Enhancement of ethanol production and cell growth in budding yeast by direct irradiation of low-temperature plasma

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Ethanol production by budding yeast was compared between direct and indirect plasma irradiation. We observed enhancement of ethanol production and cell growth not by indirect plasma irradiation but by direct plasma irradiation. Glucose consumption was increased in

budding yeast by direct plasma irradiation. Extracellular flux analysis revealed that glycolytic activity in the budding yeast was elevated by direct plasma irradiation. These results suggest that direct plasma irradiation enhances ethanol production in budding yeast by elevating the glycolytic activity.

1. Introduction

Low-temperature plasma (LTP) has been widely used for medicine and agriculture[1-7]. Indirect plasma treatments such as plasma-activated medium (PAM) as well as direct plasma treatments have broadened the ways to apply the LTP[8-11]. Since reaction products among LTP and solutions play some roles on physiological responses, indirect plasma treatments are important, however, the effects by UV/VUV and direct interactions between short-lived reactive species and biological surface are brought by direct plasma treatments. Thus, it is essential to investigate effects by both direct and indirect plasma irradiations.

The budding yeast (*Saccharomyces cerevisiae*) is an important eukaryotic model organism with well-developed genetics and molecular biology[12-14]. The effects of LTP on budding yeast have been investigated to understand molecular mechanisms common to eukaryotic cells[15-20]. For example, Hashizume et al., showed that the growth of budding yeasts' cells was regulated primarily in response to the total dose of oxygen atoms[17]. LTP induces apoptosis through reactive oxygen species (ROS) on budding yeast cells as well as mammalian cells[18]. There are two superoxide dismutases in budding yeast: Sod1p and Sod2p. Increased LTP resistance was observed in strains overexpressing *SOD1* or *SOD2*[18]. On the other hand, increased LTP sensitivity was observed in strains deleting *SOD1* or *SOD2*[19]. These results suggest that ROS generated by LTP are prominent factor in killing of budding yeasts. In addition to studies in basic science, budding yeast has been used in fermentation processes for making bioethanol[21-24]. LTP might be useful to improve the production of bioethanol by budding yeast[25].

In this study, we investigated ethanol production of budding yeast treated with LTP directly and plasma-activated medium (PAM). The ethanol production was increased not by PAM but by direct plasma irradiation. We further demonstrated glucose consumption and glycolytic activity were increased by direct plasma irradiation of budding yeast cells. These results suggest that LTP is a promising strategy for producing bioethanol.

2. Experimental methods

2.1 A yeast strain, culturing conditions, and plasma irradiations

Yeast (a W303a strain) cells were cultured in rich Yeast extract Peptone Dextrose (YPD) medium (10g/L Yeast extract, 20g/L Peptone, 20g/L Glucose) for 18 h at 30°C as previously

described[26] (Figure 1a). They were diluted to 10^6 cells/mL, and 3 mL of them was treated with LTP (direct plasma irradiation). On the other hand, 3 mL of YPD medium was treated with LTP, and the plasma-irradiated YPD medium was replaced into the YPD medium (indirect plasma irradiation). The plasma source was connected to a 9kV of 60 Hz AC high voltage power source with argon gas flowing at a rate of 2 standard liters per min (slm) as previously described[27]. The distance between the plasma source and the samples was fixed at L=13 mm as previously described[10]. After LTP treatments, those yeast cells were cultured in YPD medium for 6, 12, 18, 24, and 30 hours, and ethanol concentration, number of cells, and glucose concentration were measured. Number of cells were measured by using Disposable hemocytometer (4-chambers, Funakoshi, Tokyo, Japan).

2.2 Ethanol concentration

The ethanol concentration in medium was measured using the Alcohol Assay Kit (Cell Biolabs Inc., San Diego, CA, USA), according to the manufacturer's instructions. Absorbance was measured at 570 nm with a microplate reader (POWERSCAN HT; DS Pharma Biomedical).

2.3 Cell viability assay

Cell viability was assessed by staining trypan blue. Viability was examined under microscope. Viable cells were colorless, and dead cells were blue.

2.4 Glucose consumption assay

The glucose concentration in medium was measured using the Glucose Assay Kit-WST (Dojindo Laboratories, Kumamoto, Japan), according to the manufacturer's instructions. Absorbance was measured at 450 nm with a microplate reader (POWERSCAN HT; DS Pharma Biomedical).

2.5 Extracellular flux analysis

Glycolytic activity was assessed using the Agilent Seahorse XFp Glycolysis Stress Test Kit (Agilent Technologies 103017-100). The extracellular acidification rate (ECAR) was monitored with a Seahorse XFp Analyzer (Agilent Technologies) following the manufacturer's instructions[28]. Yeast cells were directly irradiated with LTP for 20 s. The cells (10^6 cells/well) were seeded in an 8-well plate. The YPD medium for culture was replaced with YPD medium for analysis (10g/L Yeast extract, 20g/L Peptone, 20g/L Glycerol). After 1 h, the glycolysis-stress test was performed following the standard protocol.

Glucose, oligomycin (OM) and 2-deoxyglucose (2-DG) were added to the medium at final concentrations of 10 mM, 1 μM, and 50 mM, respectively. After the completion of the glycolysis-stress test, cell numbers were determined using a Keyence BZ9000 microscope.

2.6 Statistical analysis

All data are presented as the mean ± SD. Statistical analysis of differences between groups was performed using the Student's t-test. A P-value < 0.05 (*) was considered to indicate significant difference.

3. Results and discussion

3.1 Ethanol production was increased by direct plasma irradiation

To investigate the effects of LTP on ethanol production by budding yeast, we monitored ethanol concentrations after direct plasma irradiation (Figure 1b). Enhancement of ethanol production by direct plasma irradiation was observed 24 h after plasma treatment. To optimize the efficiency of the ethanol production, we changed the plasma irradiation time (Figure 1c). When the yeast cells were irradiated with plasma for 20 s, the ethanol production 24 h after plasma irradiation was maximized. Ethanol production normalized by cell number also increased by the direct irradiation for 20 s although cell proliferation has significant contribution (Figure 1d). LTP generally affects cells directly and indirectly. To investigate whether the enhancement of ethanol production was direct or indirect effect, we also treated the yeast cells with plasma-activated YPD medium (Figure 1c). Enhancement of ethanol production by the indirect plasma irradiation was not observed. These results suggest that long-lived reactive species such as hydrogen peroxide, nitrites, and nitrates in the medium are not responsible for the enhancement of ethanol production, but UV/VUV and/or short-lived reactive species are responsible for it.

To investigate the effects of plasma irradiation on number of cells, we counted number of cells 24 h after plasma irradiation (Figure 2a). When the yeast cells were directly irradiated with plasma for 20 s, the number of cells 24 h after plasma irradiation was maximized. We also monitored the number of cells cultured for 6, 12, 18, 24, and 30 hours after plasma irradiation for 20 s (Figure 2b), and we found that the number of cells were significantly increased 24 h and 30 h after plasma irradiation. Indirect plasma irradiation did

not affect the number of cells. To investigate whether UV/VUV in direct plasma irradiation affects enhancement of ethanol production or not, we irradiated UV/VUV through a MgF_2 glass from direct plasma irradiation (Figure 3a). The UV/VUV from the direct plasma irradiation did not affect number of cells (Figure 3b). These results suggest that short-lived reactive species are responsible for the enhancement of ethanol production by direct plasma irradiation.

We reasoned that the enhancement of ethanol production by direct plasma irradiation was because the ethanol resistance was elevated by direct plasma irradiation. To investigate the hypothesis, we compared the ethanol resistance between control cells and plasma-irradiated cells. Cell viabilities were measured 24 h after incubation of cells in culture medium with ethanol (5, 10, 20 %) in control cells and plasma-irradiated cells (Figure 2c). Any differences in ethanol resistance were not observed. These results suggest that enhancement of ethanol production by direct plasma irradiation was not because of the enhancement of ethanol resistance.

3.2 Direct plasma irradiation of budding yeast increased glucose consumption and glycolytic activity.

Next, we reasoned that the enhancement of ethanol production by direct plasma irradiation was because the glycolytic activity of the budding yeast was enhanced by direct plasma irradiation. To investigate the hypothesis, we measured glucose concentrations in medium after direct plasma irradiation (Figure 4). Consistent with our hypothesis, the glucose consumption was increased by direct plasma irradiation 24 h after plasma irradiation (Figure 4b). Glucose consumption normalized by cell number was also increased by the direct plasma irradiation although cell proliferation has significant contribution (Figure 4c).

To further investigate the hypothesis, we measured glycolytic activity using an extracellular flux analyzer (Figure 5). Glycolysis is the metabolic pathway that converts glucose into pyruvate and a hydrogen ion (Figure 5a). In alcoholic fermentation, pyruvate is converted to ethanol and CO_2. Extracellular acidification rate (ECAR) represents a hydrogen ion produced by glycolytic activity and alcoholic fermentation. Cells were incubated in YPD medium for analysis which does not contain glucose. We monitored ECAR of control cells and plasma-irradiated cells using an extracellular flux analyzer (Figure 5b). Before adding

glucose, the ECAR of both control cells and plasma-treated cells were about 40 mpH/min/cells. After adding glucose, the ECAR which represents glycolysis was significantly higher in plasma-irradiated cells than in control cells (Figure 5c). These results suggest that direct plasma irradiation of budding yeast enhanced its glycolytic activities to enhance the ethanol production.

3.3 Discussion and future directions

In this study, we investigated ethanol production in budding yeast that are subjected to direct and indirect LTP irradiations. It is interesting that ethanol production of budding yeast cells was elevated when they were directly irradiated with LTP for 20 s and 30 s (Figure 1c). We have previously reported the dose-dependent effects of neutral oxygen radicals on proliferation as well as the inactivation of budding yeast cells[17]. Proliferation was promoted at moderate doses, and cells were inactivated at higher doses. Our results reproduced the dose-dependent effects in terms of cell proliferation (Figure 2a). In mammalian cells. such dose-dependent effects are generally observed[29]. These results suggest that the dose-dependent effects are common to eukaryotic cells.

We investigated the intracellular molecular mechanisms that direct LTP irradiation enhanced the ethanol production of budding yeast. There are some budding yeast strains which enhance the ethanol resistance and some mutations enhance the ethanol resistance of budding yeast[30, 31]. However, direct plasma irradiation did not enhance the ethanol resistance (Figure 2b). It was reported that activity of glycolytic enzymes was elevated by plasma treatment[25]. An extracellular flux analyzer is a powerful tool to measure the glycolytic activity[28, 32, 33]. We measured glucose consumption and glycolytic activity using the extracellular flux analyzer, and we found that direct plasma irradiation enhanced glucose consumption (Figure 4) and ECAR related in glycolytic process (Figure 5). These results are consistent with the results that the activity of glycolytic enzymes was elevate by plasma treatment.

Not indirect LTP irradiation but direct LTP irradiation enhanced the ethanol production and cell proliferation (Figure 1c, 2a). Direct LTP irradiation through MgF_2 glass did not enhance the ethanol production (Figure 3). These results suggest that not UV/VUV but short-lived reactive species are responsible for the enhancement of the ethanol

production. To demonstrate this possibility, further experiments that replace fresh medium with the medium after direct plasma irradiation were needed. It is pointed out that short-lived reactive species play important roles on plasma-induced cell membrane permeabilization[34, 35]. Further studies to measure such short-lived reactive species are needed.

Yeasts have a long-standing relationship with humankind and they have been used for the production of bioethanol, beer, and wine[24]. Our results suggest that LTP is a promising strategy for enhancing yeast alcoholic fermentations.

4. Conclusions

We found that ethanol production of budding yeast was enhanced by not indirect plasma irradiation, but direct plasma irradiation. Short-lived reactive species in LTP might be responsible for the enhancement of ethanol production. Glucose consumption and glycolytic activity were increased by direct plasma irradiation of budding yeast. These results suggest that LTP is a promising tool for producing bioethanol from budding yeast.

Acknowledgments

The authors would like to thank professors Takayoshi Tsutsumi, Hiroki Kondo, and Makoto Sekine for fruitful discussion. This work was partly supported by a Grant-in-Aid for Specially Promoted Research (No. 19H05462) and a Grant-in-Aid for Scientific Research (B) (No. 21H01072) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, as well as the joint usage / research program of the Center for Low-temperature Plasma Science, Nagoya University, and the Plasma-bio consortium.

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Figure Captions

Fig. 1. Ethanol production of budding yeast was increased by direct plasma irradiation (a) Experimental setup to assess ethanol production of budding yeast irradiated with plasma (b) Ethanol concentration dynamics after direct irradiation of plasma for 20 seconds. Values are presented as mean ± SD (n=3). * P<0.05. (c) Ethanol concentration at 24 h after plasma irradiation. Values are presented as mean ± SD (n=3). * P<0.05. (d) Ethanol concentration normalized by number of cells at 24h after plasma treatment (irradiated for 20 seconds). Values are presented as mean ± SD (n=3). * P<0.05.

Fig. 2. Effects of ethanol in budding yeast viability (a) Comparison of number of cells 24h after plasma treatment between direct and indirect plasma irradiation. Values are presented as mean ± SD (n=3). * P<0.05. (b) Growth curves (control and directly plasma irradiated cells for 20 seconds). Values are presented as mean ± SD (n=3). * P<0.05. (c) Effects of ethanol in budding yeast (control and directly plasma irradiated cells). Values are presented as mean ± SD (n=3). * P<0.05.

Fig. 3. Cell growth was not affected by UV/VUV. (a) Experimental setup to evaluate the effect of UV/VUV in direct plasma irradiation (b) UV/VUV from direct plasma irradiation did not affect cell growth 24 h after plasma irradiation. Values are presented as mean ± SD (n=3). * P<0.05.

Fig. 4. Glucose consumption was increased by direct plasma irradiation. (a) Glucose consumption was monitored in control cells and directly plasma irradiated cells. Values are presented as mean ± SD (n=3). * P<0.05. (b) Glucose concentrations in control cells and directly plasma irradiated cells 24h after plasma irradiation. Values are presented as mean ± SD (n=3). * P<0.05. (c) Glucose consumption normalized by number of cells 24 h after plasma irradiation (control and directly plasma irradiated cells for 20 seconds). Values are presented as mean ± SD (n=3). * P<0.05.

Fig. 5. Extracellular flux analysis of plasma irradiated cells (a) Schematics of glycolysis and

bioethanol production in budding yeast. (b) Budding yeast cells irradiated with and without plasma were subjected to glucose (G), oligomycin (OM), and 2-deoxyglucose (2-DG) during the monitoring of ECAR. Values are presented as mean ± SD (n=3). * $P<0.05$. (c) Extracellular acidification rate (ECAR) which represents glycolysis in control cells and plasma irradiated cells. ECAR (at 24 min) – ECAR (at 0 min) was compared so that ECAR which represent glycolysis was evaluated. Values are presented as mean ± SD (n=3). * $P<0.05$.

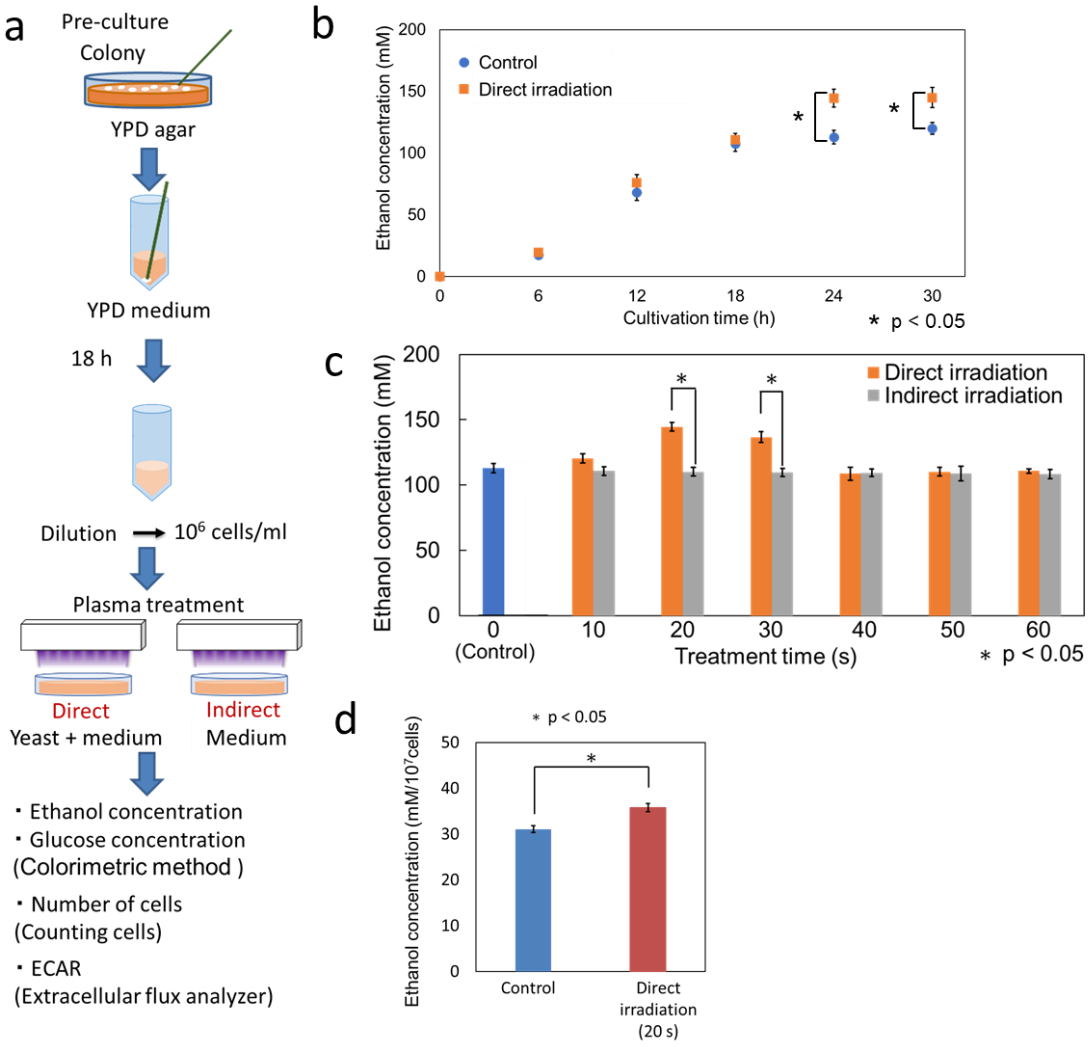


Fig.1. Ethanol production of budding yeast was increased by direct plasma irradiation

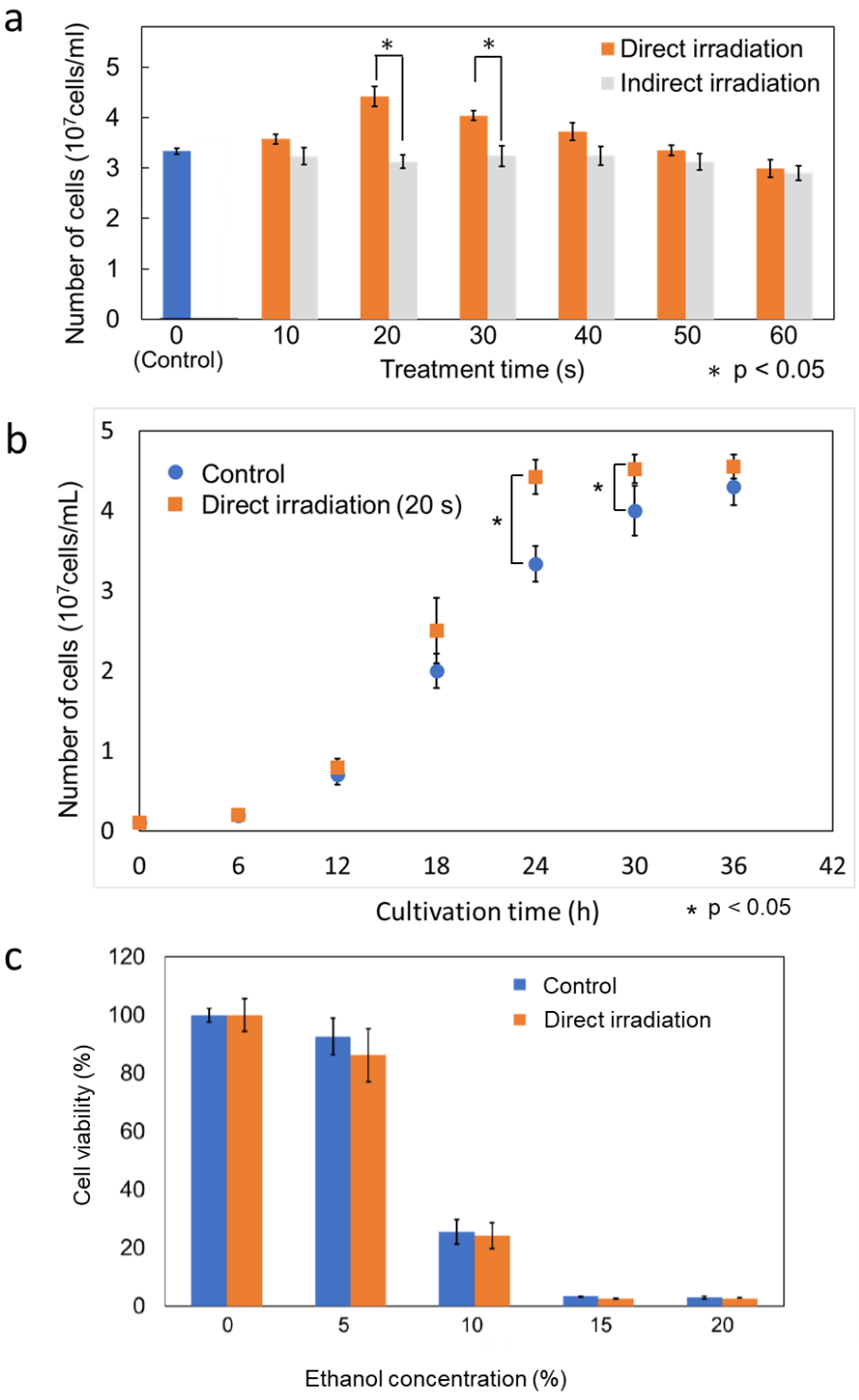


Fig. 2. Effects of ethanol in budding yeast viability

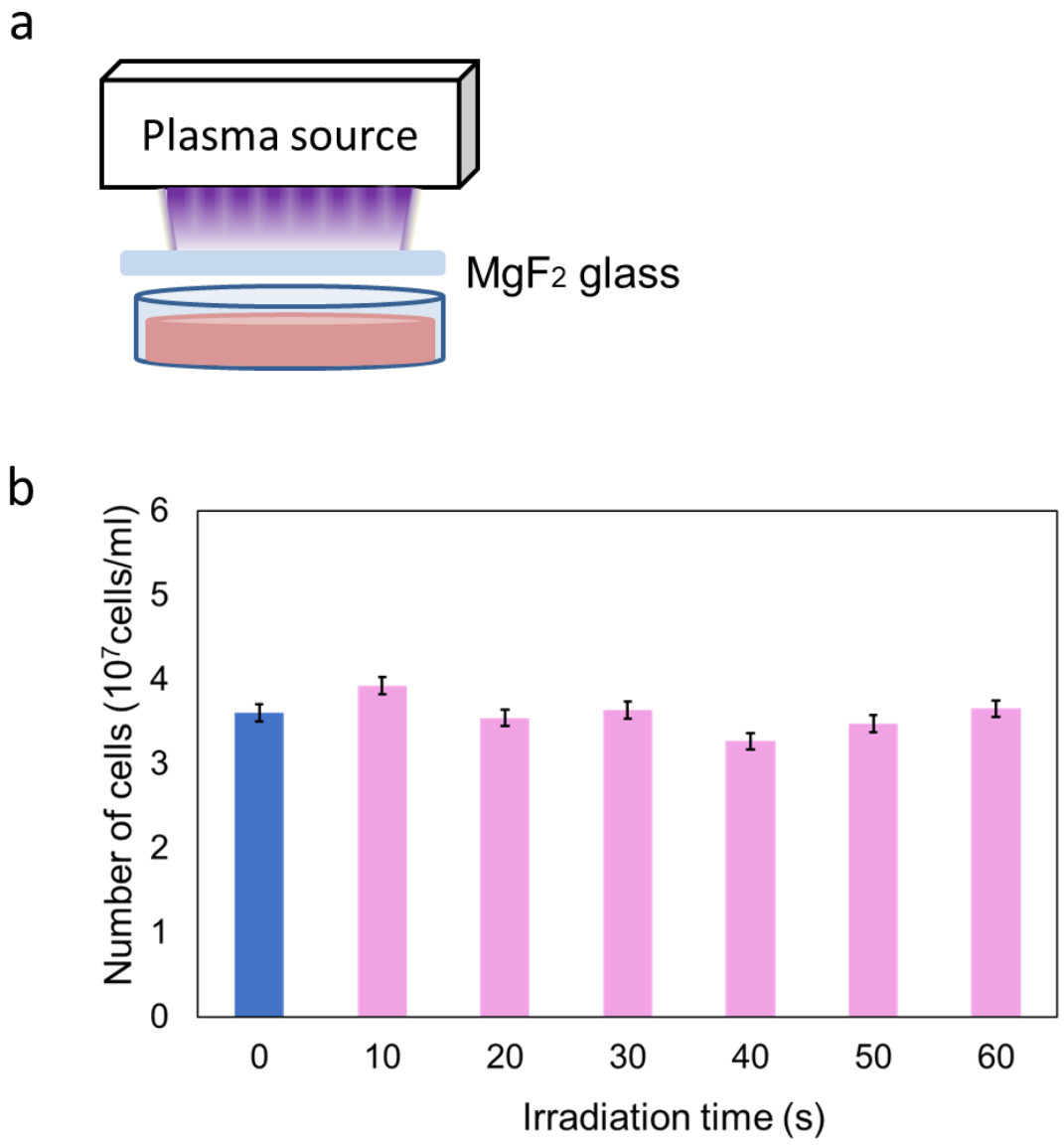
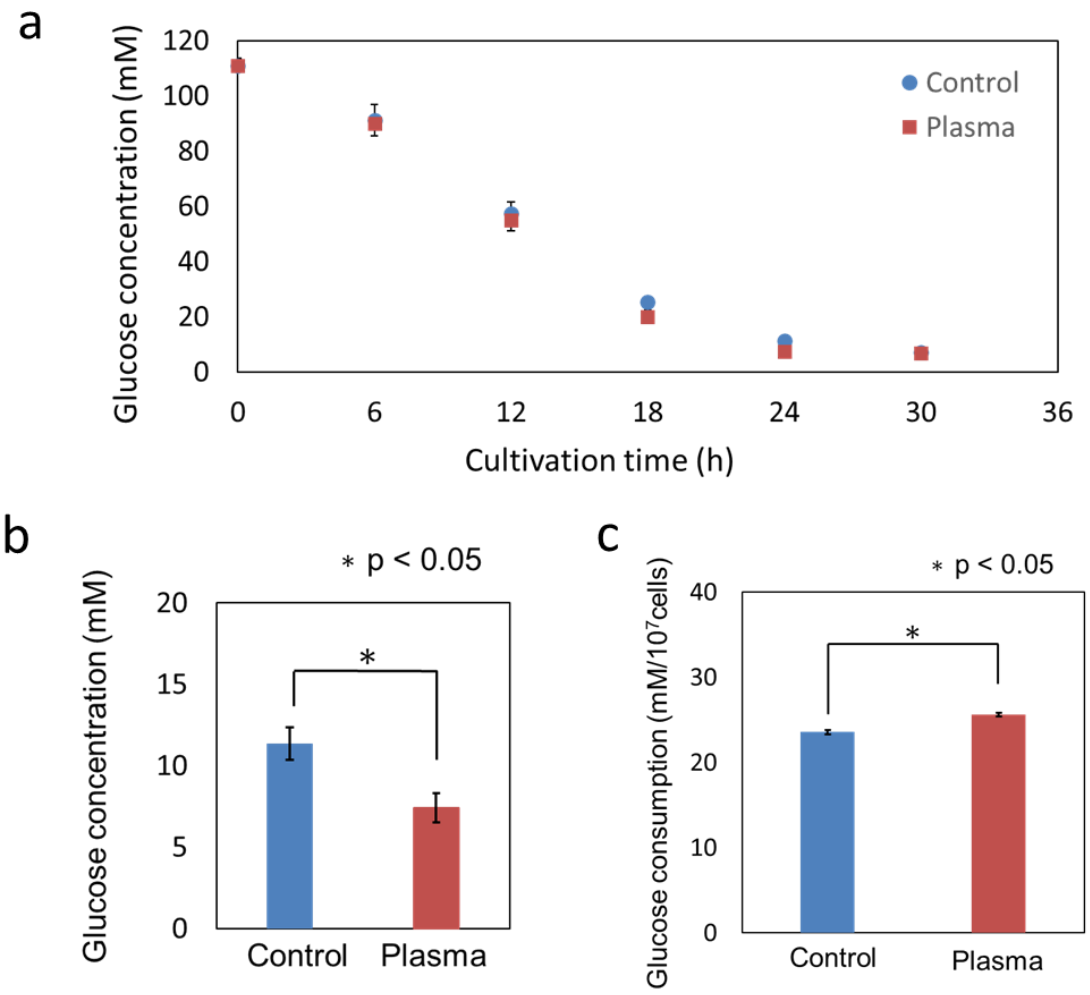


Fig. 3. Cell growth was not affected by UV/VUV.



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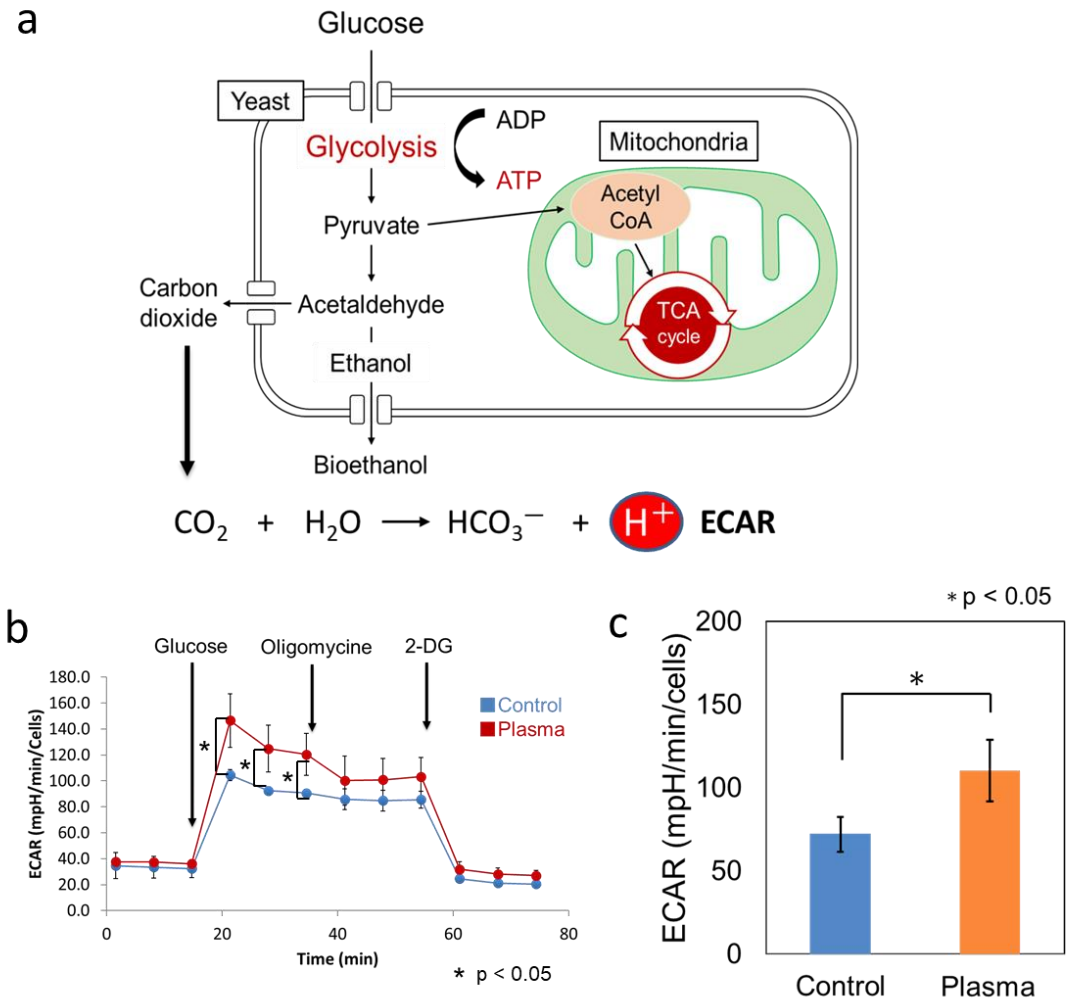

Fig. 5. Extracellular flux analysis of plasma irradiated cells