MASTER'S THESIS

Production Study of the Functional Bioactive Compound from *Lactobacillus rhamnosus*

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(Supervised by Professor Dong-Sun Lee)

A thesis submitted in partial fulfillment of the requirement

for the degree of Master of Advanced Convergence Technology and Science

August, 2017

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LIST OF ABBREVIATIONS

DBD	Dielectric barrier discharge
µM	Micromole
min	Minute
hrs	Hours
Fe^{3+}	iron (III)
mM	millimole
MRS agar	deMan, Rogosa and Sharpe agar
CFU	Colonies forming units
NOD	non- enzymatic decarboxylation

LIST OF FIGURES

ABSTRACT

Diacetyl is a high value product that can be significantly used as a food ingredient. Based on the knowledge of involving in diacetyl production, various strategies have been characterized to improve the growth rate and diacetyl production by lactic acid bacteria. The lactic acid bacteria such as *Lactobacillus rhamnosus* can be used to produce flavour compounds like diacetyl. In this study, diacetyl production of *L. rhamnosus* GG strain was increased as *L. rhamnosus* GG was cultured with glucose and pyruvate medium than in MRS media. In order to increase diacetyl production, 20 mM Fe^{3+} was added to the fermentation broth both on MRS and glucose and pyruvate medium at the optimal time. Furthermore, recently a method using surface sterilization Dielectric Barrier Discharge (DBD) air plasma can be considered to make mutagenesis of bacteria. The DBD air plasma method at atmospheric pressure was performed to induce mutation of *L. rhamnosus* GG. In this study, we treated *L. rhamnosus* GG using cold atmospheric plasma at Petri dishes with cultured bacterial by a DBD reactor. Sterilization of *L. rhamnosus* GG for less than 5min was carried out. Thus, a concentration of diacetyl was increased by using the plasma treatment mutagenesis at 150V at 2min on *L. rhamnosus* GG strain.

Keywords: Diacetyl, *Lactobacillus rhamnosus*, Metal ions (Fe^{3+}), Glucose and pyruvate medium, Dielectric barrier discharge, Plasma sterilization.

INTRODUCTION

Originally microorganism produces natural flavor compounds through fermentation. *Lactobacillus rhamnosus* is the heterolactic acid bacteria and is extensively used in food industry. Several studies indicate that this organism is a possible candidate for industrial synthesizing flavor compound such as diacetyl [1-5]. Diacetyl is an important flavor compound and essential for the flavor of dairy industry [6-10]. Based on the knowledge of involving in diacetyl production, various strategies methods have been characterized to improve the growth rate and diacetyl production by lactic acid bacteria [11&12]. Previous bacterial studies on *L. rhamnosus* have been reported that the use of multiple substrates in the medium, especially glucose and pyruvate have been reported to affect bacteria growth as well as increase the yield of diacetyl production. Through the metabolic engineering approach described, diacetyl was synthesized with a high yield by using glucose pyruvate as substrate [13-18].

In addition, diacetyl production in heterolactic acid bacteria have indicated that in order to achieve optimal production of diacetyl α-acetolactate can be converted to diacetyl in the presence of metal ions (Fe^{3+}). Metal ions Fe^{3+} was selected as an accelerator for the decarboxylation of α-acetolactate to diacetyl [19-21]. Moreover, such as *Lactobacillus* mutant was able to growth and produce overproduce with a final concentration of diacetyl production [22-25].

Moreover, presently, some general methods for lactic acid bacterial mutation are used to improve diacetyl formation. Accordingly, plasma treatment processing is one of the leading technologies in material engineering. Non-equilibrium plasma source that can be operated with different gasses at elevated pressures (up to atmospheric pressure) is called the dielectric barrier discharge (DBD). DBD usually consists of a large number of short-living microchannel (filaments) that are randomly distributed over entire area of the dielectric barrier. Therefore, a DBD can be applied in direct contact with biological objects without causing any damage [26-29].

Although a few bacterial studies on *L. rhamnosus* have been reported, yet the physiological reasons for the production of diacetyl is not clear to understand and its mutation studies on dielectric barrier discharge (DBD) air plasma have not been explored. In this study, increased production of diacetyl by *L. rhamnosus* GG strain was checked with MRS and glucose pyruvate medium. And, 20 mM Fe^{3+} was added to the fermentation broth both on MRS and glucose pyruvate medium at the optimal time in order to increase diacetyl. Furthermore, in this work we describe DBD reactor, which generated cold atmospheric plasma with bacterial culture. All plasma treatments were conducted at same discharge power (150V) with different exposure time. All bacterial cells were killed for less than 5 mins of DBD treatment. The possible induction of mutation in *L. rhamnosus* GG strain by DBD air with bacterial culture was verified to improve a final concentration of diacetyl.

MATERIALS AND METHODS

1. Microorganism

Lactobacillus rhamnosus (GG), a strain obtained from KCTC was stored at -80 ^{0}C in de Mann Rogosa Sharp (MRS) medium with 50% glycerol in the laboratory and used for all the experiments.

2. Maintaining cultured Cells

The cultured cells were maintained at 4℃ on slants consisting of *Lactobacillus rhamnosus* (GG) MRS Medium (Hi-Media). The bacteria was grown in *Lactobacillus rhamnosus* (GG) MRS Broth at 37℃ for 24 hours without shaking.

3. Inoculum and fermentation media

3.1 MRS medium

The different sets of media were sterilized at 121 ^{0}C for 15 min. The inoculum was grown in 250 ml Erlenmeyer flasks containing 100 ml of the sterilized medium at 30^{0}C and 37^{0}C at 120 rpm for 24 hours. The optical density (OD) of inoculum was adjusted to approximately 0.5 at 600 nm in each case and the fermentation media were inoculated with 1% of the inoculum.

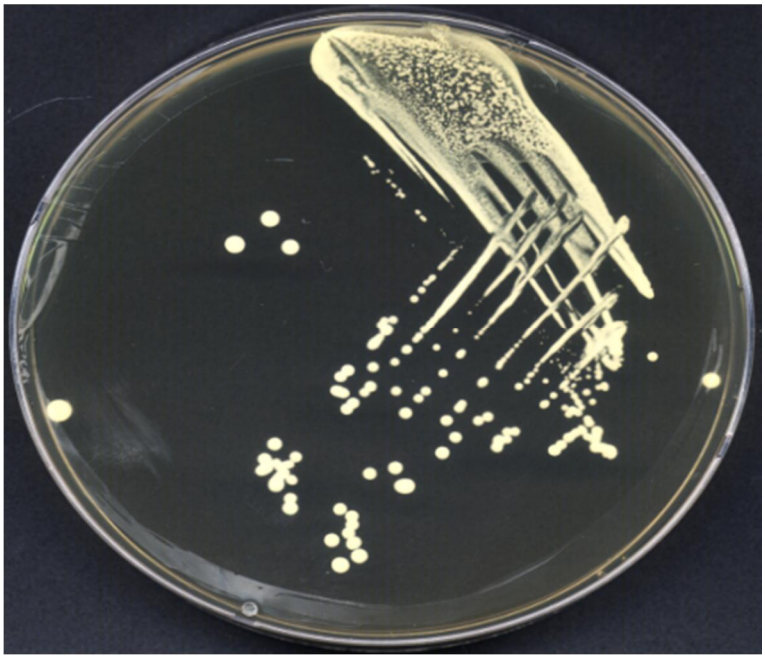


Figure 1. Colony morphology of *L. rhamnosus* strain GG cultured aerobically on MRS agar at 37°C for 48 hrs.

3.2 Glucose and Pyruvate medium

The medium used for inoculum and fermentation was the same. The glucose and pyruvate medium for shake flask experiments was contained of yeast extract, 5.0 g/1 ; $MgSO_4.7H_2O$: 0.1 g /1 ; $MnSO_4.H_2O$: 0.05 g /1 ; NaH_2PO_4 : 2.0 g /1 ; Tween-80 : 1.0 g/ l ; glucose : 6.7 g/1; pyruvate : 1.7 g /1.

3.3 Iron (III) addition

The cell suspension was added to the flask contained 50ml of medium (pH 6.5). 20 mM Fe^{3+} was added in to the medium and the media were adjusted at the pH (pH 6.5) using the 1M NaOH.

4 Diacetyl Assay

Diacetyl: A basic solution of 5µL diacetyl was adulterated in 50ml distilled water directly. The main solution is freshly prepared for each experiment.

Solution B: This solution contains 3% NaOH and 3.5% α-naphthol (Sigma). Diacetyl and solution B were both protected from light.

Creatine: Creatine (Sigma) was dissolved in distilled water to produce a saturated solution.

Development of assay. To determine optimal amounts of each reagent, the concentration of the reagent being tested was varied in each set of experiments

while keeping the other reagents constant. The development of the color was also assessed with time at room temperature.

The final procedure adopted was as follows: In a 1-ml final volume are added 325μL of the creatine solution, 150μL Solution B (3.0% NaOH and 3.5% α-naphthol), diacetyl (1-5μg), and distilled water. After standing at room temperature for 30 min, the absorbance was measured in a VERSA max Tunable Microplate Reader at 525 nm [30].

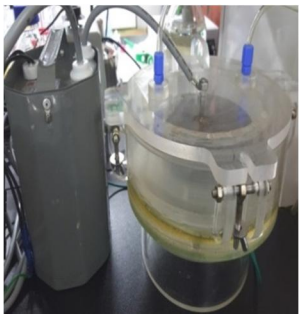
5. Analytical of DBDs

DBD bacterial sterilization already used in the experimental arrangement was shown in Figure 3. The inside of petri dishes containing bacteria was covered with low-temperature plasmas at atmospheric pressure, which was partially filled with 10 ml agar culture medium leaving air spacing about 5mm**.** Suspensions of *L. rhamnosus* GG was standardized to the concentration by optical spectrophotometry 600 nm. Portions of 0.1 ml of the initial suspensions were evenly spread on the surface of the MRS agar medium and finally treated by DBD plasma. The discharge was driven by a low-frequency high voltage power supply operating at 60 Hz. The high voltage electrode was placed on the Petri dish and the electrode under the Petri dish was grounded.

The discharge and the applied voltage were shown in Figure 3. To obtain the charge flowing through the gap the resistor was substituted by a 1 μF capacitor. The mean power is obtained by multiplying the energy by the source

frequency. During all experiments, the magnitude of the applied voltage was fixed at 150V, which corresponded to mean discharge energy per cycle (electric power of 1.0 W). To analyzing the DBD sterilization efficiency of the plasma display time was varied from 1 min to 5 mins. The Petri dishes were incubated at 37°C for 24 hours after the treatment with plasma. All experiments were done in duplicate. Next step, the exact amount of colonies was counted. The mean values were useful in the bacterial survivor curves and also the results were expressed in the high values of colonies forming units (CFU).

Gas /Argon/



Dielectric barrier discharge plasma generator

Voltage controller

Figure 2. Apparatus of plasma mutagenesis.

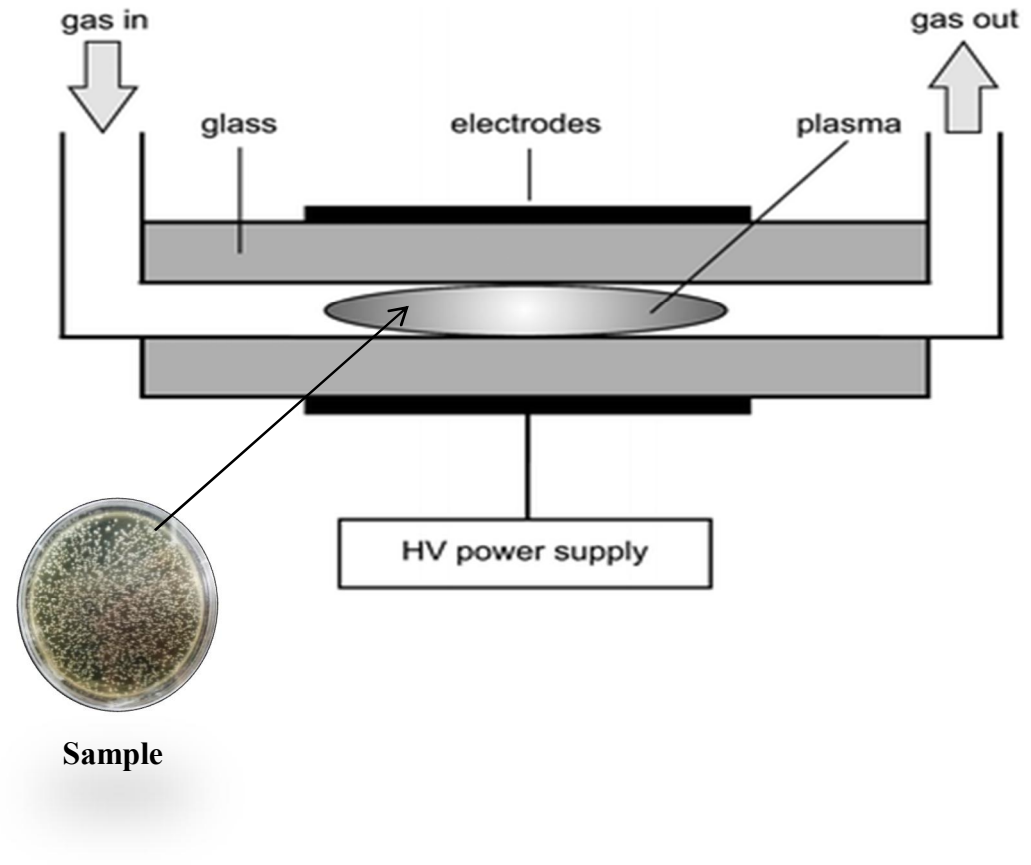


Figure 3. Schematic diagram of experimental set up of atmospheric pressure discharge (DBD) plasma treatment.

RESULTS

1. Optimization of incubation period, temperature and diacetyl measurment

Figure 4 shows the relations between growth of bacteria and diacetyl production at 30ºC and 37ºC. The growth (OD600 nm) of the *L. rhamnosus* strain GG increased drastically between 24 hrs to 58 hrs after inoculation and bacteria didn't increase at all until 72hrs of incubation. The biosynthesis of diacetyl increased concomitantly with the growth 37°C, with a maximal production at 24 hrs of incubation. At 24 hours of incubation, the levels of diacetyl formed was higher at 37°C than at 30°C. So it can be inferred that the *L.rhamnosus* GG showed the growth stability and maximum diacetyl production at 37°C.

As for the effect of incubation temperature, the bacterial growth was most significant when cultured at 37ºC. The production of diacetyl showed higher in MRS media at 37ºC than the incubation temperatures, 30ºC. So the bacteria cultured at 37ºC was chosen as the temperature for the further experiment.

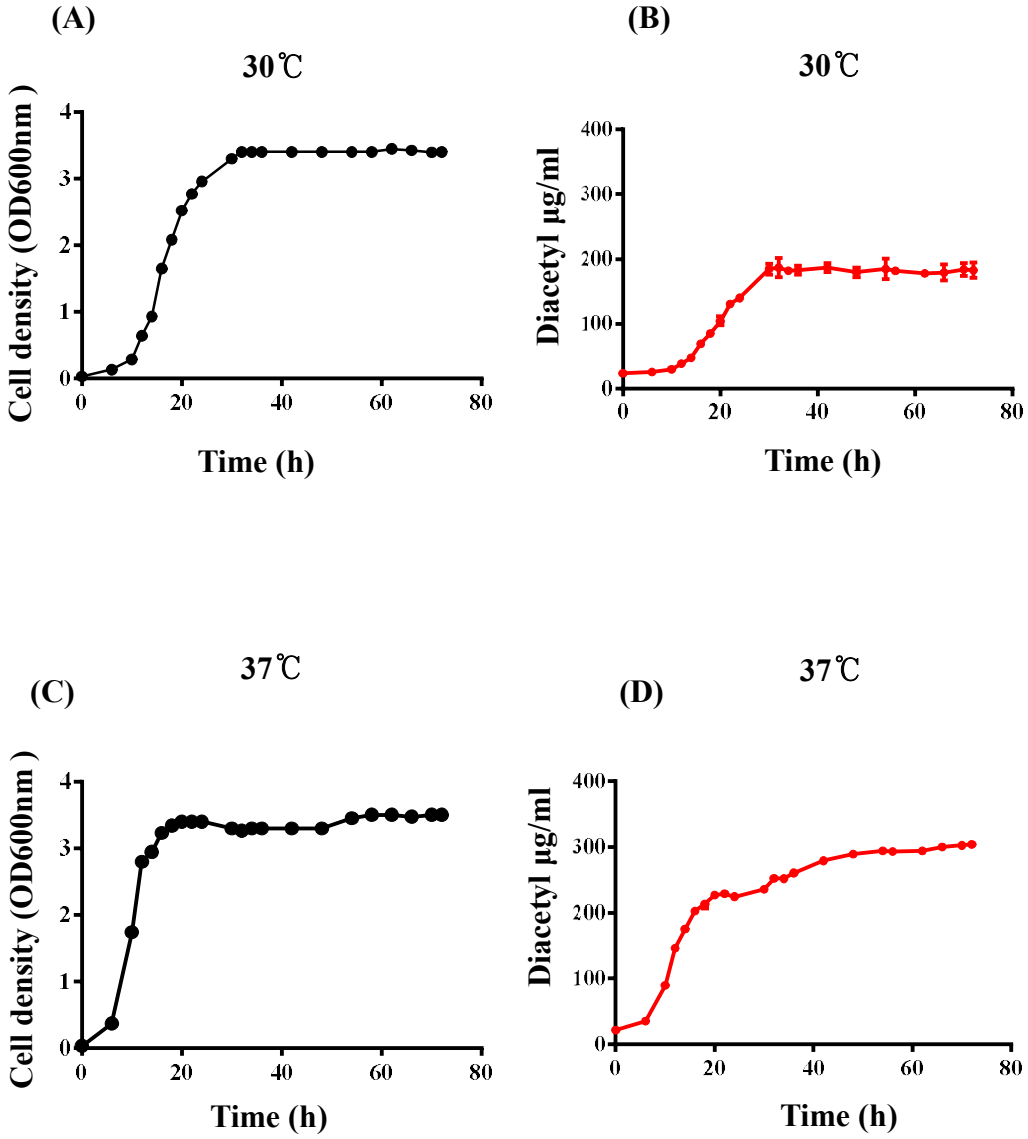


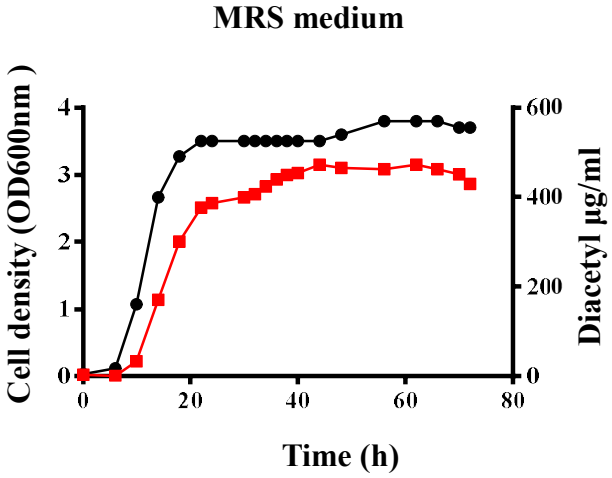
Figure 4. Growth curve and diacetyl production of *L. rhamnosus* GG in MRS medium at 30℃ and 37℃. The growth curve (A) and production of diacetyl (B) at 30 ^{0}C. The growth curve (C) and production of diacetyl (D) at 37 ^{0}C. Symbol indicate : : growth curve, : diacetyl production.

2. Optimization of Fe^{3+} addition

In order to achieve optimal production of diacetyl alone, rather than co-production of α-acetolactate and diacetyl, nonenzymatic decarboxylation (NOD) of α-acetolactate to diacetyl should be enhanced. Mohr et al. [20] reported that α-acetolactate can be converted to diacetyl and consequently greater production of diacetyl under 20 mM Fe^{3+}.

After added 20 mM Fe^{3+} the growth (OD600 nm) of the *L. rhamnosus* strain GG increased highly and the biosynthesis of diacetyl was increased extensively with a maximal production at 24 hrs of incubation as compared with control (MRS medium without adding after 20 mM Fe^{3+}) . As shown in Figure 5, addition of 20 mM Fe^{3+} in MRS medium led to increase in diacetyl production. These results suggested that nonenzymatic decarboxylation of α-acetolactate to diacetyl could be enhanced by the addition of 20 mM Fe^{3+}.

(A)



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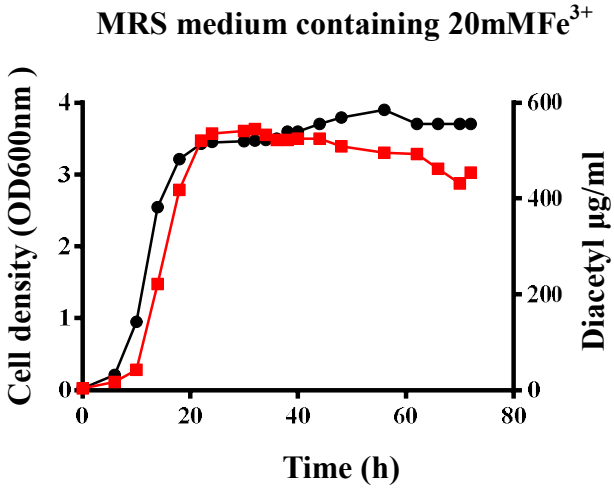
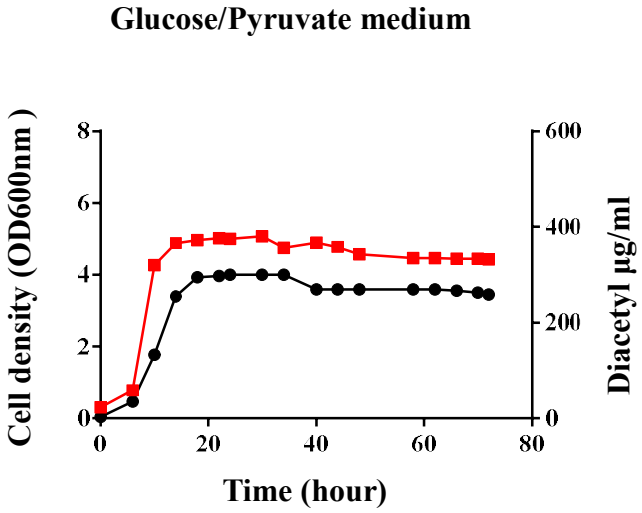


Figure 5. Growth curve and diacetyl production of *L. rhamnosus* GG in MRS medium containing 20 mM Fe^{3+} at 37^0C. (A) Growth curve and diacetyl production of *L. rhamnosus* GG in MRS medium at 37^0C. (B) Growth curve and diacetyl production of *L. rhamnosus* GG in MRS medium containing 20mM Fe^{3+} at 37^0C. Symbol indicate : ●—●: growth curve, ■—■ : diacetyl production.

3. Effect of medium composition and Fe^{3+} on bacteria growth and diacetyl production

Glucose with sodium pyruvate was tested for diacetyl production on the growth of *L.rhamnosus* GG. A specific increase of bacteria growth rate was obtained on glucose and pyruvate medium as compared with MRS medium. The diacetyl also increased extensively with high yields during growth of *L. rhamnosus* GG after adding 20 mM Fe^{3+}. As shown in Figure 6, addition of 20 mM Fe^{3+} in the medium led to increase in diacetyl. It can be hypothesized that the excess pyruvate provided in the medium was converted to increased amounts of flavour compounds as a diacetyl. Thus, glucose with sodium pyruvate was chosen as a carbon source for further experiment.

(A)



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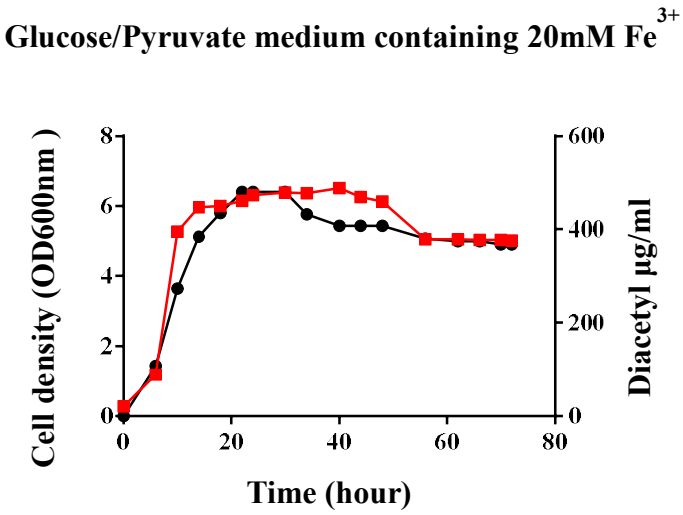


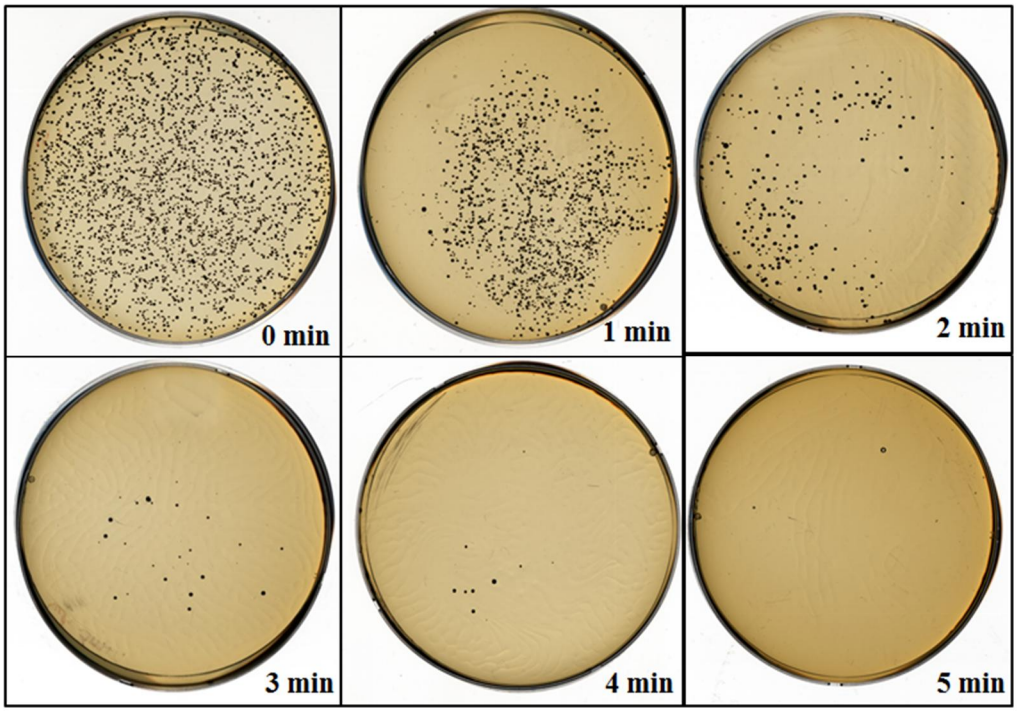
Figure6. Growth curve and diacetyl production of *L. rhamnosus* GG in glucose and pyruvate medium at 37^{0}C. (A) Growth curve and diacetyl production of *L. rhamosus* GG in glucose pyruvate medium at 37^{0}C. (B) Growth curve and diacetyl production of *L. rhamnosus* GG in glucose pyruvate medium containing 20mM Fe^{3+} at 37^{0}C. Symbol indicate: ●: growth curve, ■ : diacetyl production.

4. The mutagenesis of *Lactobacillus rhamnosus* GG using plasma mutagenesis by gas pressure dielectic barrierd discharge (DBD)

In order to increase production of diacetyl, the possible induction of mutation in *L. rhamnosus* GG by DBD argon plasma was verified if the resulting mutant of *L. rhamnosus* GG is able to improve diacetyl production. The dielectric barrier discharge (DBD) is a non-equilibrium plasma source that can be operated with different gasses at atmospheric pressure. Therefore, a DBD can be used directly to connect with living tissues and open injuries without causing any damage.

Dielectric barrier discharge reactor generates argon plasma inside a petri dish of bacteria culture and placed between two metal electrodes connected to a high-voltage transfer (60 Hz). The DBD sterilization efficiency and the plasma exposure time was varied from 1min to 5mins.

(A)



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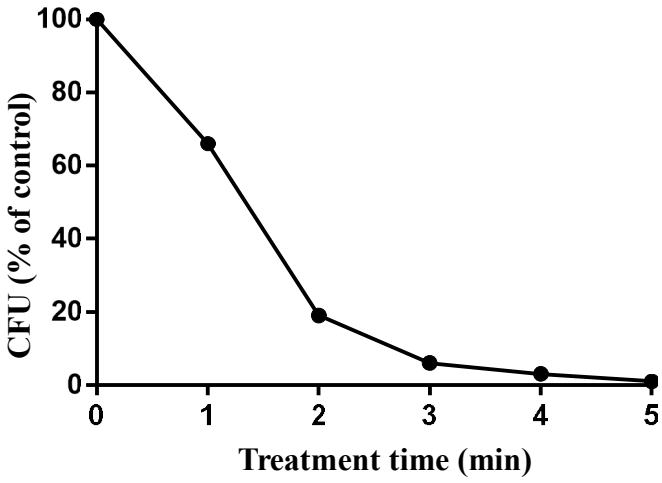


Figure 7. Image of Petri dishes showing the viable colonies of *L.rhamnosus* GG after plasma treatment for 0, 1, 2, 3, 4 and 5 min (A) and the *L.rhamnosus* GG survivor curve (B).

5. Effect of dielectric barrier discharge (DBD) plasma-treated on bacteria growth and diacetyl production

The initial bacterial suspensions were spread on agar inside six Petri dishes, one was kept for control (without plasma exposure) and five Petri dishes were treated by DBD. We chosed five treatment time intervals of 1, 2, 3, 4 and 5 min at a fixed applied voltage of 150V. The visible bacterial colonies were counted after the plasma treatment. Then samples were incubated for 24hrs at 37°C. All experiments were done in duplicate. Photographs of *L. rhamnosus* GG colonies inside Petri dishes are shown in Figure 7 (A) while Figure 7 (B) presents the bacterial survival rate as a function of the plasma exposure time. As it can be seen from this figure bacterial sterilization was achieved in less than 5 mins.

The time interval necessary to reduce the bacterial population by DBD plasma exposure was 2 mins and the bacterial killing affect showed a considerably higher value at 3 mins. Thus, we chose the plasma exposure time at 2 mins for the further experiment.

6. Processing of diacetyl assay on *L. rhamnosus* GG using plasma mutagenesis

The activity of diacetyl production in *L. rhamnosus* GG was investigated after using plasma mutagenesis**.** The initial bacterial suspensions were spread on agar inside Petri dishes. After using plasma treatment 150V for 2mins then the bacterial colonies were incubated in MRS agar media at 37^0C for 24-48 hours. The bacterial single colony was selected and spread on agar inside square petri dish. Then bacterial was incubated in MRS broth at 37^0C for 24 hours in 96 well plates. And then we checked the concentration of diacetyl at Figure 8.

After checking diacetyl production, the result in Figure 9 shown that 2nd plate gave higher amount of diacetyl production then 1st and 3rd plate.

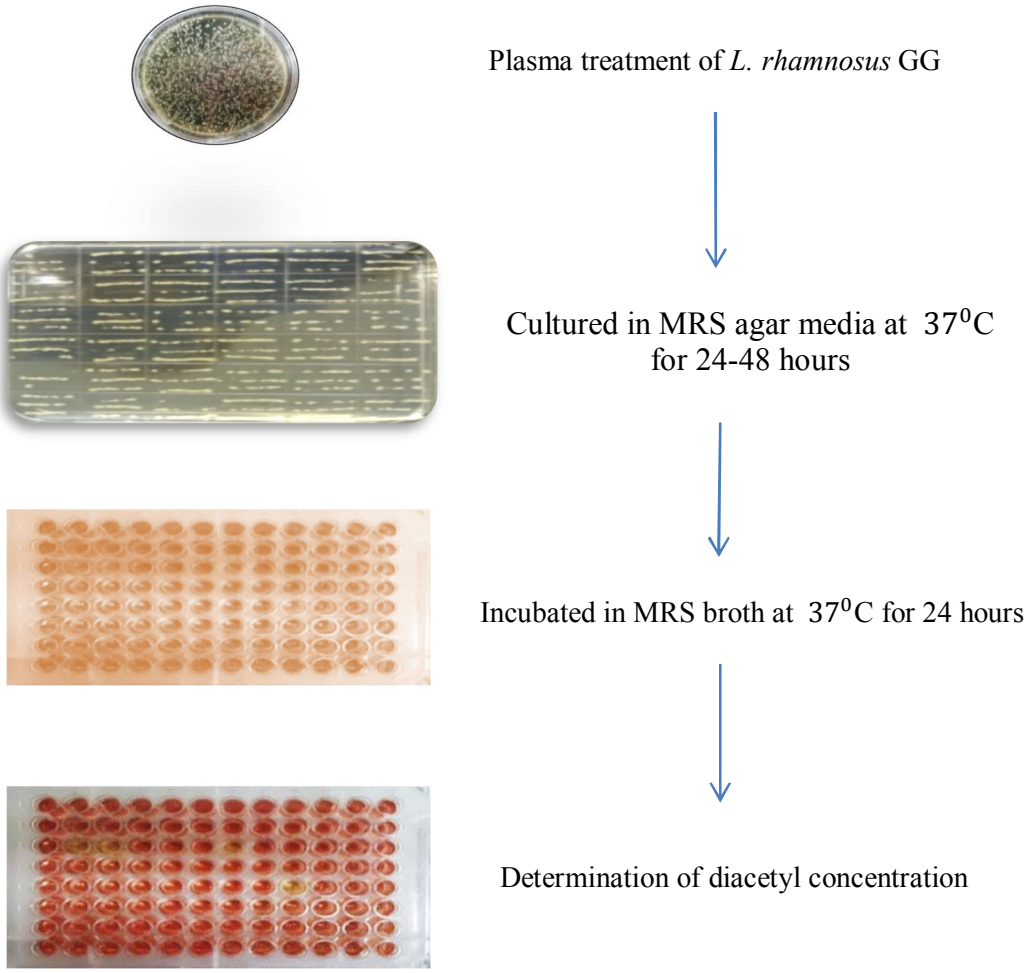


Figure 8. Processing of diacetyl assay on *L. rhamnosus* GG after using Plasma mutagenesis

Plate1

	1	2	3	4	5	6	7	8	9	10	11	12
A	2.68	2.41	2.50	2.29	2.50	2.50	2.62	2.32	2.62	2.58	2.61	2.54
B	2.58	2.51	2.47	2.42	2.46	2.48	2.57	2.62	2.57	2.42	2.49	2.60
C	2.68	1.78	1.68	2.54	2.47	2.55	1.67	2.46	2.64	2.53	2.59	2.72
D	2.79	2.64	2.59	2.41	2.46	2.53	2.56	2.46	2.53	2.44	2.50	2.67
E	2.78	2.68	2.58	2.50	2.49	2.59	2.61	2.58	1.64	2.51	2.61	2.52
F	2.69	2.61	2.52	2.57	2.59	2.47	2.52	2.42	2.33	2.31	2.34	2.46
G	3.16	2.62	2.59	2.60	2.55	2.56	2.58	2.33	2.45	2.39	2.41	2.33
H	2.58	2.50	2.46	2.55	2.38	2.60	2.22	2.12	2.47	2.26	2.37	2.27

Plate2

	1	2	3	4	5	6	7	8	9	10	11	12
A	2.87	2.98	3.02	2.86	3.10	3.00	3.02	2.93	2.92	2.90	3.13	3.19
B	3.16	2.82	3.08	2.99	2.95	3.09	2.90	2.90	2.96	3.10	3.19	2.89
C	3.07	3.15	3.10	2.94	2.96	3.04	2.63	2.68	2.98	3.82	2.94	3.05
D	2.98	2.82	3.38	3.13	2.77	3.01	3.03	3.06	3.08	3.05	2.82	2.77
E	3.36	2.84	2.88	2.85	2.83	2.51	2.94	3.00	2.97	3.00	2.96	3.30
F	3.56	2.86	2.88	2.88	2.91	2.88	2.79	3.05	2.93	3.18	3.02	3.31
G	2.75	2.69	2.92	2.87	2.65	1.91	2.73	2.64	3.18	3.02	3.11	3.20
H	2.51	2.33	2.43	2.30	2.32	2.40	2.43	2.53	3.06	3.25	3.03	3.22

Plate3

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.85	1.90	1.96	1.84	1.89	1.82	1.78	1.76	1.94	1.91	1.83	1.77
B	2.04	1.99	2.06	1.87	1.88	2.01	1.32	1.89	1.85	1.87	1.82	1.84
C	2.01	1.84	2.03	1.88	1.88	1.87	1.85	1.93	1.39	1.91	1.97	2.01
D	1.82	1.82	2.03	1.89	1.91	2.00	1.95	2.03	1.96	1.94	2.02	1.96
E	1.94	1.37	1.99	1.90	1.81	2.03	1.79	1.99	1.94	1.27	2.05	1.89
F	2.14	1.97	2.05	1.89	1.80	2.03	1.95	2.11	1.92	2.00	2.20	1.84
G	1.91	2.06	2.11	1.93	1.87	1.94	2.03	2.04	1.95	2.04	2.15	2.14
H	1.80	2.02	1.94	1.96	1.78	1.82	1.79	1.97	2.06	1.94	2.03	2.02

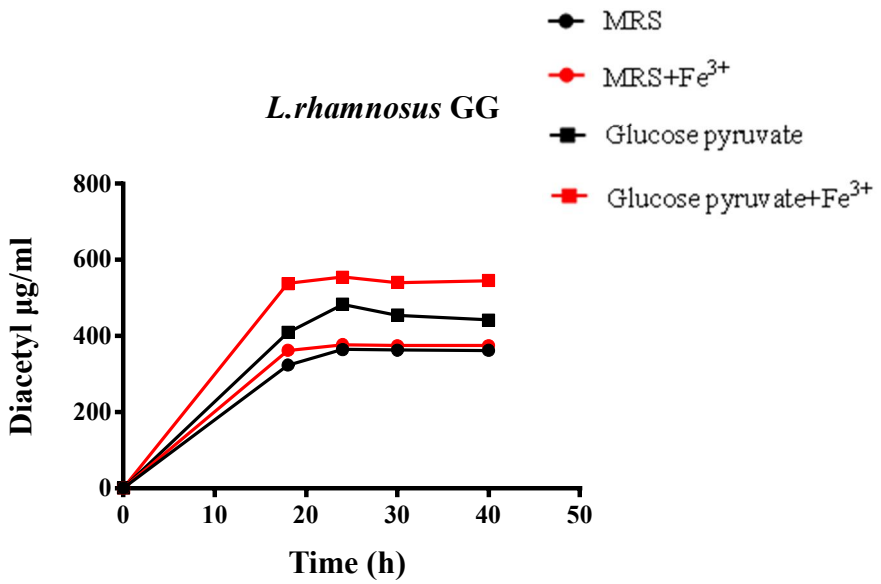
Figure 9. Production of diacetyl on *Lactobacillus rhamnosus* GG using Plasma mutagenesis

7. Diacetyl production by addition of Fe^{3+} after plasma treatment

In order to achieve increase production of diacetyl the characteristics of the engineered strain GG in MRS and glucose with Pyruvate medium containing 20 mM Fe^{3+} after plasma treatment were studied. As shown in Figure 10, the diacetyl production in MRS and glucose with pyruvate medium after plasma treatment led to increase in diacetyl production.

In summary, the result of diacetyl formation was increased after using plasma mutagenesis by argon pressure dielectric barrier discharge plasma. So DBD plasma treatment at 150V 2 mins gave higher amount of diacetyl production as compared with parents strain.

(A)



(B)

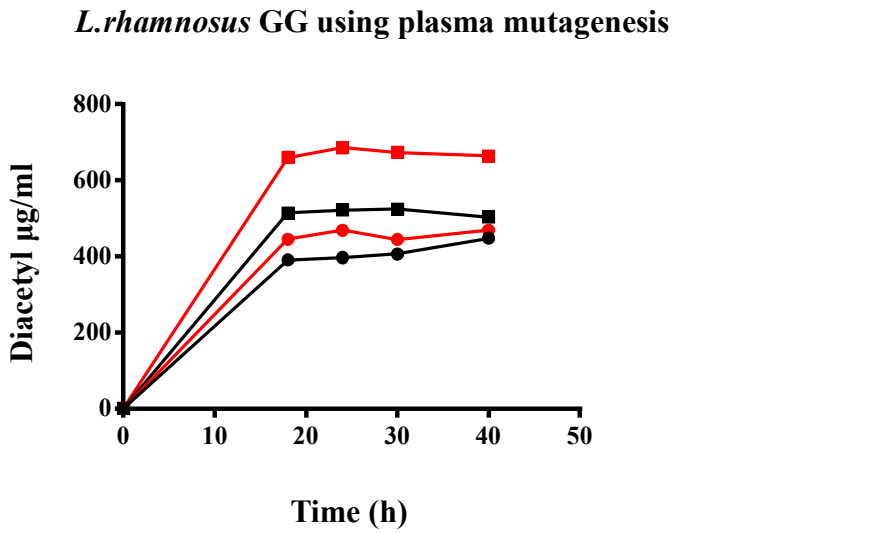


Figure 10. Diacetyl concentration of *L. rhamnosus* GG and plasma-treated *L. rhamnosus* GG with MRS and glucose pyruvate medium at 37^{0}C. (A) Diacetyl concentration in MRS and glucose pyruvate medium containing Fe^{3+} at 37^{0}C. (B) Diacetyl concentration in MRS and glucose pyruvate medium containing Fe^{3+} using plasma-treated bacteria at 37^{0}C

DISCUSSION

In this study we showed the production of diacetyl on *L. rhamnosus* GG after culture in MRS and glucose pyruvate medium (Figure 2&3). And the best growth was observed when the bacteria grown on glucose pyruvate medium. Base on this result, this is consistent with previous study. Regarding to the effect of carbohydrates, Liew *et al*. [17] showed a slight effect of glucose on the cell number of *Lactobacillus rhamnosus* and Bajpaj-Dikshit *et al*. [18] reported that *L. rhamnosus* increased the maximum growth rate nearly 1.4-fold when it co-metabolized sodium pyruvate with glucose. It appears that among the various carbon sources studied, the production diacetyl particularly was affected by the increase in the concentration of glucose with sodium pyruvate which is the preferred substrate for the growth of *L. rhamnosus* GG strain.

The conversion of α-acetolactate to diacetyl could be achieved by addition of Fe^{3+} studied by Gao et al. [21]. The previous studies have been reported that the most of engineered strains was described the exhibit co-production of α-acetolactate and diacetyl following an inefficient NOD of α-acetolactate to diacetyl [19-21]. Mohr et al. [20] showed that Fe^{3+} caused more significant decarboxylation of α-acetolactate to diacetyl and it was indicated that Fe^{3+} would also influence the glucose consumption and might decrease the diacetyl production during the fermentation process. The addition of 20 mM Fe^{3+} is also optimized in the present study. And this observation is agreement with these research. As shown in Figure 5&6, 20 mM Fe^{3+} could accelerate the NOD of α-acetolactate, and accumulate the highest concentration of diacetyl.

Moreover, Laroussi 1996 [31] first demonstrated that discharge plasma generated at atmospheric pressure was a very effective sterilization agent. Since then, plasma treatment has been reported to be effective for inactivation of a variety of microorganisms; however, its effectiveness differs owing to differences in membrane structure reported by Lu et al. [32] Non-thermal atmospheric plasma has been used for different biological applications including sterilization of a wide range of bacteria [33-36]. Recently, the air plasmas are excellent sources of reactive oxygen and nitrogen species such as atomic oxygen, ozone, and hydroxyl group, NO, NO_2 etc. These highly reactive species have direct impact on the microorganisms, and especially on their outmost membranes by chemical etching [39]. In this study, we investigate the effects of plasma exposure on the bacterial by using DBD. Suspensions of *L. rhamnosus* GG was inoculated on the Petri dish was put inside a DBD reactor. The samples were treated for 5 mins at applied voltage of 150V. Since the voltage applied in this experiment is quite high (150V), the kinetic energy of the ions may become high enough to produce some structural damage on the cells membrane upon hitting a bacterium. The time interval necessary to reduce the bacterial population is 2 mins and the bacterial killing effect showed a considerably higher value about 3 mins. After using DBD Plasma treatment at 150V at 2 mins, *L. rhamnosus* GG mutant gave higher amount of diacetyl production as compard with a *L. rhamnosus* GG without plasma treatment.

CONCLUSION

Diacetyl production was maximally produced when *lactobacillus* cells were grown on glucose pyruvate medium as compared to MRS medium. The cells may be channeling excess pyruvate towards the flavor compounds as a diacetyl. The productivity of diacetyl in the case of growth on glucose and pyruvate medium was higher than in MRS medium. In addition, the addition of 20 mM Fe^{3+} to the medium enhanced the conversion of α-acetolactate to diacetyl and accumulated the reflecting higher production for diacetyl in both MRS and glucose pyruvate medium. Furthermore, in this paper bacterial sterilization using DBD plasma was investigated. The effects of DBD plasma on bacteria sterilization was achieved in less than 5 mins. It was also shown that, the result of diacetyl formation was increased after using argon plasma mutagenesis. Therefore, diacetyl production was higher with the *L.rhamnosus* GG mutants than with the parents strain.

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ACKNOWLEDGEMENTS

For these two years, I would love to express my deepest gratitude to my supervisor Prof. Dong-Sun Lee for his kind supervisions, advices, supports, and guidance from the beginning until I successfully completed my master course. I also would like to express my profound gratitude to Dr. Hack Sun Choi for his kind advices, technical supports, caring and encouragement during these two years. I would love to give my special thanks to all my kind lab mates, Kang Jiyoung, Kim Jihyang, Kim Solim, and Deung Hong Won who dedicated their time and energy for teaching and supporting me not only in the lab but the way how to live in Jeju. I would like to say thank you so much my Korean teacher Han Hogyeong and Cambodian junior Soth Sereirath and Chin sreytoch and my family for their love, help, encouragement, and warmest friendship. I also express my deepest gratitude to all professors, lecturers, and staff members of College of Applied Life Sciences, Jeju National University, South Korea, for their guidance, support, teaching and encouragement.