Chapter 8

Enhanced Ethanol Production of *Saccharomyces cerevisiae* Induced by Cold Plasma at Atmospheric Air Pressure

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Additional information is available at the end of the chapter

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Abstract

In this study, cold plasma at atmospheric pressure, as a novel approach of bioprocess intensification, was used to induce yeast for the improvement of ethanol production. Response surface methodology (RSM) was used to optimize the discharge-associated parameters of cold plasma for the purpose of maximizing the ethanol yield achieved by cold plasma-treated *S. cerevisiae*. The resulting yield of ethanol reached to 0.48 g g^{-1} under optimized parameters of plasma exposure time of 1 min, power voltage of 26 V, and an exposed sample volume of 9 mL, which represented an increase of 33% over control. Compared with non-exposed cells, cells exposed with plasma for 1 min presented a notable increment in cytoplasmic free Ca^{2+}, when these exposed cells showed the significant increase in membrane potential. At the same time, ATP level decreased by about 40%, resulting in about 60% reduction in NADH. Taken together, these data suggested that the mechanism that air cold plasma raised plasma membrane potential, which led to increases in cytosolic Ca^{2+} concentration. Furthermore, the cofactor metabolism, such as ATP and NADH, was subjected to regulation that was mediated by Ca^{2+}, ultimately improving yeast productivity. This may have a underlying and broad utilization in enhancing bioconversion capability of microbe in the next few years.

Keywords: ethanol, *Saccharomyces cerevisiae*, cofactor metabolism, bioprocess intensification, cold plasma at atmospheric pressure

1. Introduction

Bioethanol is currently being commercially produced as an alternative to petroleum-based transportation fuels, since it is clean, renewable, carbon-neutral and environmentally friendly [1–3].

Saccharomyces cerevisiae is one of dominant strains of bioethanol production. During fermentation, various factors such as cell membrane barrier, intracellular enzyme activity, the multiple inhibitions of products and substrates, limit the yeast growth and reduce microbial viability, and consequently cause a decrease in ethanol yield [4]. Among those factors, cell membrane permeability is main influence factor that restricts the rates of substrate uptake and release of metabolic products. It has become a focus of global attention to develop a novel method to control membrane permeability for improving yeast capacity in bioconversion of ethanol.

Pretreatment technologies have been developed to intensify bioethanol production, including physical, chemical, biological and physicochemical technologies [5–9]. Furthermore, the methods to control the membrane permeability have also been established, such as microwave, electric field, oxidative stress [10–12]. However, these methods have several drawbacks. For example, the chemical methods could generate enormous amounts of hazardous waste, while physical methods are difficult to apply at large scales. It is therefore necessary to develop a novel approach to change cell membrane permeability for improved bioethanol yield.

Cold plasma at atmospheric air pressure has recently been regarded as a new and advantageous pretreatment technology result from its superior features of high efficiency, low energy consumption and environmentally friendly. Air cold plasma could present various biological effects on the microbes, such as activation effect, sterilization effect and mutagenesis effect, due to the changes in the concentration of reactive species caused by different parameters associated with the plasma discharge [13]. Therefore, the discharge-associated parameters for improved ethanol yield need to be optimized. In this study, the response surface method (RSM) was performed to optimize experimental parameters that could cause the increase in the yield of ethanol generated by *S. cerevisiae*.

Saccharomyces cerevisiae has been widely used in the production of bioethanol by transforming glucose in industry. The glucose metabolic pathway in *S. cerevisiae* during anaerobic fermentation is shown in **Figure 1**. The tricarboxylic acid cycle (TCA) pathway occurs as two branches in the cytosol [14], but does not operate as a cycle in the mitochondrion as most of the earlier reports.

The cell membrane is the first barrier that the substrate enters into the cytoplasm. Thus the improved membrane permeability would promote the glucose utilization and even ethanol release. The rapid consumption of glucose could disturb the cofactor metabolism (such as ATP, NADH et al.) and the re-distribution of carbon flux in glycolysis pathway [15]. In addition, the open of ion channels is the one of mechanisms that the cell membrane permeability is improved. Especially, calcium ion channel administers the alterations of cytoplasm calcium ion concentration ($[Ca^{2+}]_{cyt}$). Ca^{2+}, as a key secondary messenger, is importantly responsible for cell metabolism and activities of some categories of ATPase [16]. As shown in **Figure 1**, a raise of $[Ca^{2+}]_{cyt}$ can be result of improved inflow of extracellular Ca^{2+} by Cch1 protein/Mid1 protein (Cch1/Mid1 p) on cell membrane or as a result of outflow of vacuolar Ca^{2+} into the cytoplasm through vacuole membrane-located Yvc1 protein (Yvc1p) channel [17–20]. Until now, little knowledge has been obtained on the relationship among air cold plasma, cell membrane permeability, cofactor metabolism and ethanol yield.

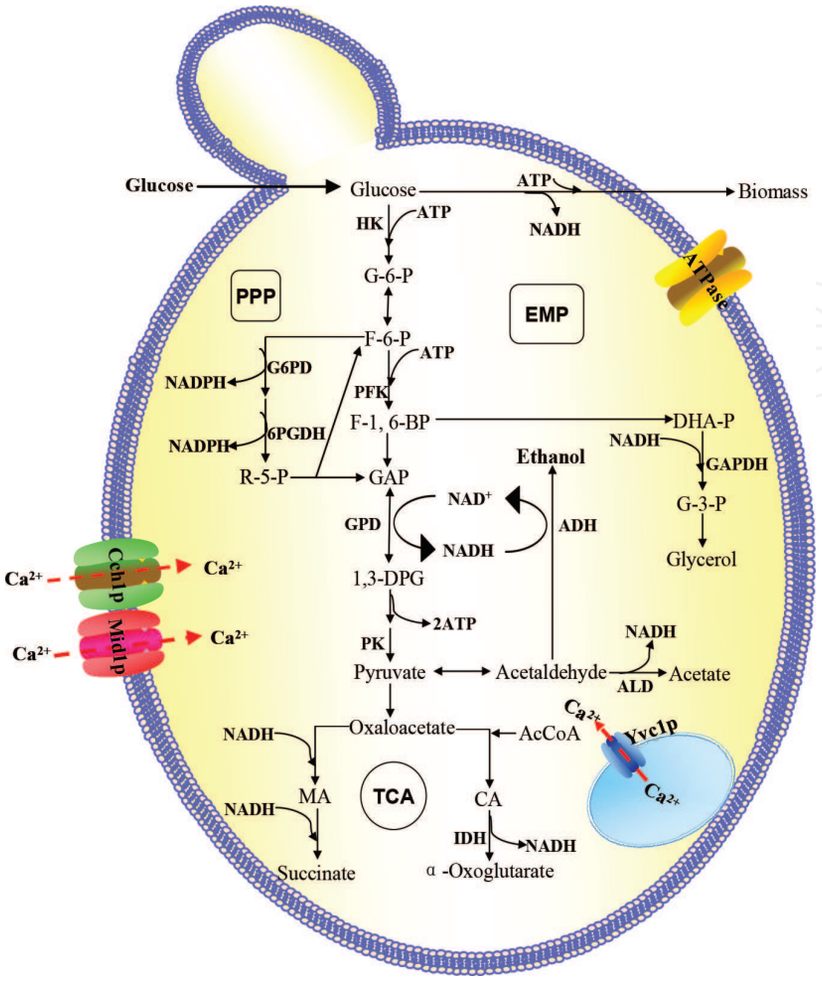

Figure 1. Major pathway of glucose metabolism in *S. cerevisiae* under anaerobic conditions.

The object of this study was to achieve the maximum yield of ethanol by optimizing parameters associated with plasma discharge. Moreover, the mechanism of intensified yield of ethanol produced by *S. cerevisiae* was explored. These data will provide the valuable theory base for developing a novel bioprocess intensification technology in biochemical engineering industry.

2. Results and discussion

2.1. Parameter optimization associated with plasma discharge for enhanced ethanol yield

2.1.1. Influence of plasma treatment time on ethanol yield

To achieve the maximum ethanol-yield, plasma treatment time was set at five different time intervals, from 1 to 5 min. Ethanol yield at 3 min reached to the maximum (0.45 g/g), and it presented an increase of 29% over the control (**Figure 2**). This indicated that a plasma treatment

time of 3 min was appropriate for maximal ethanol production. Thus, 3-min treatment time was chosen as the treatment time for studying the influences of various power supply voltages and volumes of yeast suspension on ethanol yield. In our earlier research, the highest yield of 1,3-propanediol produced by *Klebsiella pneumoniae* was got when the cell suspension was treated by dielectric barrier discharge for 4 min [21], proposing that different species microbes tend to respond differently to different times of plasma treatment. It was clear that 3-min is optimal for *S. cerevisiae* to obtain the maximum ethanol yield in glucose fermentation.

2.1.2. Influence of power supply voltage on ethanol yield

The influence of the power supply voltage in plasma treatment on ethanol yield is shown in **Figure 3**. Ethanol yield raised with raising power supply voltages, up to 0.42 g/g, then dropped with further increase in power supply voltage. The maximum yield of ethanol was achieved at 26 V.

It has been reported that charged particles in low-temperature plasma play a key role in the alterations of the outer structure of *Candida albicans* [22]. Raising the power supply voltages also causes an increase of the electric field in the gap distance. This might cause the microbial cell membrane to depolarize and become permeabilized, making it easier for the substrate to enter into the cells and for the products to release out the cells, which accordingly forming 27% increase in ethanol yield over the control. However, further increment in voltage results in a reduction of ethanol yield. This might be attributed to the neutralization of the negative charges, which could lead to cytoplasm leakage and cell death [13].

2.1.3. Influence of treated suspension volume on ethanol yield

The influence of various sample volumes on the ethanol yield was studied for the maximal ethanol yield. As shown in **Figure 4**, a sample volume of 5 mL enhanced ethanol yield by 28% for

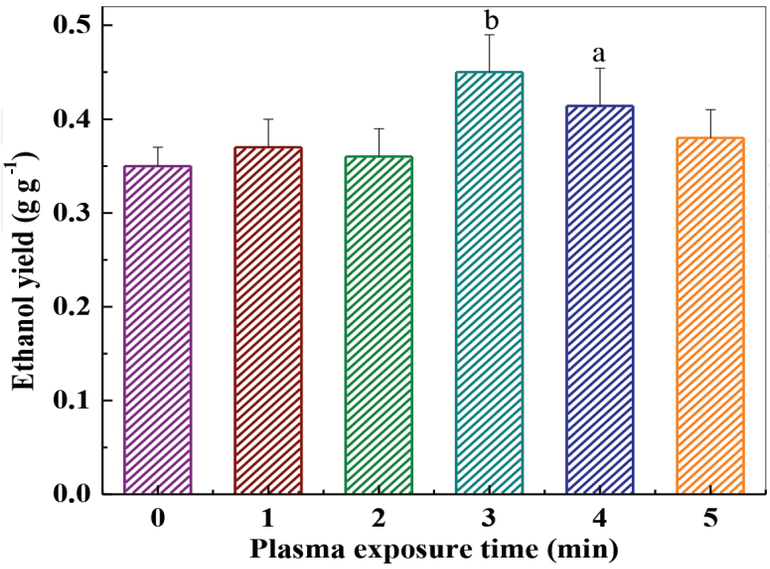

Figure 2. Influence of plasma treatment time on ethanol yield. Data are expressed as mean ± SES. 'a' and 'b' indicate $P < 0.05$ and $P < 0.01$, respectively.

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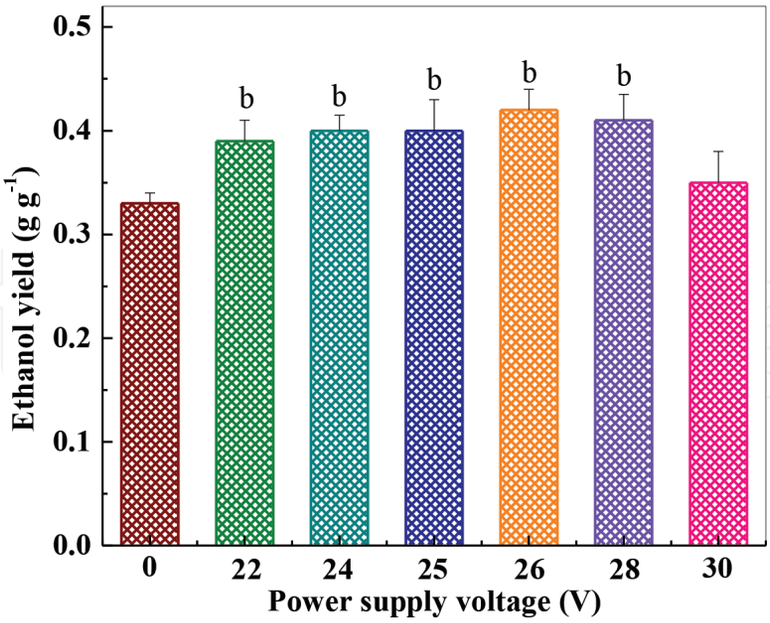

Figure 3. Influence of power supply voltage on ethanol yield. Data are expressed as mean ± SES. 'a' and 'b' indicate $P < 0.05$ and $P < 0.01$, respectively.

the plasma pretreated cells over the control. Cell suspension also constitutes a dielectric layer. A larger suspension volume means that the thickness of the dielectric layer would increase in a Petri dish of 60-mm diameter, and any alteration about dielectric properties would also caused a alteration in discharge characteristics, especially for the power voltage [23]. As a result, a sample suspension volume of 5 mL could show an impactful augment in ethanol yield.

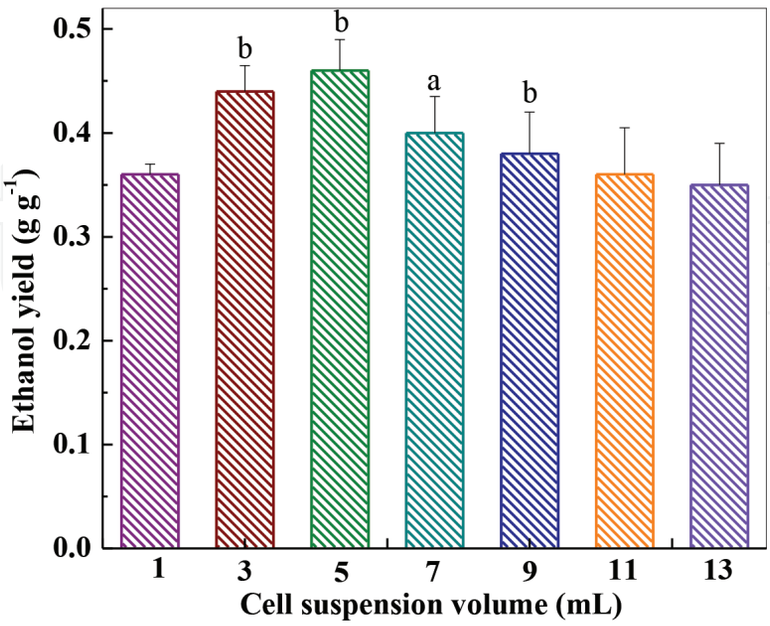

Figure 4. Influence of yeast suspension volume on ethanol yield. Data are expressed as mean ± SES. 'a' and 'b' indicate $P < 0.05$, $P < 0.01$, respectively.

2.1.4. Predictive response model

The design matrix and the corresponding experimental data were presented in **Table 1**. These values were fitted to the next second-order polynomial equation and the results were presented in **Table 2**.

$$Y = 0.22 - 0.12\,X_1 - 0.046\,X_2 + 0.11\,X_3 - 0.039\,X_1X_2 + 0.044\,X_1X_3 - 0.039\,X_2X_3 + 0.05\,X_1X_1 - 0.04\,X_2X_2 + 0.07\,X_3 \quad (1)$$

The adequacy of the model was checked using analysis of variance (ANOVA), which was tested using Fisher's statistical analysis [24]. The Model F-value of 6.09 indicated model significance. Value of "Prob > F" less than 0.05 indicated that the model terms were remarkable, whereas values greater than 0.10 indicated no significance. ANOVA resulted in a value of 0.85 for the coefficient of determination (R^2) and 0.71 for the adjusted coefficient of determination

Run	X_1	X_2	X_3	*Y*
1	−1	−1	−1	0.27
2	1	−1	−1	0.04
3	−1	1	−1	0.35
4	1	1	−1	0.03
5	−1	−1	1	0.48
6	1	−1	1	0.49
7	−1	1	1	0.47
8	1	1	1	0.27
9	−1	0	0	0.49
10	1	0	0	0.02
11	0	−1	0	0.31
12	0	1	0	0.02
13	0	0	−1	0.22
14	0	0	1	0.29
15	0	0	0	0.23
16	0	0	0	0.22
17	0	0	0	0.23
18	0	0	0	0.22
19	0	0	0	0.22
20	0	0	0	0.22

[Y] Observed Ethanol yield (g/g)

Table 1. Experimental design and results for the central composite design.

Source	Sum of squares	*df*	Mean square	*F*-value	*P*-value
Model	0.37	9	0.041	6.09	0.005
X_1	0.15	1	0.150	21.92	0.001
X_2	0.02	1	0.021	3.12	0.108
X_3	0.13	1	0.130	18.47	0.002
X_1X_2	0.01	1	0.012	1.77	0.213
X_2X_3	0.02	1	0.015	2.26	0.164
X_2X_3	0.01	1	0.012	1.77	0.213
X_1X_1	0.006	1	0.007	1.01	0.338
X_2X_2	0.003	1	0.004	0.65	0.440
X_3X_3	0.013	1	0.013	1.98	0.189
R^2	–	–	0.85	–	–
Adj-R^2	–	–	0.71	–	–

Table 2. AVOVE for response surface quadratic model for ethanol production.

(R^2_{adj}). The R^2_{adj} value was close to 1, which indicated a high degree of correlation between the observed and predicted values [25]. *P*-values were used to check the significance of each variant. Each of the *P*-values also indicated the interaction strength between any two of the independent variants; the smaller the *P*-value, the higher the significance of the corresponding variant [26]. As with the interaction between any of the two variants, the smallest *P*-value was seen with X_1X_3. This suggested that among the three parameters investigated, maximum interaction occurred between plasma treatment time and the volume of the induced sample.

2.1.5. Influence of various experimental parameters on ethanol yield

The influences of the independent parameters, including plasma treatment time, power supply voltage and induced-sample volume, on ethanol yield were analyzed by three dimensional response surface plots (**Figure 5**). **Figure 5(a)** presented the ethanol yield based on a combination of plasma treatment time and power supply voltage. The predicted ethanol yield showed to increases at 1 min and from 25 to 27 V. **Figure 5 (b)** presents the interaction between plasma treatment time and sample suspension volume on ethanol yield. The highest ethanol yield was achieved when 9-mL sample suspension was treated by dielectric barrier discharge (DBD) plasma for 1 min. The predicted ethanol yield of *S. cerevisiae* reached to a maximum when 9-mL sample was treated under the range of the power supply voltage from 22 to 26 V (**Figure 5(c)**). These three-dimensional plots offer a visual interpretation of the interaction between two parameters and promote the location of optimum experimental parameters. The optimized conditions for the three experimental parameters (as obtained from the maximal point of the model) were calculated by the Design expert software to be 1 min, 26 V and 9 mL, respectively, corresponding to plasma exposure time, power voltage, and volume of exposed cell suspension. The model forecasted a highest response of 0.49 g/g ethanol yield for this point.

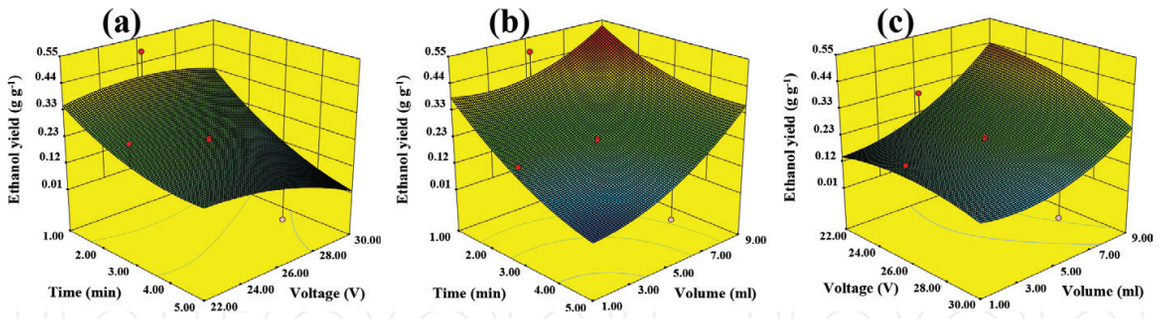

Figure 5. (a) Response surface plot of the interaction between plasma-treatment time and power supply voltage on ethanol yield; (b) response surface plot of the interaction between plasma-treatment time and induced sample volume on ethanol yield; (c) response surface plot of the interaction between power supply voltage and volume of induced sample on ethanol yield.

2.1.6. Confirmation of optimum parameters

Optimum conditions of the parameters achieved from the above analysis were verified by carrying out flask fermentation with *S. cerevisiae* from 9-mL sample suspension that had been exposed with plasma for 1 min and a power supply voltage of 26 V. As shown in **Table 3**, the ethanol yield reached to 0.48 g/g, which was very close to the predicted value of 0.49 g/g, and represented a 33% increase compared with the yield of the untreated sample (0.36 g/g). The outstanding correlation between the predicted and the measured values confirmed that the model was feasible and that an optimal point for increasing ethanol yield could be obtained. The ethanol concentration in the fermentation also raised by 42% and the biomass raised by 24% over those obtained from fermentation by untreated sample (**Table 3**).

To enhance the concentration of ethanol, different methods have been used to improve the productivity of the correlative microorganism strains, including construction of genetic engineering strain [27], mutagenesis and breeding [28], as well as metabolism control by changing the osmotolerance of the external environment [29]. Up to now, little study has been reported about the application of cold plasma at atmospheric pressure in intensifying ethanol yield of *S. cerevisiae*. It has been early found that growth of *K. pneumoniae* could be enhanced by air cold plasma, causing an increment in productivity of 1,3-propanediol [21]. In addition, the application of plasma discharge could also lead to the degradation of the biomacromolecules that constitute the cell-envelope, such as polysaccharides and protein [30]. Cell membrane permeability is influenced as a result of alterations in the cell envelope composition. This then leads to alterations in metabolic products as well as in the physiological activity of the cells. Yonson et al. has discovered that human hepatocytes (HepG2) cells could become provisionally permeabilized

Groups	Biomass (g/L)	Glucose consumption (g/L)	Ethanol (g/L)	Ethanol yield (g/g)
Control group	5.4 ± 0.9	132.0 ± 8.3	47.5 ± 2.7	0.36 ± 0.02
Optimized group	6.7 ± 1.1	141.0 ± 10.8	67.5 ± 4.2	0.48 ± 0.03

Table 3. Comparison of flask fermentation by ***S. cerevisiae*** under optimized and untreated conditions.

when they are induced by a miniature atmospheric-pressure glow-discharge plasma torch [31]. Therefore it is thought that the permeability of the cell membrane in *S. cerevisiae* might probably promote the diffusion of substrates into the cell as well as the export of products out of the cells, causing an alteration in the metabolic process. This could also be the reason why the improved ethanol yield could be observed with plasma treated *S. cerevisiae* in this research.

The optimized parameters (1 min, 26 V, 9 mL) achieved by the central composite design experiment were different from the optimized parameters (3 min, 26 V, 5 mL) achieved by single-factor experiment. This may be due to the following reasons. Firstly, response surface methodology reflected the influences of interaction among the three parameters employed with the other parameter maintained at its respective zero level on ethanol yield. In this research, the dielectric layer became thick when the volume of the test sample was increased in an unchanged 60-mm-diameter Petri dish, causing an alteration in the power voltage. Therefore, the three parameters (plasma exposure time, test sample volume, power supply voltage) underwent a simultaneous alteration. Secondly, the plasma discharge device was directly laid in air at room temperature, and the discharge was affected by various environmental factors, such as air humidity and ambient temperature. Finally, experimental errors were observed during the operation. For example, the gap distance between electrodes was widened again and again for putting the sample on the bottom electrode before every experiment, and then the distance between electrodes was recovered.

2.2. Mechanism study about enhanced ethanol yield of *Saccharomyces cerevisiae* with cold plasma

2.2.1. Plasma membrane permeability

The alterations in membrane permeability exhibited by *S. cerevisiae* cells following their exposure to plasma and subsequent culturing under fermentation conditions are shown in **Figure 6**. After plasma treatment for 1 min, the membrane permeability reduced compared with that of untreated cells, but raised when the samples were treated respectively from 2 to 4 min, and fell back to the level of untreated cells when the sample was induced for 5 min. The membrane permeability of the treated cells reached to a maximum when the sample treated for 4 min were cultured for 9 h, producing a 1.2-fold increase over that of untreated cells. As for sample that was cultured for 21 h, a significant increase in membrane permeability only occurred for those that were derived from samples treated to plasma for 1 and 5 min.

2.2.2. Plasma membrane potential

The membrane potential was measured with the aid of the fluorescence probe Rh123 (**Figure 7**). The fluorescence intensity of Rh123 was positively correlated with plasma membrane potential. These data indicated that the plasma membrane permeability was increased (20%) when the samples were treated for 1 min, but was decreased when they were treated for 2–5 min. When the treated samples were cultured for 9 h, only the membrane potential of the sample treated for 1 min reduced relative to that of non-treated sample. Other

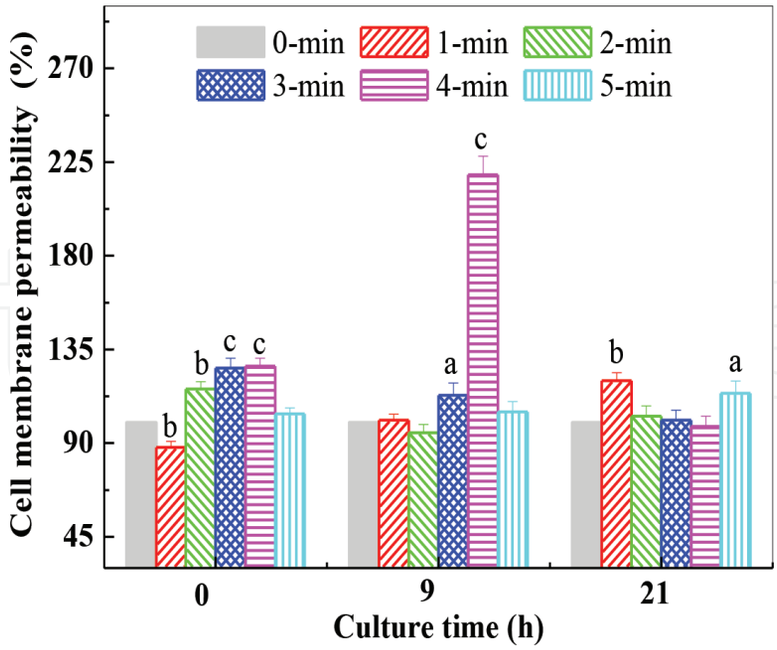

Figure 6. Influence of plasma treatment on cell membrane permeability of *S. cerevisiae* before and after fermentation. Data are expressed as mean ± SES. 'a', 'b', and 'c' indicate $P < 0.05$, $P < 0.005$ and $P < 0.001$, respectively.

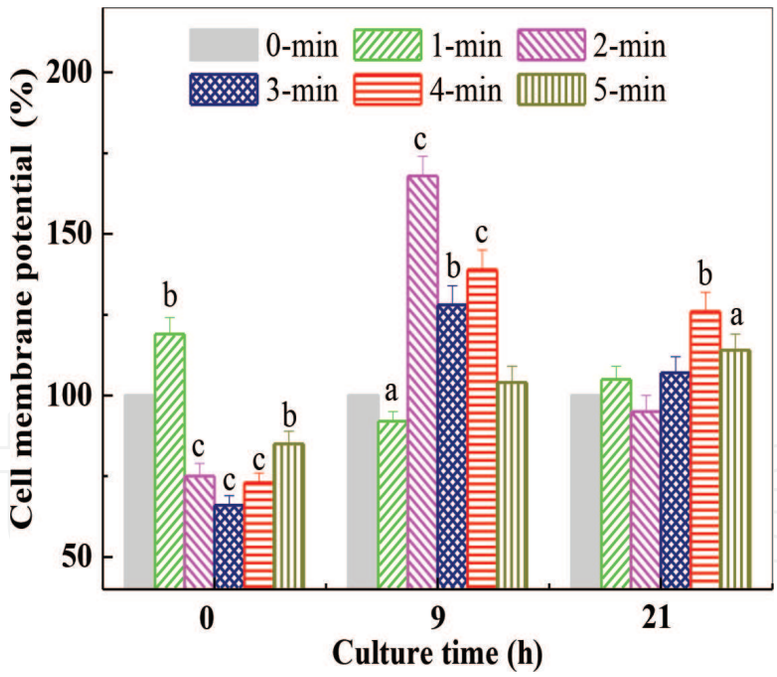

Figure 7. Influence of plasma treatment on cell membrane potential before and after fermentation. Data are expressed as mean ± SES. 'a', 'b', and 'c' indicate $P < 0.05$, $P < 0.005$ and $P < 0.001$, respectively.

exposure times gave various increases in membrane potential, among which 2 min exposure yielded the maximum increase (70%) compared with non-treated sample. In the case of 21-h fermentation, 4- and 5-min exposures gave remarkable improvements in membrane potential over non-treatment. These data seemed to show that cold air plasma discharge could either increase or decrease the plasma membrane potential of *S. cerevisiae* cells.

2.2.3. Cytoplasmic calcium concentration

The intracellular calcium concentration of plasma-treated samples was detected using the fluorescence probe Fluo-3 AM (**Figure 8**). The calcium concentrations in the cytoplasm were improved with plasma treatment time, with 5 min treatment giving the maximal increase, about 36% more than the concentration measured in the non-treated cells. After 9 h of fermentation, cytoplasmic Ca^{2+} concentrations were significantly increased in the sample of 1- or 2-min plasma treatment over non-treatment of plasma, but in the samples from 3- to 5-min plasma treatment, Ca^{2+} concentrations were less compared with non-treatment of plasma.

2.2.4. Extracellular ATP concentration

The influence of plasma treatment on extracellular ATP concentration was most significant prior to fermentation (0 h) and at the 9-h stage of fermentation following plasma exposure (**Figure 9**). Prior to fermentation, some significant reductions in extracellular ATP concentration were measured when *S. cerevisiae* cells were treated by plasma for 1 and 2 min, but the remarkable increases in ATP concentration happened when the samples were treated by plasma for 3–5 min over non-treated sample. At the 21-h period of fermentation, however, the extracellular ATP concentrations in 1- and 5-min treatments appeared to be somewhat lower than that of non-treated cells. Thus the data showed that the plasma treatment might change the concentration of extracellular ATP either immediately after treatment or when the treated samples were permitted to reproduce for a moderate stage of time under normal fermentation conditions.

2.2.5. Extracellular NADH concentration

Differences in extracellular NADH concentrations between non-treated and plasma-treated *S. cerevisiae* samples were less uniform for all the three periods of measuring. The differences were more noticeable between non-treated sample and treated samples before fermentation or at the 21-h stage of fermentation (**Figure 10**). Before fermentation, 1-min treatment induced a decrease of 60%, but 2- and 3-min treatments led to 0.8- and 1.8-fold increases, respectively, in extracellular NADH concentration. At the 9-h fermentation stage, the extracellular NADH concentrations of treated samples were either similar to or significantly lower than those of non-treated sample. However, the sample that were treated with plasma for 1 min represented a noticeably higher extracellular NADH concentration than that of non-treated sample at the 21-h fermentation stage, although it remained much lower than that of non-treated sample in the other two stages (0 and 9 h). In addition, the samples treated for 2 to 5 min also showed remarkably higher extracellular NADH concentration than non-treated sample at the 21-h fermentation stage. Taken together, these results indicated that plasma treatment can change the extracellular NADH concentration, either quickly after treatment or in subsequent fermentation, depending on the exposure time.

In this research, we have proved that remarkable decrease in membrane permeability of live cells were distinct after the sample was treated by plasma for 1 min (**Figure 6**). At the 21-h periods of fermentation, the membrane permeability was increased showing that the effect of air cold plasma on membrane permeabilization was temporary and non-inheritable. This

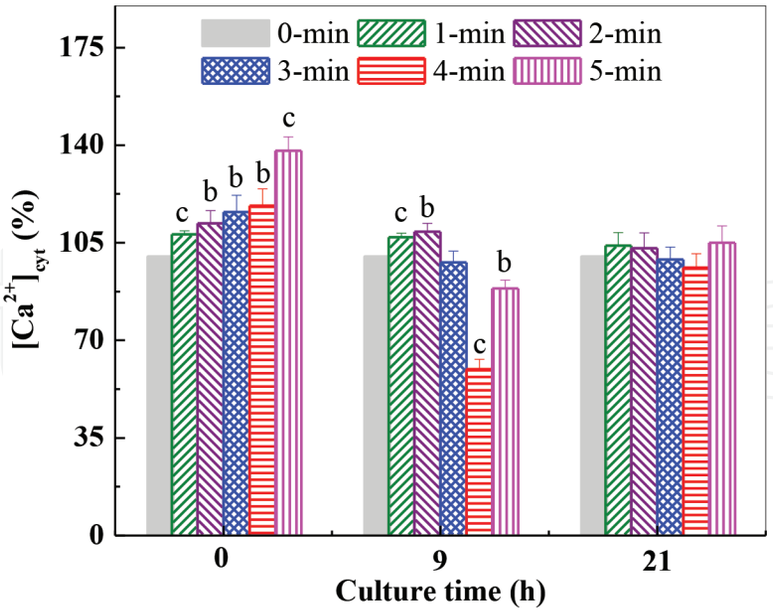

Figure 8. Influence of plasma treatment on $[Ca^{2+}]_{cyt}$ before and after fermentation. Data are expressed as mean ± SES. 'a', 'b', and 'c' indicate $P < 0.05$, $P < 0.005$ and $P < 0.001$, respectively.

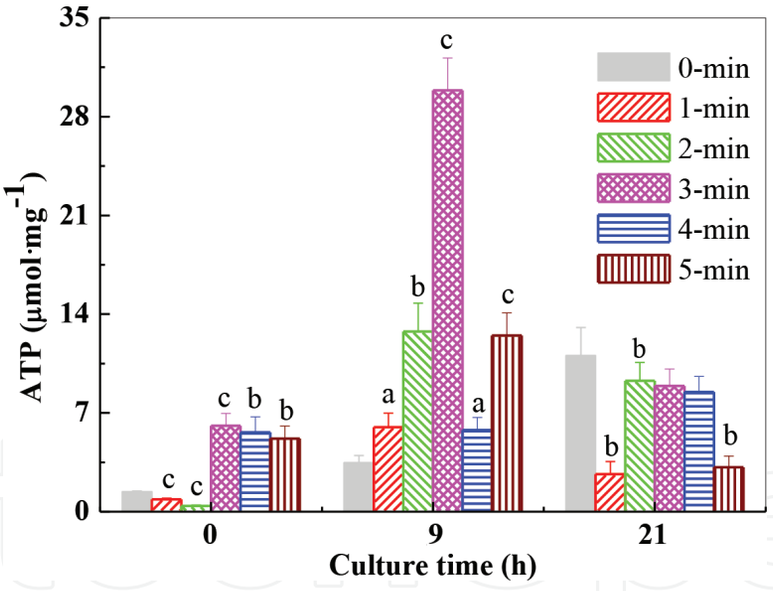

Figure 9. Influence of plasma treatment on extracellular ATP before and after fermentation. Data are expressed as mean ± SES. 'a', 'b', and 'c' indicate $P < 0.05$, $P < 0.005$ and $P < 0.001$, respectively.

result was in accordance with the study of Yonson *et al.*, who reported that cell membrane permeability is temporarily improved by a miniature atmospheric pressure glow discharge plasma torch [31].

Membrane potential is an important factor in cellular functions such as signaling and transport, which can eventually affect cell metabolism [32]. An alteration in membrane potential can be positively detected by an alteration in fluorescence intensity of Rh123. When discharge

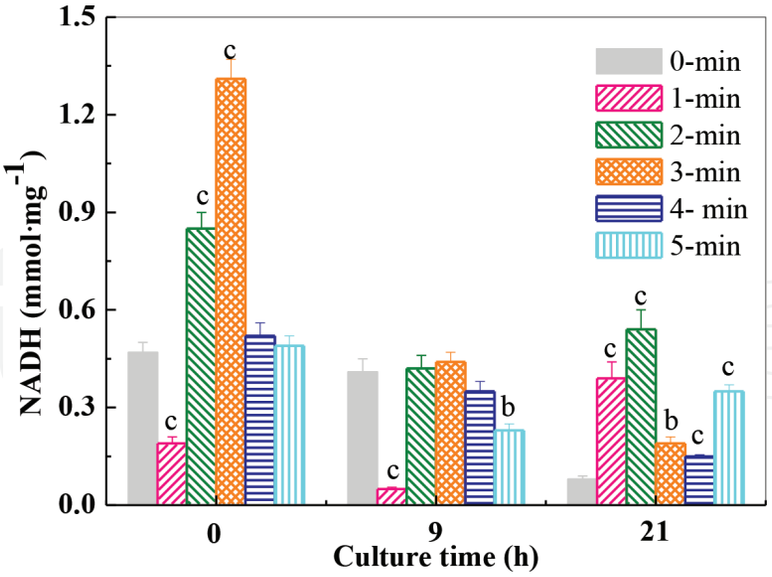

Figure 10. Influence of plasma treatment on extracellular NADH before and after fermentation. Data are expressed as mean ± SES. 'a', 'b', and 'c' indicate $P < 0.05$, $P < 0.005$ and $P < 0.001$, respectively.

plasma occurs over the solution surface, a variety of physical and/or chemical processes are activated. Many active species such as oxygen, hydrogen, hydroxyl and hydroperoxyl radicals are produced. These reactive species can diffuse in the surrounding liquid and induce the redistribution of charges on the inner and outer surfaces of the cell membrane, leading to an increase or reduction of membrane potential. Such change of the membrane potential would directly affect the plasma membrane permeability. After *S. cerevisiae* cells were treated by air cold plasma, the change in the membrane potential quickly contrasted with the change in membrane penetrability (**Figure 7** versus **Figure 6**). The cell membrane was depolarized due to the lowered potential, finally improving the permeability of the membrane. More inorganic and organic ions can then pass freely through the cell membrane as a result of this enhanced permeability [33]. After the 9- and 21-h stages of fermentation, the increase in membrane potential led to membrane hyperpolarization, and accordingly enhanced the membrane permeability.

The change of cell membrane potential could activate the voltage-dependent Cch1p channel, causing more influx of Ca^{2+} from the extracellular environment into the cytoplasm (**Figure 1**). Therefore, the calcium level in the cytoplasm of treated cells was enhanced after plasma treatment. Air cold plasma slightly improved the cytoplasmic calcium concentration of the sample following treatment for 1 min. This might result from the increase in plasma membrane potential (**Figure 7** versus **Figure 8**, at 0-h culture), causing cell membrane hyperpolarization and opening of Ca^{2+} channels. But the opening of Ca^{2+} channels did not cause an increase in cell membrane permeability (**Figure 6**). This result suggests that the increment in cell membrane permeability might be controlled by more than one channel modulator.

The alteration trend of ATP concentration was different from the alteration tend in membrane permeability with plasma discharge. This shows that change of extracellular ATP concentration is a direct consequence of alterations in intracellular ATP. Before fermentation, the

lower concentrations of ATP at 1 and 2 min plasma treatment might be due to 6.8 and 10% increments in calcium concentration, respectively. The increased calcium concentration promoted the hydrolysis of ATP to adenosine diphosphate (ADP) (**Figure 9**). A Ca^{2+} concentration gradient from 1 to 10 μM, could improve the cell function that regulates cell growth and metabolism to eventually enhance microbial productivity. However, the high concentrations of intracellular Ca^{2+} can induce cell injury or death [34, 35]. The higher concentrations of ATP in the samples treated by plasma for 3–5 min might be due to an inhibition of ATP hydrolysis caused by the higher cytoplasmic calcium concentration (**Figures 8** and **9**). In addition, any disturbance in environmental conditions would influence the activities of catabolic enzymes, thereby accelerating the accumulation of ATP or ADP [35]. Air cold plasma might lead to the accumulation of ADP in the treated samples within 1–2 min of treatment, and of ATP in the treated samples within 3–5 min of treatment, as suggested by the data in **Figure 9**. The accumulation of ATP or ADP might have immediately affected the glycolysis rate [36], producing different ATP concentrations at the 9- or 21-h period of fermentation, depending on the plasma treatment time (**Figure 9**).

Air cold plasma produces different reactive species in the gas phase [37]. These active species further react with water and produce a variety of biologically active reactive species (RS) in the liquid phase, including long-lifetime RS (ozone, hydrogen peroxide and nitrate ions) and short-lived RS (superoxide, hydroxyl radicals and singlet oxygen) [38]. In our research, these reactive species could increase or decrease the cell membrane potential and open Ca^{2+} channels, consequently improving $[Ca^{2+}]_{cyt}$ (**Figures 7** and **8**, at the beginning of culture). Ca^{2+} supplementations of 0.5 and 1.5 mM have been shown to induce the increment in ATPase activity [29]. The enhanced ATPase activity would then promote the generation of proton motive force through hydrolysis of ATP [29, 39]. A reduction in the intracellular ATP level can result in the up-regulation of the activities of phosphofructokinase (PFK) and pyruvate kinase (PK) [40]. This would accelerate the glycolytic flux and enhance the NADH level in the central metabolic pathway [41]. At the same time, NADH-dependent alcohol dehydrogenase (ADH) activity might be improved, leading to up-control of the oxidation of NADH to NAD^+ [40, 42] (**Figure 1**). Therefore, the NADH concentration obtained from 1 min treatment was reduced over the control because of the lower level of ATP (**Figure 10** 1 min versus **Figure 9** 1 min). The oxidation of NADH to NAD^+ would lower the activity of NADH-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH), causing decreased glycerol production and ultimately causing more carbon flux from glycolysis being funneled to ethanol [42–44].

3. Conclusion

Experimental parameters associated with cold plasma discharge at atmospheric air pressure for enhancing ethanol yield of *S. cerevisiae* has been successfully optimized in this research. The maximum theoretical ethanol yield of 0.49 g/g was predicted by the response model under three optimized parameters (1 min of exposure time, 26 V of power voltage and 9 mL of test sample volume), which was closely consistent with the experimental yield of 0.48 g/g. The model may be used as a reference for modulating the experimental parameters related with dielectric barrier discharge at air atmospheric pressure and a novel approach for improving ethanol yield in bio-manufacturing industry.

Furthermore, the potential mechanism that air cold plasma alters the cofactor metabolism of *S. cerevisiae* was explored by analyzing the changes in plasma membrane potential, cytoplasmic calcium concentration and the two cofactors of ATP and NADH. The sample of 1-min treatment presented a notable increment in plasma membrane potential, whereas the sample of 2-min treatment presented a distinct reduction in plasma membrane potential. In addition, the calcium concentrations for the samples treated by plasma for 1–5 min were remarkably improved prior to the beginning of the fermentation compared with that for the untreated sample. An increase of 7.0% in calcium concentration led to the remarkable reductions of 40% in ATP and 60% in NADH in the sample of 1-min treatment. At 9-h culture, the ATP concentration of treated sample for 1 min increased by 72%, whereas NADH concentration decreased by 88% relative to those of the control. Briefly, the mechanism that plasma promoted alterations in cofactor level in *S. cerevisiae* showed to be by improving the cell membrane potential, which then caused increases in cytosolic free Ca^{2+} concentrations within the cells, eventually enhancing microbial productivity. This may a potential and broad application in intensifying the biotransformation capability of microorganisms in the future.

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Conflict of interest

The author declares no financial or commercial conflict of interest.

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