of microbial products. Ethanol is produced as one of the renewable source of energy for which one can use even waste products like lignocellulosic biomass. Selection of best ethanol producing yeast strain and best ethanol tolerant yeast cell is first priority for optimized production. Out of eight yeast strains isolated in Central Department of Biotechnology (CDBT). strain CDBT 2 showed good ethanol tolerance with maximum growth even at 8% ethanol. Thebest ethanol tolerant strain CDBT 2 found to grow till 18% ethanol. Total ethanol production by CDBT 2 was found to enhance by about 15±0.12% than control without supply of 4V external energy when glucose was used as carbohydrate source. Drastic increase in ethanol production by 129±0.877% was seen when external voltage was supplied in electrochemical cell with alginate immobilized yeast cells on porous graphite cathode with or withput external voltage supply. However total enhancement is 7.0±0.056% than control w/o immobilization of yeast strain in cathode. 9.0±0.225% increament was observed when cathode was immobilized with neutral red with and without supply of external voltage. 30.64±0.30% and 28.67±0.344% increament in ethanol were observed when cathode were immobilized with neutral red only and neutral red followed by yeast immobilization than with normal graphite felt cathode without external voltage supply. The best ethanol tolerant yeast strain, CDBT 2 was grown in electrolytic cell at best electrode combination with lignocellulosic biomass, *Saccharum spontanum*, pretreated with ammonium hydroxide followed by hydrolysis with hydrochloric acid gave the ethanol production of 16.67% greater than without external voltage supply.

1. Introduction

A microbial electrolytic cell is like a biological system in which microbes do not directly transfer their produced electrons to their characteristic electron acceptors instead, they are subsequently transferred through a resistance or power source to cathode (Korneel Rabaey *et al*, 2003). Current technologies to produce ethanol largely depend upon sugarcane and/or starch based grains and tubers, however, the increasing stress on food prices and food security has evolved lignocellulosic materials as a source of ethanol. Significant efforts on the development of second

generation processes of ethanol production from lignocellulosic materials rich in their lignin, cellulose, hemicelluloses and pectin contents are being made. Lignocellulosic materials for this purpose includes lignocellulosic biomass like corn stover, wheat straw, sugarcane bagasse, rice straw, rice hull, corn fiber, cotton stalk; energy crops such as switch grass and Alfa-Alfa and various weeds such *Saccharum spontaneum, Lantana camara, Eichhornia crassipies* etc. Cellulose is the major component of lignocellulosic biomass and its concentration ranges from 40-50% of dry weight (Zhang *et al*, 2004), hemicelluloses is present at a concentration of 25-35% (Saha *et al*, 2007), lignin being the third major component is present at a concentration of 20-35% (Joshi et al, 2011) and pectin in trace amounts of 1-2% . Pretreatment is the key technology to reduce the cost of cellulosic ethanol (Yang B., 2008)

As compared to the use of prokaryotes in the biofuel cell, much less research have been carried out on the use of eukaryotes such as yeasts as biocatalysts in the electrolytic cell. Yeasts such as *Saccharomyces cerevisiae* and *Candida melibiosica* have the electrochemical capabilities to use as biocatalysts in a biofuel cell (Hubenova.Y and Mitov .M, 2008). *S. cerevisiae* was genetically modified for enhancement in production of ethanol in normal fermentation, Result showed very less improvement [David et al, 2010]. However, *S. cerevisiae* found to enhance ethanol production when grown in cathode with 1.5V supply [Shin et al, 2002].

Most microorganisms are not anodophiles i.e.; cannot transfer electrons directly to the electrodes, electron mediators to enhance the power output are used. Various endogenous and synthetic electron mediators are commonly used in cells. Neutral red, methylene blue, thionine, iron (III) EDTA, Meldola's blue, Mn^{4+} are the synthetic mediators and humic acids, Anthraquinone, the oxyanions of sulphur (sulphate and thiosulphate) are the endogenous mediators (Park *et al*, 2000). The endogenous mediators transfer electrons from inside the cell membrane to the anode but there are only a limited number of organisms that can transfer electrons across the membrane by themselves to the anode. Microorganisms majorly bacteria such as *Geobacteraceae sulferreducens, Geobacter metallireducens, Shewanella putrefaciens* (Kim *et al*, 2010) and *Rhodoferax ferrireducens* (Chaudhari and Lovely, 2003) are known to efficiently form film on the anode surface and transfer electrons directly to the electrode across the membrane minimizing the use of electron mediators that are known to be toxic (Hahn-Hägerdal et al., 2006). This paper studied the improvement in ethanol production by electrochemical redox combination of yeast cells.

2. Materials and Methods

2.1. Isolation and Characterization of Yeast Strains

All the chemicals were purchased from HiMedia unless stated. Murcha samples (locally used Fermentation starter ie; the local yeast sources specifically used for ethanol production) were collected from Bhaktapur and Lubu area of Kathmandu valley, Central Nepal. They were serially diluted and used pour plate for isolation in Yeast Maltose Agar (YMA) media with composition Yeast extract (3 gm/l), Malt extract (3 gm/l), Peptone (5 gm/l) and Glucose (10 gm/l). Media adjusted to pH 4.5. The isolated strains were sub-cultured for getting pure strains [T. Karki et al 1999; Middelhoven et al 2002; Middelhoven et al 1998]. Pure cultures were stored as glycerol (15%) stock for further use.

2.2. Study of Ethanol Tolerance of Yeast Cells

All isolated yeast cells were cultured in Peptone Yeast Extract Nitrogen (PYN) media supplemented with Peptone 3.5 gm/l, Yeast extract 3 gm/l, KH2PO4 2 gm /l, MgSO4 1 gm/l, $(NH_4)_2SO_4$ 1 gm/l and Glucose 50 gm/l with variable concentrations of ethanol ranges from 0 to 22%. Culture conditions were maintained at 28^{o}C, pH 4.5 for 3 days to see effect on growth pattern. Microbial growth pattern were observed spectrophotometrically [Genesis] at 615 nm against medium blank. The best ethanol tolerant strain was sent for molecular characterization.

2.3. Effect of pH on Ethanol Production

The effect of pH on the fermentation process was determined by adjusting the pH of the PYN media. The pH of the media were adjusted in the range from 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 to 7.0 with 1N HCl and 1N NaOH. The media was then inoculated with 4% (18 hrs culture) of the inoculum and incubated at optimum temperature for 72 hrs. Ethanol concentration was measured after solvent extraction with tri- n-butyl phosphate and treating with acidified potassium dichromate as described by Seo et al, 2009.

2.4. Effect of Temperature on Ethanol Production

The effect of temperature on the fermentation process was determined by inoculating the inoculum to PYN medium and incubating at different temperature. The culture was incubated at temperatures 24^{o}C, 26^{o}C, 28^{o}C, 30^{o}C and 32^{o}C respectively for 72 hrs. The temperature at which maximum ethanol production was observed as described by Seo et al, 2009.

2.5. Effect of Different Nitrogen Supplements on Ethanol Production

The effect of various nitrogen sources on fermentation was determined by incorporating various nitrogen sources in the fermentation media. The nitrogen sources used were ammonium sulphate, ammonium nitrate, ammonium chloride, ammonium oxalate, ammonium acetate, yeast extract, peptone and urea. The media were then inoculated and incubated at optimum temperature for 72 hrs. The best source of nitrogen for ethanol production was then determined.

2.6. Ethanol Production in Electrochemical Cell

Immobilization of Neutral red to Graphite Electrode

The graphite felt soaked in methanol was dipped in 1% polyvinyl alcohol solution for 3 to 4 hours. Dry in oven at about 80^{o}C for 24 hours. The completely dried graphite felt was then soaked in pure chloroform containing 10% thionylchloride and 0.01% neutral red (Sigma Co.) for 6 hours. The graphite felt was then left for 12 hours for air dry. It was then autoclaved and washed in running water till color persist. Finally dried at 60^{o}C for 1 day and used as NR graphite electrode (Jeon et al, 2009).

Immoblization of Yeast Cells on Graphite Electrode

2.4 ml of 18 hrs. culture of yeast cells were centrifuged and pellet mixed with 10 ml of 25 mM phosphate buffer (pH 7.0) containing 4% sodium alginate and absorbed into the graphite electrode for 30 mins. The NR-graphite electrode containing alginate and yeast cells was then soaked in a chilled 100 mM $CaCl_2$ solution for 30 mins to induce calcium alginate coagulation and washed with a 25 mM phosphate buffer.

Construction of Electrolytic Cell

A two compartment electrolytic cell was designed to induce the electrochemical oxidation and reduction reaction simultaneously. The anode and cathode compartments were separated by a porcelian membrane and cellulose acetate film. Anode is made from Platinum electrode (0.2 mm diameter, Sigma) and cathode is made specifically from graphite fiber (Immoblized and unimmoblized). Small scale working volume of the cathode compartment was adjusted to 60 ml each and observed the product concentration at optimized condition.

Ethanol Production in Electrochemical Cell Using Glucose as Carbohydrate Source

Potent yeast strain was observed for improvement in ethanol production in electrochemical cell with PYN media. Electrochemical cell without electrical connection was taken as control.

Ethanol Production in Electrochemical Cell Using Pretreated Lignocellulosic Biomass Hydrolysate as Substrate

Lignocellulosic biomass was pretreated with 0.5M ammonium hydroxide for 24 hours at 65^{o}C. The pretreated biomass was then hydrolyzed with 0.5M hydrochloric acid. The hydrolysate was adjusted to optimized pH and used as substrate for ethanol fermentation by CDBT 2 in electrically enhanced cell.

3. Results and Discussions

Isolation and Characterization of Yeast Isolates

Yeast strains were isolated from Murcha samples collected from Lubhu and Bhaktapur area using PYN media. Eight yeast isolates were selected and maintained pure cultures.

Fig. 1. *Yeast strains isolated from Murcha samples in PYN media. Left: CDBT 1 and right: CDBT 2.*

All the yeasts were creamy white, round or irregular margin with smooth raised or flat surface (Fig.1). Most of the yeast showed budding characteristics.

Ethanol Tolerance of Yeast Cells

All the eight yeast strains found to grow well till 4 % (Fig. 2). However CDBT 2 showed regular growth till 6% ethanol concentration. According to the report by Kyung, 2003, ethanol tolerance of 5% is considered as good yeast. Almost all yeasts tolerated ethanol up to 14%. Beyond this there was sharp decrease in the growth ie; almost nil, which was similar to the ethanol tolerance showed by most of *Saccharomyces cerevisiae* i.e. 14.5 % (v/v) (Teramoto et al., 2005).

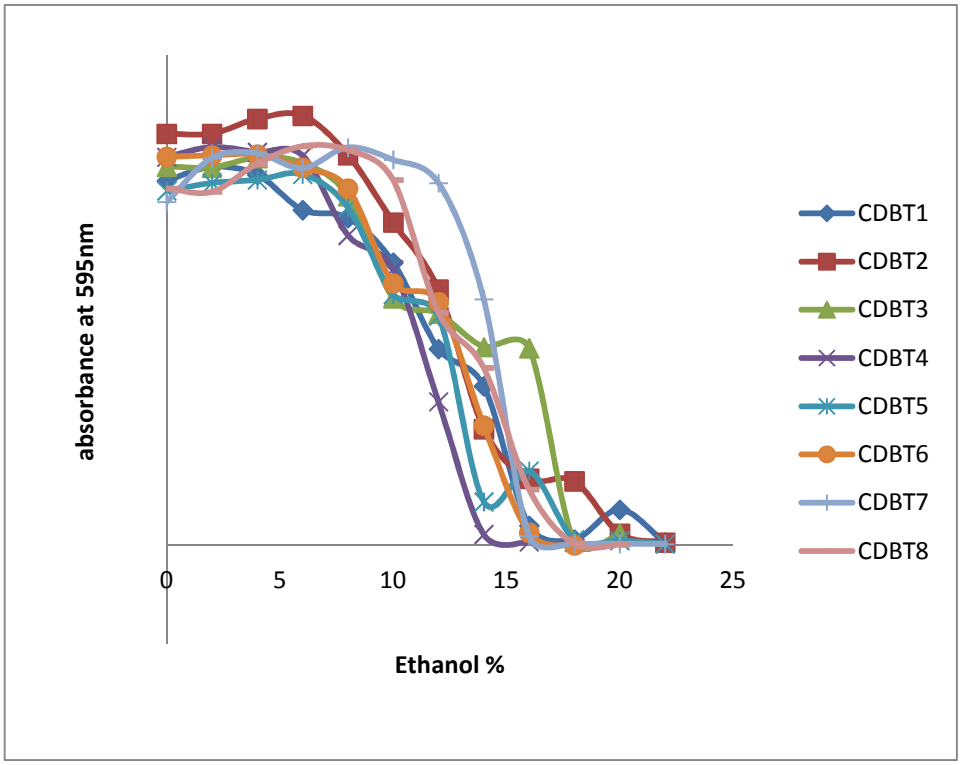

Fig. 2. *Strains CDBT 1 to CDBT 8 grown at different initial concentration of ethanol ranges from 0 to 22% in PYN media for 3 days at pH 4.5 and temperature 28°C. [The data is average of three repeated results]*

Yeast strain CDBT 2 found to grow till 18% ethanol. Ethanol tolerance is an advantage when yeast is being used for the industrial production of ethanol (Ekunsanmi and Odunfa, 1990). During fermentation it is difficult to avoid the ethanol tolerance but substrate inhibition can be overcome by the stepwise addition of substrate. However, temperature (Casey and Ingledew, 1986; D'Amore and Stewart, 1987), natural habitat and the origination of isolation area (Torija et al, 2003) may be the factor for the resistance of strain to ethanol. The ethanol tolerance may be contributed by the physiological factors such as medium composition and the mode of substrate feeding intracellular ethanol accumulation, temperature and osmotic pressure (D'Amore and Stewart 1987). Gonzalez et al 2002 verified that the changes in the expression of several genes concerned with the synthesis of cell envelop components may contribute to increased ethanol tolerance of yeasts. According to Logothetis et al, 2007, water molecules due to its hydrophilic nature can penetrate the lipid bilayer and form hydrogen bonds with the polar groups of phospholipids maintaining the structure of the biological membrane. Ethanol displaces water molecules altering the position of molecules on the membrane affecting the interaction between the lipids and proteins, ultimately damage the structure and function of the membrane. Trehalose has the ability to retain water in the yeast membranes and hence stabilizes the membrane.

According to Gunclave lab, the D1D2 sequencing of CDBT 2 strain was found to resemble *Saccharomyces cereviceae*.

Optimization of Ethanol Production

Effect of pH on Ethanol Production

Fig. 3 showed the effect of pH on ethanol production. Maximum production of ethanol was found at pH 5.5 with 12.37±0.44 mg/ml.

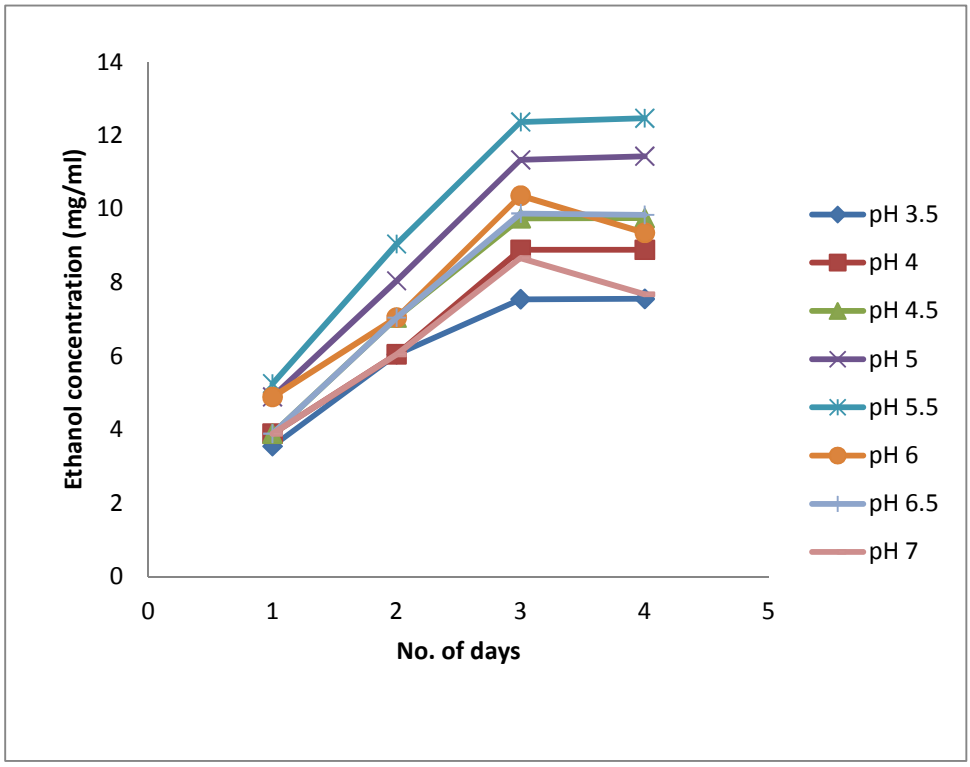

Fig. 3. *Ethanol production by strain CDBT 2 grown in PYN media adjusted at different pH ranges from 3.5 to 7.0 at 28°C . [The data is average of three repeated results]*

Effect of Temperature on Ethanol Production

Ethanol production was found to be optimum at 30°C with 12.55± 0.70 mg/ml yield.

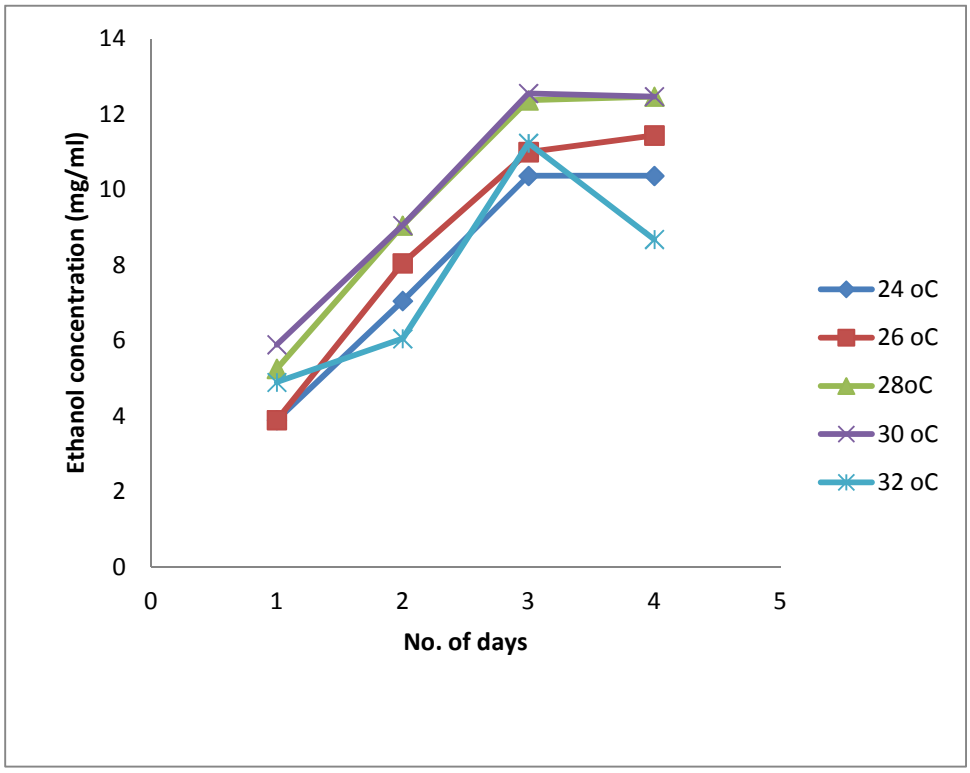

Fig. 4. *Ethanol production by strain CDBT 2 grown in PYN media adjusted at different temperature ranges from 24 to 32°C at pH 5.5. [The data is average of three repeated results]*

Effect of Different Nitrogen Supplements on Ethanol Production

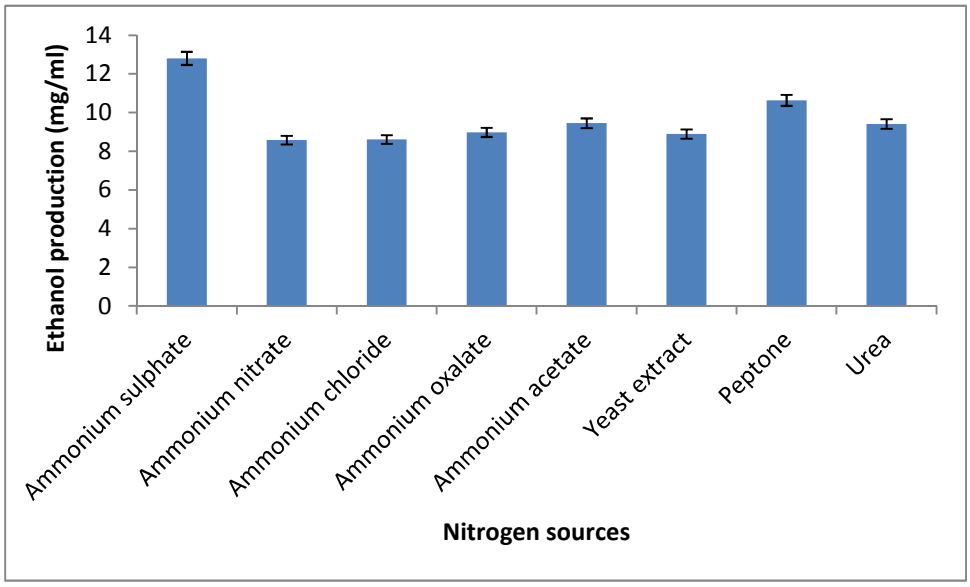

Fig. 5. *Ethanol production by strain CDBT 2 grown in YNB media supplemented with different nitrogen sources adjusted at pH 5.5 and temperature 308°C .*

Out of different nitrogen supliments, ammonium sulphate was found to be the best nitrogen source for ethanol production. The efficiency of ethanol production was found to be in the order 12.82 ± 0.34 mg/ml.

Ethanol Production in Electrochemical Cell

Several types of Electrochemical cells were designed for studying ethanol production. PYN media with 5% glucose adjusted at optimized condition of pH 5.5, temperature 30°C were inoculated with yeast cells and cultured for 3 days. Platinum wire (Sigma) with diameter 0.2 mm were used as anode. Cathodes were made with graphite fibre of thickness 10 mm (Nippon company, Japan) with several modifications. Graphite fibre without immobilization of yeast strain CDBT2 ie; *S. cereviceae*, yeast strain immobilized graphite fibre, neutral red immobilized graphite fibre and neutral red and *S. cereviceae* both immobilized graphite fibre respectively and were separately used as cathode electrode in combination with Pt anode. Graphite felt has more surface area to use as electrode and neutral red immobilized graphite is a good electron donor for reduction of NAD to NADH in vitro, hence to produce more ethanol [Jeon Bo, 2009, Park et al, 1999]. Ethanol production efficiencies were studied for all possible cathode arrangements.

Ethanol Production in Electrochemical Cell with PYN Media Using Different Combination of Electrode

Ethanol was allowed to produce in 4V external energy supply which is optimum for *S. cereviceae* to produce ethanol [Jeon Bo et al, 2009]. When ethanol was allowed to produce in electrochemical cell with 4V external electricity supply, using normal graphite felt as cathode and Platinum as anode, total production was found to enhance by about 15±0.12% than control (without supply of electricity) which was quiet near to 12% enhancement in ethanol production of *S. cereviceae* as reported by Shin et al, 2002 when cultured in cathode. Ethanol production was 15.5±0.124 mg/ml and 13.5± 0.108 mg/ml in anode and cathode respectively where as total ethanol production in control was 12.63±0.076 mg/ml only (Fig 6). Drastic increase in ethanol production from 5.12±0.378 mg/ml to 15.0± 0.09 mg/ml was seen when external voltage was supplied in electrochemical cell with alginate immobilized yeast cells on cathode than ethanol production by control system with yeast cell immobilization on graphite electrode which was around 129±0.877% increment. However total enhancement is 7±0.056% less than control without yeast immobilization.

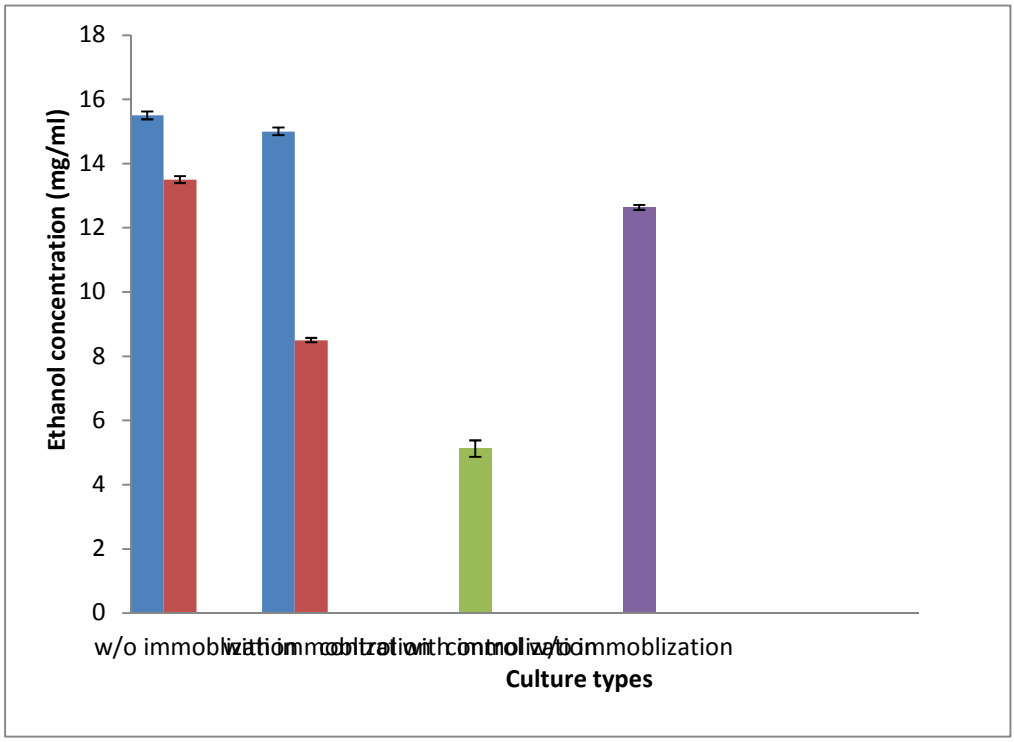

Fig 6. *Ethanol production by strain CDBT 2 in electrochemical cell with or without immobilization of yeast cells in graphite felt cathode. Pt wire was used as anode. Culture was incubated in PYN media adjusted at pH 5.5, temperature 30 °C for 3 days . Blue: ethanol produced at cathode, Red ethanol produced at anode and green and purple: control without electricity supply respectively.*

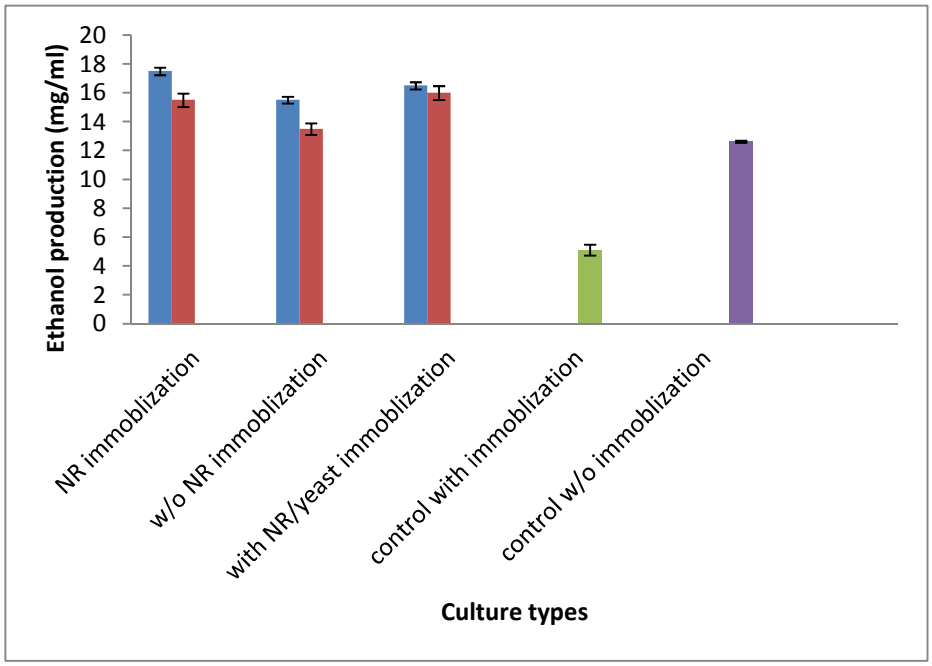

Fig. 7. *Ethanol production by strain CDBT 2 in electrochemical cell with or without immobilization of yeast cells, with or without immobilization of neutral red or with or without immobilization of neutral red followed by yeast cells in graphite felt cathode. Pt wire was used as anode. Culture was incubated in PYN media adjusted at pH 5.5, temperature 30 °C for 3 days . Blue: ethanol produced at cathode, Red ethanol produced at anode and green and purple: control without electricity supply respectively.*

Fig.7 showed the effect of neutral red and followed by yeast cell immobilization on ethanol production. Total ethanol production in anode (17.51± 0.343 mg/ml) and cathode (15.50 ± 0.465 mg/ml) were found to be enhanced by about 9 ± 0.225 % when cathode was immobilized with neutral red in comparison to 15.12± 0.378 mg/ml without voltage supply. While the total ethanol production was enhanced by 30.64±0.30% than control without neutral red immobilization and without supply of external voltage. Whereas the average total ethanol production in anode

(16.51± 0.066 mg/ml) and cathode (16.0±0.128 mg/ml) was enhanced by only 28.67±0.344% than normal fermentation ie control when NR and yeast both were immoblized in cathode which showed that yeast cell immobilization in cathode electrode is not necessary in electrochemical cell. According to Jeon Bo et al. immobilization of yeast cells on cathode does not enhance ethanol production. In the two compartment electrochemical bioreactor, the anode functions as working electrode and cathode functions as counter electrode. There was drastic improvement in ethanol production found in anode compartment than in cathode compartment when cultured at 4V. This indicates that oxygen is absolutely required for normal growth and fermentatively metabolism [Sablayrolles et al, 1996]. At this voltage anaerobic condition have been reported to cause the NADH/NAD+ ratio to be imbalance and limit sugar in the fermentative metabolism of *Saccharomyces* spps.[Sims et al, 1978]

Ethanol Production in Electrochemical Cell Using Pretreated Lignocellulosic Biomass Hydrolysate

The washed and oven dried biomass was pretreated with 0.5M Ammonium hydroxide followed by 0.5M Hydrochloric acid hydrolysis. The hydrolysate was adjusted to optimized pH of 5.5 and kept in electrochemical cell with neutral red immobilized cathode. Fermentable sugar converted to ethanol by *S. cerevisiae* found be 11.10± 0.067 mg/ml and 10.0 ± 0.04mg/ml in anode and cathode respectively which was 16.66±0.0833% more than non electrolytic condition (Fig. 8). The decrease in ethanol production may be due to generation of salt during neutralization. However use of electrically enhanced method found to enhance lipid biosynthesis hence increases salt tolerancy [Rosenfeld et al, 2003]

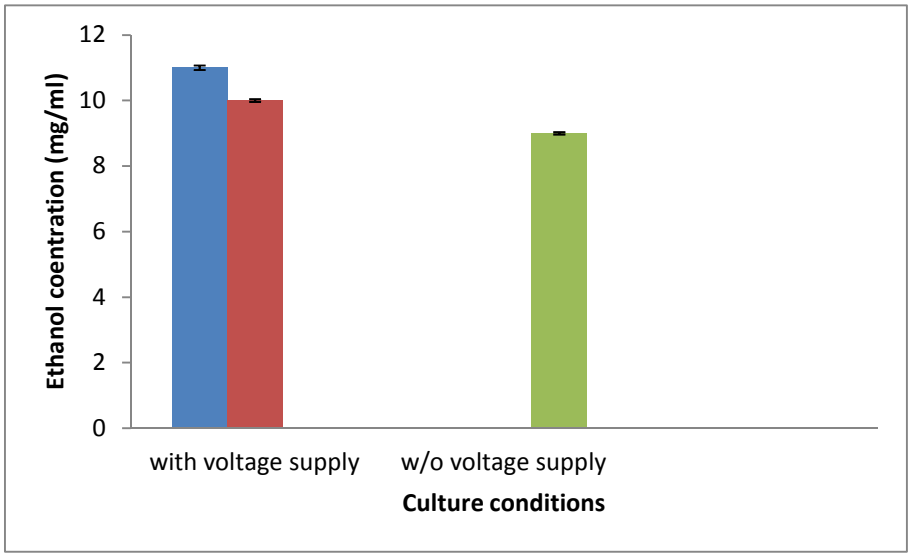

Fig. 8. *Ethanol production by Saccharum spontanum hydrolysate using strain CDBT 2 in electrochemical cell with or without supply of external voltage. Graphite felt immobilization of neutral red was used as cathode and Platinumt was used as anode. Blue: ethanol produced at cathode, Red ethanol produced at anode respectively.Green:control w/o voltage supply.*

Acknowledgement

We would like to express our sincere gratitude to University Grants Commission for supporting us financially and Central Department of biotechnology for creating environment to accomplish work. We are thankful for Prof. Paula Goncalves laboratory, Portugal for helping us identifying yeast strain. We are also thankful to Prof. Doo Hun Park, Department of Biological Engineering, Seokyeong University, Seoul, South Korea; Dr. Amar Yadav, Central Department of Chemistry, Tribhuvan University, Nepal and Renewable Nepal, Kathmandu University, NORAD, SINTEF Programme for providing additional help.

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