# The New Era has Begun: Renewable Bioenergy Production as a Progressing Interdisciplinary Research Approach

Yolina Hubenova[1], Mario Mitov[2,3]

[1)] Department of Biochemistry and Microbiology, "Paisii Hilendarski" University, Plovdiv, Bulgaria

[2)] Department of Chemistry, South-West University "Neofit Rilsky", Blagoevgrad, Bulgaria

[3)] Institute of Electrochemistry and Energy Systems – Bulgarian Academy of Sciences, Sofia, Bulgaria

The aim of this study is a verification of possibilities for introducing budding yeast cells as a microreactor into biofuel cells. The investigation includes optimization of conditions for high efficiency utilization of in vivo produced electrons within the main biochemical pathways, which naturally cover the energy needs of the living cells. We trace out the three stages of cellular respiration - glycolysis, citric acid cycle and oxidative phosphorylation in eukaryotic cells, aspiring to shuttle the electrons between the biological system and the fuel cell anode by using appropriate mediator. The challenge is to use the biological energy from subcellular level. We modulate the biofuel cell/cell suspension elements in such manner that the mediator could capture the electrons from the biological electron transport chains and transfer them subsequently through the double mitochondrial membrane and outer membrane of intact cells and/or from cytosol under anaerobic conditions and finally, to the electrode, where they are transmuted into electricity.

## *Introduction*

One of the greatest challenges at the onset of the 21st century is to solve the problem of permanently increasing energy consumption. On the one hand, because of the progressively enlarging population on our planet and the rapidly developing technical progress as well as regardless of consequences the constant exhaustion of the natural fuel resources. On the other hand, the use of traditional carbon-containing fuels aimed at satisfying energy needs today has led to a hazardous increase of the environmental pollution and climate changes. In this regard, the application of ecologically friendly energy sources and innovative converters has turned into one

alpine space - man & environment, vol. 7: Global Change and Sustainable Development in Mountain Regions
, ISBN 978-3-902571-97-7

of the most urgent and primary tasks of our time. This includes research, development and demonstrations (European Commission C 5765, 2007) aiming to:

- Improve energy efficiency throughout the energy system taking into account the global environmental performance;
- Accelerate the penetration of renewable energy sources;
- Decarbonise power generation and, in the longer term, substantially decarbonise transport;
- Reduce greenhouse gas emissions;
- Diversify Europe's energy mix;
- Enhance the competitiveness of European industry.

From all developed up-to-now energy converters, hydrogen fuel cells possess the highest efficiency, reaching up to 85%, when the electric and the heat energy are co-generated. The production and application of the different fuel cells types is still quite limited, mainly due to the high price of hydrogen production and storage and to the use of expensive catalysts. However, in the next 10-15 years fuel cells are expected to replace a big share of the currently used energy converters, not only in the transport and mobile applications, but also in the stationary ones.

As alternative to the conventional fuel cells we are looking for new ways for sustainable energy production combining the biotechnological principles with those of electrochemistry. Biofuel cells are a perspective approach for overcoming both the dramatically increasing energy demands and the irreversible environmental pollution caused by the use of non-renewable carbon-based fuel resources. In addition, biofuel cells potentially offer solutions to the trend towards miniaturization and portability of computing, communications as well as implantable electrically operated devices.

The use of entire microorganisms as microreactors in fuel cells eliminates the need for isolation of individual enzymes and allows the active biomaterials to work under conditions close to their natural environment, thus at high efficiency. Microorganisms have the ability to produce electrochemically active substances that may be metabolic intermediates or final products of anaerobic respiration.

The most investigations up-to-now include the usage of prokaryotes as a proton/electron source. Those are hydrogen gas producing bacteria (Das and Veziroglu, 2001), (Maness and Weaver, 2001), bacteria, which generate fuels such as methane (Kim et al., 2002), ethanol, methanol (Kosaric and Velikonja, 1995) from waste products, sulphate reducing bacteria (Cooney et al., 1996), etc.

The aim of this study is a verification of possibilities for introducing budding yeast cells (eukaryotes) as a microreactor into biofuel cells. The investigation includes biochemical and electrochemical studies of conditions for high efficiency utilization of *in vivo* produced electrons within the main biochemical pathways, which naturally cover the energy needs of the living cells.

## *Experimental*

In this *ab initio* study, the log-phase of growth during *Saccharomyces cerevisiae* yeast cell cycle was examined under aerobic as well as anaerobic conditions. Different amounts of yeast were cultivated in suspension media containing carbohydrates and phosphate buffer pH 7. The assimilation levels of monosaccharide glucose and disaccharide sucrose in the yeast medium were quantified by 3,5-dinitrosalicilic acid (DNS) colorimetric method (Kozłowska et al., 2007). In parallel, the quantity of the inorganic phosphate converted into organic one in the progress of cell cycle was analyzed by means of Molybdenum blue phosphorous method (He and Honeycutt, 2005). The values of glucose and phosphate concentration in the cell suspensions were determined by standard calibration curves methods.

Electrochemical experiments were performed in a model two-compartment cell. Suspension of 0.1 g/ml yeast, 0.25 mol/l glucose or sucrose, methylene blue as an electron mediator and phosphate buffer was used as an anolyte. The suitable pH of buffer solution was verified as described by Benetto (1990). The neutral pH choice was considered as an optimal value for the cell growth. Solution of 0.1 mol/l $K_3[Fe(CN)_6]$ or $NH_4VO_3$ in phosphate buffer (pH 7) was used as a catholyte. For accomplishment of a galvanic cell both solutions were poured into separate closed vessels connected with a salt bridge. Segmented graphite rods were used as electrodes.

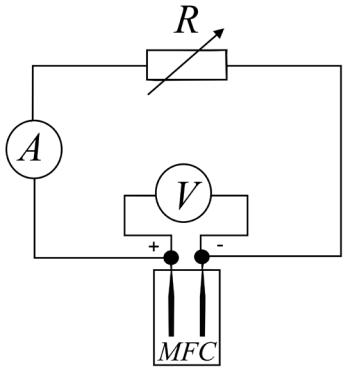


*Fig.1: Circuit diagram for realization of polarization measurements: MFC – model fuel cell, R – variable resistor, A – ammeter, V – voltmeter.*

Polarization characteristics were achieved by realization of electric scheme, drawn in Fig. 1. Varying the resistance R values, pairs of voltage and current were recorded and the corresponding volt-ampere as well as power curves were plotted. Discharge curves at constant ohmic resistance were also monitored.

## *Results and discussion*

The three stages of cellular respiration - glycolysis, citric acid cycle and oxidative phosphorylation in eukaryotic cells, were traced out in the present study, aspiring to shuttle the electrons between the biological system and the fuel cell anode by using appropriate mediator. Conditions for optimal electron harvest during the cell cycle development of *Saccharomyces cerevisiae* were searching for. To realize that, the biofuel cell/medium elements should have been modulated in such manner that the used mediator could capture the electrons from the biological electron transport chains and transfer them subsequently throughout the double mitochondrial membrane, cytosol and outer membrane of intact cells and finally, to the electrode.
The results from quantitative analyses, showing the progress of yeast cell development, are summarized in Table 1.

*Table 1: Quantitative determination of glucose and inorganic phosphate by DNS and Molybdenum blue phosphorous methods during yeast cultivation.*

| Yeast suspension | 0.1 g/ml yeast + 250 mM glucose/ 67 mM phosphate buffer, pH 7 | | | | 0.1 g/ml yeast + 250 mM sucrose/ 67 mM phosphate buffer, pH 7 | | | |
|---|---|---|---|---|---|---|---|---|
| Conditions | aerobic | | anaerobic | | aerobic | | anaerobic | |
| Incubation time (at 30°C), minutes | glucose mM | $PO_4^{3-}$ mM | glucose mM | $PO_4^{3-}$ mM | glucose mM | $PO_4^{3-}$ mM | glucose mM | $PO_4^{3-}$ mM |
| 20 | 11 | 48 | 9 | 20 | 40 | 20 | 39 | 21 |
| 30 | 9 | 20 | 8 | 19 | 40 | 16 | 34 | 15 |
| 40 | 5 | 19 | 7 | 19 | 40 | 16 | 32 | 15 |

The time limit, during which enough adenosine 5‘-triphosphate (ATP) amount had been synthesized without inhibiting effect for the glycolysis regulation enzymes, was determined. In such manner, we indirectly proved the stage of further running of processes involved in Krebs cycle and the conversion of the inorganic phosphate into ATP as well as the transfer of electrons by the electron-carriers nicotineamide-adenine-dinucleotide (NAD) and flavine-adenine-dinucleotide (FAD) into respi-

ratory chains under aerobic conditions. The same analysis was made under anaerobic conditions, by which carbohydrates metabolize to ethanol. In general, this is of importance for the fuel cell application because if oxygen is present then it will collect the electrons as it has a greater electronegativity than the mediator.

Under aerobic conditions, the oxidation of glucose in the examined yeast strain runs more intensively than the further pyruvate transmutation in citric acid cycle and oxidative phosphorylation. In a contrast, the glycolysis and the processes of formation of ATP obviously take place with similar rates even at the first 20 minutes under anaerobic conditions.

The rate of phosphate assimilation was also verified by adding disaccharide into the yeast suspension. Due to sucrose hydrolysis and participation of fructose in the cell catabolism identical quantity of phosphate under both aerobic and anaerobic conditions was determined at the end of the first generation time.

The iso-osmotic conditions for cell culture development were chosen for running of electrochemical assays. Typical polarization voltage-current dependences and the corresponding power curves, obtained with the model biofuel cell by using *Saccharomyces cerevisiae* budding yeast, are presented in Fig. 2.

Both polarization characteristics and power curves are comparable with those reported for other types of microorganisms (prokaryotes) in literature (Bullen et al., 2006). The open circuit voltage exceeds 500 mV and the output power tends to 15 μW. These results demonstrate the operational principles of biofuel cells utilizing yeast and could be used as a base for further investigations.

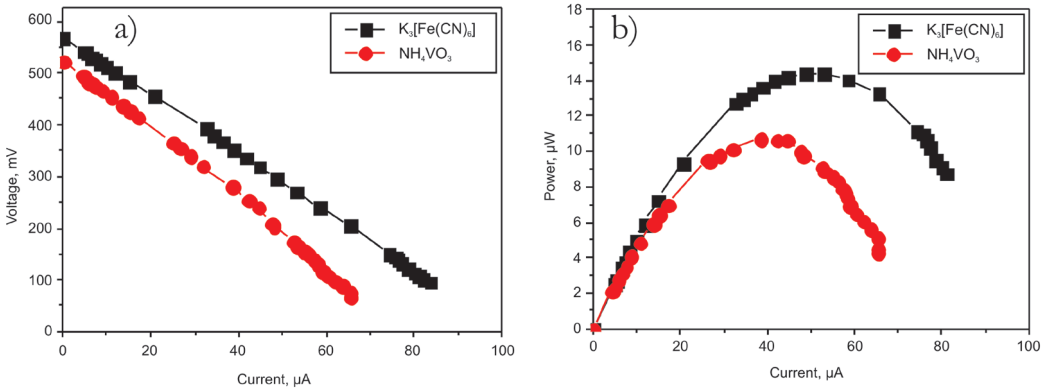


*Fig. 2: a) Polarization characteristics and b) power curves obtained with 0.1 g/ml yeast + 250 mM glucose/67 mM phosphate buffer, pH 7 as an anolyte and 100 mM $K_3[Fe(CN)_6]$ or $NH_4VO_3$/67 mM phosphate buffer, pH 7 as a catholyte.*

As seen from Fig. 2, at equal other conditions better performance was observed with $K_3[Fe(CN)_6]$ in comparison with ammonium vanadate solution as a catholyte. By this reason, the potassium ferricyanide solution was further used in the subsequent experiments.

Discharge of the model biofuel cell at a constant resistance equal to this, at which maximum power had been obtained, was also carried out. Resulting curves, presenting the change of generated current within time, are plotted in Fig. 3. The electrooxidation of carbohydrates (glucose or sucrose) without yeast suspension has been monitored as control test (blank) and the corresponding discharge curves are also shown in Fig. 3.

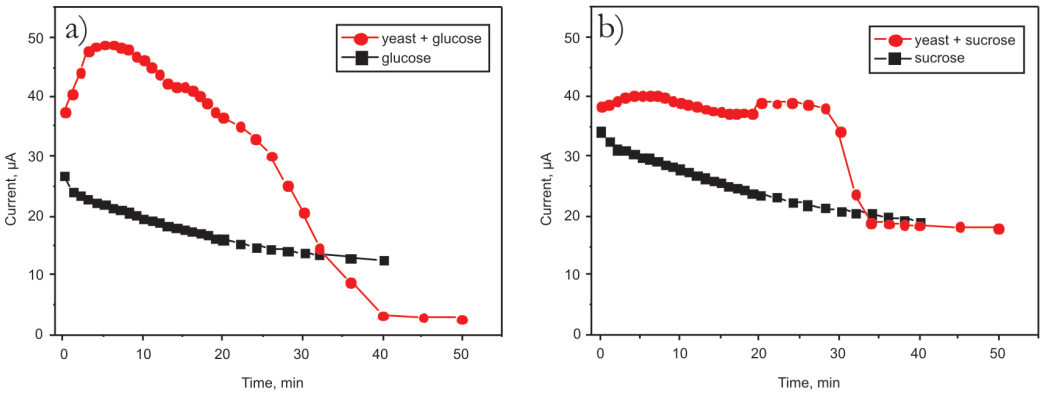


*Fig. 3. Current vs. time curves obtained at constant ohmic resistance (1000 Ω) by using: a) glucose; b) sucrose as a substrate.*

As expected, most intensive current generation was observed during the log-phase growth of yeast cells, determined by biochemical analysis. Probably, the slower assimilation of sucrose, used as a carbohydrate source, leads to more uniform levels of generated current. However, after 30 minutes the current values significantly decrease and become comparable and even lower than those obtained with pure substrates. The obtained results indicate that the generated electricity is mainly due to the electrons gained from the biological electron-carriers in the processes of glycolysis and anaerobic respiration. For comparison, values of current and cell voltage obtained with yeast-carbohydrate suspension as well as carbohydrate solutions without yeast are summarized in Table 2. The calculated differences between measured values are presented in separate columns.

*Table 2: Experimental values of current and cell voltage. (Sg-Bg – calculated differences between Sample glucose and Blank glucose data; Ss-Bs – calculated differences between Sample sucrose and Blank sucrose data).*

| | Blank glucose | | | | Sample glucose | | | | Sg-Bg | | | | Blank sucrose | | | | Sample sucrose | | | | Ss-Bs | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Anolyte content | 250 mM glucose/ 67 mM $PO_4^{3-}$ | | | | 10g yeast + 250 mM glucose/ 67 mM $PO_4^{3-}$ | | | | | | | | 250 mM sucrose/ 67 mM $PO_4^{3-}$ | | | | 10g yeast + 250 mM sucrose/ 67 mM $PO_4^{3-}$ | | | | | | | |
| Time, minutes | 0 | 20 | 30 | 40 | 0 | 20 | 30 | 40 | 0 | 20 | 30 | 40 | 0 | 20 | 30 | 40 | 0 | 20 | 30 | 40 | 0 | 20 | 30 | 40 |
| Current, µA | 26,9 | 16,1 | 13,9 | 12,6 | 37,4 | 37,6 | 20,5 | 2,1 | 10,5 | 21,5 | 6,6 | -10,5 | 34,2 | 23,6 | 21,0 | 19,1 | 38,4 | 39,2 | 34,3 | 18,6 | 4,2 | 15,6 | 13,3 | -0,5 |
| Voltage, mV | 40 | 23 | 20 | 18 | 57 | 57 | 30 | 2 | 17 | 34 | 10 | -16 | 51 | 35 | 31 | 28 | 58 | 59 | 52 | 27 | 7 | 24 | 21 | -1 |

The electrochemical characteristics proved that at least 250 mM monosaccharide have to be present into the primary yeast cell suspension because the current values decrease significantly after the glucose amount falls down up to approximately 5 mM at the 40$^{th}$ minute (*see* Table 1).

## *Conclusions*

The results from this investigation confirm the possibility for electricity generation by using *Saccharomyces cerevisiae* budding yeast as a microreactor into biofuel cells. Although the values of generated current and power are relatively low, they demonstrate the basic principle of how our model fuel cell converts the *in vivo* produced electrons into electricity.

Further interdisciplinary cooperation between researchers in biochemistry, microbiology, electrochemistry and engineering is required for optimisation of microorganisms-mediator-electrode interaction as well as improvement of biofuel cell operational characteristics. The expected economical and ecological effect is related both to green electricity production and waste water treatment technologies.

## *References*

Bennetto HP, Delaney GM, Mason JR, Roller SD, Stirling JL, Thurston CF (1985) The sucrose fuel cell: efficient biomass conversion using a microbial catalyst. Biotechnol. Lett. 7: 699-705.

Bennetto HP (1990) Electricity generation by microorganisms. Biotechnology Education 1 (4): 163-168.

Bullen RA, Arnot TC, Lakeman JB, Walsh FC (2006) Biofuel cells and their development. Biosens Bioelectron. 21 (11): 2015-2045.

Cooney MJ, Roschi E, Marison IW, Comninellis C, Stockar U (1996) Physiologic studies with the sulfate-reducing bacterium *Desulfovibrio desulfuricans*: evaluation for use in a biofuel cell, Enzyme Microb. Technol. 18 (5): 358-365.

Das D, Veziroglu TN (2001) Hydrogen production by biological processes a survey of literature. Int. J. Hydrogen Energy 26: 13-28.

Gascon S, Lampen JO (1968) Purification of the internal invertase of the yeast. J. Biol. Chem. 243: 1567-1572.

Hartwell LH, Mortimer RK, Culotti J, Culotti M (1973) Genetic Control of the Cell Division Cycle in Yeast: Genetic Analysis of cdc Mutants. Genetics 74 (2): 267-286.

He ZQ, Honeycutt CW (2005) A modified molybdenum blue method for orthophosphate determination suitable for investigating enzymatic hydrolysis of organic phosphates. Communications in Soil Science and Plant Analysis, 36 (9/10):1373-1383.

Kilkenny BC, Hinshelwood C (1951) The Utilization of Carbon Sources by Certain Yeast Strains. Proc. R. Soc. Lond. B Biol. Sci. 138 (892):375-385.

Kim JK, Cho HC, Lee JS, Hahm KS, Park DH, Kim SW (2002) Mass production of methane from food wastes with concomitant wastewater treatment. Appl. Biochem. Biotechnol. 98-100:753-764.

Kosaric N, Velikonja J (1995) Liquid and gaseous fuels from biotechnology: challenge and opportunities. FEMS Microbiology Reviews 16:111-142.

Kozłowska M, Rybus-Zając M, Stachowiak J, Janowska B (2007) Changes in carbohydrate contents of Zantedeschia leaves under gibberellin-stimulated flowering. Acta Physiologiae Plantarum 29 (1):27-32.

Liberatore PA, Cintra 10e – Enhanced Sensitivity from 600-1,200 nm, Analysis of Phosphorus to Sub-ppb Levels. GBC Scientific Application Notes – UV-Visible, 35-40.

Maness PC, Weaver PF (2001) Evidence for three distinct hydrogenase activities in *Rhodospirillum rubrum* Appl Microbiol Biotechnol. 57 (5-6):751-756.

Palmore GTR, Whitesides GM (1994) Microbial and Enzymatic Biofuel Cells. In: Enzymatic Conversion of Biomass for Fuels Production, Himmel ME, Baker JO, Overend RP (eds) ACS Symposium Series № 566, American Chemical Society, Washington DC, 271-290.

Prasad D, Arun S, Murugesan M, Padmanaban S, Satyanarayanan RS, Berchmans S, Yegnaraman V (2007) Direct electron transfer with yeast cells and construction of a mediatorless microbial fuel cell. Biosens Bioelectron. 22 (11):2604-2610.