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# Design of Experiment-based Testing of Air, Charged Ions, and Hydrogen Peroxide in a Direct Current Steady-State Plasma Sterilizer

Arun Balasundaram,[1] Igor Alexeff,[2]* Rapinder S Sawhney[1]

[1]Department of Industrial and Information Engineering and [2]Department of Electrical Engineering, University of Tennessee, Knoxville

*Address all correspondence to: Igor Alexeff, Department of Electrical Engineering, University of Tennessee, 315 Ferris Hall, 1508 Middle Drive, Knoxville, TN 37996-2100; alexeff@utk.edu.

**ABSTRACT:** Three primary factors, hydrogen peroxide, charged ions, and air (oxygen), were evaluated in the kill rate of *Escherichia coli* bacteria. The results were evaluated by analysis of variance and regression analysis. Air and charged ions are found to be extremely effective. These two factors in combination strongly enhance each other. The hydrogen peroxide did not enhance the kill rate.

**KEY WORDS:** plasma sterilizer; air, charged ions, hydrogen peroxide; DC barrier discharge, ANOVA test, *E. coli*

## I. INTRODUCTION

Our resistive barrier discharge (RBD) has been demonstrated to be successful on *Escherichia coli*, *Pseudomonas fluorescens* (5RL), spores, and bacteriophages. It has been tested successfully in sterilizing pagers contaminated with methicillin-resistant *Staphylococcus aureus* at the St. Jude Research Hospital in Memphis, Tennessee. Here, we evaluate 3 primary factors in the atmospheric pressure RBD: hydrogen peroxide, charged ions, and air (oxygen). The experiment used analysis of variance (ANOVA) and regression analysis. The tests used 144 Petri dishes and the *E. coli* bacteria. Hydrogen peroxide was used as a replacement for the water conductor on the RBD electrode. The charged ions were removed by a double charged wire mesh between the discharge and the Petri dish. The air was displaced by a slow flow of nitrogen into the experimental area. The basic conclusions are that air and charged ions both are extremely effective in killing bacteria. In addition, air and charged ions together strongly enhance each other. In our experiments, hydrogen peroxide did not enhance the kill rate. The effects of electric fields and ultraviolet light were not tested. Electric fields are excluded from the exposure area by the design of the apparatus. Ultraviolet light from our discharge was measured previously and was found to be very low compared with ultraviolet light from a mercury discharge tube.

Interest and research efforts in the field of atmospheric pressure nonthermal plasma have grown significantly during the past decade. The need for robust sterilization techniques that preserve surface integrity in the field of biological decontamination has only helped the cause.[1–4] Atmospheric pressure nonthermal plasma discharges are known to

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facilitate enhanced levels of ionization without the need for high temperatures of operating gas.[5] Recently, Alexeff, Laroussi, and their colleagues[6–10] demonstrated the use of a direct current (DC) steady-state atmospheric pressure RBD. The RBD has proven to be particularly successful on *E. coli, Pseudomonas fluorescens* (5RL), spores, and bacteriophages.[11]

Research claims regarding the primal role of inactivation factors such as ultraviolet (UV) light, heat, charged ingredients, and radicals in a plasma discharge varies. One of the study of the effectiveness of UV photons and oxygen atoms by Selwyn et al and Moisan et al account for such a disparity.[3,12] Previous studies by Laroussi[13] and Moisan et al[14] have quantified some of the effects of these factors through analytical and experimental methods. There has been a considerable amount of research that focuses on how inactivation agents such as UV, heat, free radicals, and charged ingredients bring about cell lysis in a sterilization cycle. Articles about plasma sterilization mechanisms and modeling such as those by Moisan et al[14] refer to the synergistic role of the agents. However, fewer publications have collectively analyzed the level of significance of all the agents and their interaction effects involved in an atmospheric pressure RBD. The advantage of an RBD over competing techniques is that it uses simple high voltage DC or alternating current and easily can be scaled up to very large units. Dielectric barrier discharges require a radio frequency power supply.

Our article attempts to establish the significance levels of 3 primary factors in a atmospheric pressure resistive plasma discharge: hydrogen peroxide, charged ions, and air (oxygen compounds such as ozone) in their roles as inactivation agents in an atmospheric plasma discharge. Our goal—to estimate significance levels of agents and study the nature of their interaction effects—was achieved by Design of Experiments, a popular statistical tool well suited for parameter selection and optimization. Factorial designs allow for the simultaneous study of the effects that several factors may have on a process. When performing an experiment, varying the levels of the factors simultaneously rather than one at a time is efficient in terms of time and cost and allows for the study of interactions between the factors. Interactions are the driving force in many processes. Without the use of factorial experiments, important interactions may remain undetected. ANOVA and regression analysis were then used to analyze and interpret the experimental dataset. ANOVA is a statistical test scheme to detect the differences in means of a set of factors as a whole at a chosen level of probability, similar to a *t* test. This article is organized into sections II, III, and IV that describe the discharge apparatus, experimental description, and the statistical significance testing and analysis, respectively.

## II. PLASMA DISCHARGE APPARATUS

The plasma discharge apparatus generates significantly large volumes of nonequilibrium resistive barrier plasma discharge at atmospheric pressure. The plasma is generated between 2 planar electrodes using a DC power source (30KV, 10mA) or with a low-frequency alternative current (120 V, 60 Hz) fed through a neon-sign step-up transformer

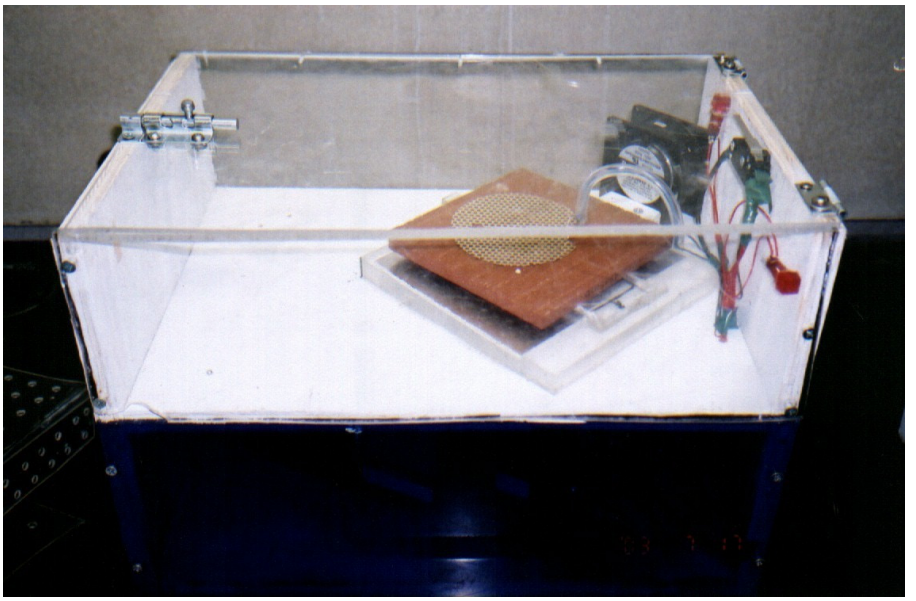

**FIGURE 1.** Direct current steady-state plasma discharge apparatus.

(output voltage of 15 kV across secondary terminals at 60 mA). Figures 1 and 3 illustrate the experimental design set up of the plasma reactor, which consists of 2 electrodes: a bottom wire mesh and a top electrode that usually rests on a highly resistive, wetted, unglazed ceramic barrier (20 × 20 × 1 cm). The ceramic is cooled and rendered conductive by using either water or hydrogen peroxide (30%), while ensuring no contact with the inoculated agar test plates. The working medium in the plasma reactor is air and traces of water vapor/hydrogen peroxide. The resistive barrier (ceramic tile) with a resistance of 1 Meg-Ohm prevents the diffuse discharge from contracting into an arc. A nonconducting frame separates the 2 electrodes, creating an air gap of ¼ inch and allowing for the placement of test plates during plasma treatment. The plasma sterilizer houses the electrode arrangement in an upper section of plastic containment, and the lower compartment of the containment houses the high-voltage circuitry, as shown in Fig. 1. A filamentary discharge with air or a diffuse discharge with helium gas can be achieved by applying a high voltage between the 2 electrodes. Figure 2 depicts a plasma reactor that can typically generate up to 1800 $cm^3$ of helium plasma.

## III. EXPERIMENTATION

### A. Cell Culture

Frozen stocks of *E. coli* were cultured in 100 mL of Luria Bertani growth medium for 4–6 hours at 37°C in an incubator equipped with a shaker. *E. coli* exposure samples

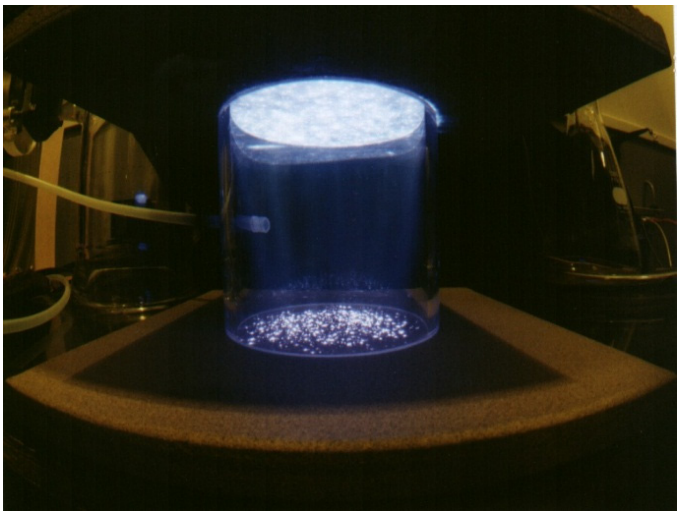

**FIGURE 2.** Helium plasma.

for the bacterial decontamination experiments were prepared by inoculating 100 μL of cell suspension containing approximately $4 \times 10^6$ cells/mL in the growth medium onto Luria Bertani–agarose plates. The concentrated cell suspension was diluted (1 to 10 serial dilution), such that it yielded about 100–150 colony-forming units (CFUs) after an overnight incubation, to facilitate manual counting. Each experimental run involved the preparation of triplicate samples of inoculated agar plates (Petri dishes) followed by plasma exposure with varying time intervals. The configuration of specimen plates is shown in Fig. 1. The plates then were allowed to age overnight in an incubator alongside with the untreated plates (control plates). The *E. coli* cells on the control plates were provided the exact same growth conditions and temperature as the treated plates. The ratio of CFUs of plasma in the treated and untreated samples yielded the percentage kill rate in each case. The temperature variations inside the discharge chamber and on the surface of the test specimens (inoculated agar plates) were monitored using a radiation thermometer before and immediately after the discharge. The temperature was within 2 to 4°C of room temperature, thus rendering it insignificant in the context of contributing to the kill rate of microbes.

## B. Test Parameters

All bacterial samples were exposed to an atmospheric pressure plasma discharge, with a specific power of 100 W, in a gas medium of air and residue from water. The residue is from hydrogen peroxide in experiments where the water-cooled ceramic resistive barrier is replaced with a hydrogen peroxide–cooled barrier electrode. The discharge products (charged ions, ozone, and residue from water/hydrogen peroxide) typically reach the surface of the specimen through diffusion. The specimen plates are usually

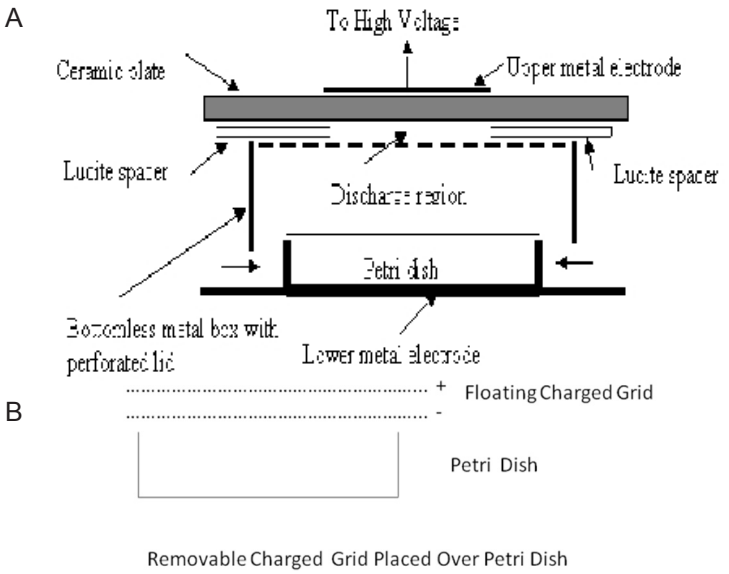


**FIGURE 3.** A: Schematics of the sterilizer configuration. B: Removable charged grid placed over the Petri dish.

placed directly below the region of discharge, as in Fig. 3, during plasma exposure. The electrostatic filter tests for the presence of charged ions. The Petri dish is away from the discharge region below a grounded mesh because of high-voltage circuit constraints. The experiment relied on plain diffusion of ozone and other species (positive and negative ions) produced by plasma onto the samples and did not use a forced draft system. The percentage ratio of the viable *E. coli* CFU count on the treated sample to that on the untreated sample was plotted against the exposure time along the horizontal axis. The experiments also included negative controls to monitor any external decontamination in the inoculation plates (medium plates).

## IV. STATISTICAL SIGNIFICANCE TESTING

### A. Screening Design

The sterilization runs were based on a 2-level full factorial screening design with 3 factors: air, charged ions, and hydrogen peroxide. The 2 levels consisted of "high/on," indicating presence of the corresponding factor, and "low/off," indicating a block of the corresponding factor. The factorial design–based testing provides a method to isolate or mask the effect of the chosen factors without changing the test environment. In addition, unlike most analytical methods, it does not necessitate expensive testing equipment. The effect of hydrogen peroxide is masked by replacing the electrode coolant with water. A simple double metal mesh connected to a high voltage (400 V) serves as an electrostatic

**TABLE 1.** Analysis of Variance for Reduction (%) in Count of Colony-Forming Units (*Escherichia coli*)

| Source | DF | Seq SS | Adj SS | Adj MS | F | *P* |
|---|---|---|---|---|---|---|
| Blocks | 2 | 301.8 | 301.83 | 150.91 | 0.52 | 0.606 |
| Main effects | 3 | 6669.9 | 6669.87 | 2223.29 | 7.65 | 0.003 |
| 2-Way interactions | 3 | 2175.8 | 2175.8 | 725.27 | 2.5 | 0.102 |
| 3-Way interactions | 1 | 13 | 13.04 | 13.04 | 0.04 | 0.835 |
| Residual error | 14 | 4069 | 4068.96 | 290.64 | | |
| Total | 23 | 13229.5 | | | | |

filter to actively filter out charged ions generated in a plasma discharge. The discharge apparatus was flushed with nitrogen gas at the rate of 10 L/min, which removes traces of oxygen-based compounds for experimental instances, wherein the effect of the "air" factor needed to be blocked. The factorial design accounts for the isolated/main effect terms for each factor as well as interaction terms. All the terms of the design are free from aliasing. The experimental design tests all possible combinations of the factors. The 3 factors are the predictor variables in the model. The response variable is the percentage kill rate obtained from the CFU count. The kill rate for each instance of factor combination is the mean from 6 experimental runs. Every experimental run included 3 replicates distributed in a random order within the run. The replicates also were used as one of the blocks in the model.

## B. Whole Model Fit

The main intent of the surface plasma treatment experiment was to analyze the nature of effects of the 3 factors. The main effects in the model refer to the isolated effects of hydrogen peroxide, charged ions, and oxygen compounds. In addition, the model also studies the 2-way interactions between the 3 factors. The model fitting and ANOVA were performed using JMP (SAS, Inc, Cary, NC) and Minitab (Minitab, Inc, State College, PA) statistical packages. The main effects of the model are significant, as shown in Table 1, where $P = 0.003$ ($<0.05$) at a 95% confidence level. The overall 2-way interactions for the whole model, however, were found to be significant only at a much lower confidence level (90%). The sequential sum of squares or type I test values corresponding to the main effects and 2-way interactions support the high statistical significance. The 3-way interactions among the factors show a significant lack of fit. The "blocks" variable representing the replicates has a lack of fit, meaning there is no significant difference in the measured means in the case of different replicates.

**TABLE 2.** Estimated Effects for Significant Factors

| Term | Estimate | Standard Error | *t* Ratio | *P* > \|t\| |
|---|---|---|---|---|
| Intercept | 85.800417 | 3.045934 | 28.17 | <0.0001 |
| CI[off] | –10.67292 | 3.045934 | –3.50 | 0.0022* |
| Air[off] | –12.78875 | 3.045934 | –4.20 | 0.0004* |
| CI[off]*air[off] | –9.390417 | 3.045934 | –3.08 | 0.0059* |

*
CI, charged ions.

**TABLE 3.** Sequential (Type I) Sum of Squares Table

| Source | Nparm | DF | Seq SS | F Ratio | *P* > F |
|---|---|---|---|---|---|
| CI | 1 | 1 | 2733.8676 | 12.2779 | 0.0022* |
| Air | 1 | 1 | 3925.2510 | 17.6285 | 0.0004* |
| CI*Air | 1 | 1 | 2116.3182 | 9.5045 | 0.0059* |

*
CI, charged ions.

## C. Reduced Model Fit

Table 2 represents the ANOVA results, which dictate that charged ions, air, and the 2-way interaction effects between the 2 factors be significant. It also provides an estimate of the intercept and coefficients of the predictor variables. As expected, the sequential sum of squares, as shown in Table 3, were found to be high for the 2 significant factors and one 2-way interaction compared with the rest of the factors.

The regression equation below explains the relationship between the factors and the measured percentage kill rate.

**Y**(% Kill rate) = 85.8004166666667 + Match( :CI, "Off", -10.6729166666667, "On", 10.6729166666667, . ) + Match( :Air, "Off", -12.78875, "On", 12.78875, . ) + Match( :CI, "Off", Match( :Air, "Off", -9.39041666666666, "On", 9.39041666666666, . ), "On", Match( :Air, "Off", 9.39041666666666, "On", -9.39041666666666, . ),.)

The equation clearly summarizes the relationship between the percentage kill rate corresponding to "on" (not masked) states for the agents charged ions and air.

## D. Significant Effects

The half normal plot in Fig. 4 is a graph of the absolute magnitude and the statistical significance of both the main and interaction effects. The plotted line indicates where the points would fall if all the effects were zero. The line separates the significant factors on the right from the insignificant ones on the left. The factor air, marked as "C" and plotted farthest to the right of the line, has the highest statistical significance in the model, followed by charged ions ("B") and the 2-way interaction ("BC"). Among the main effects

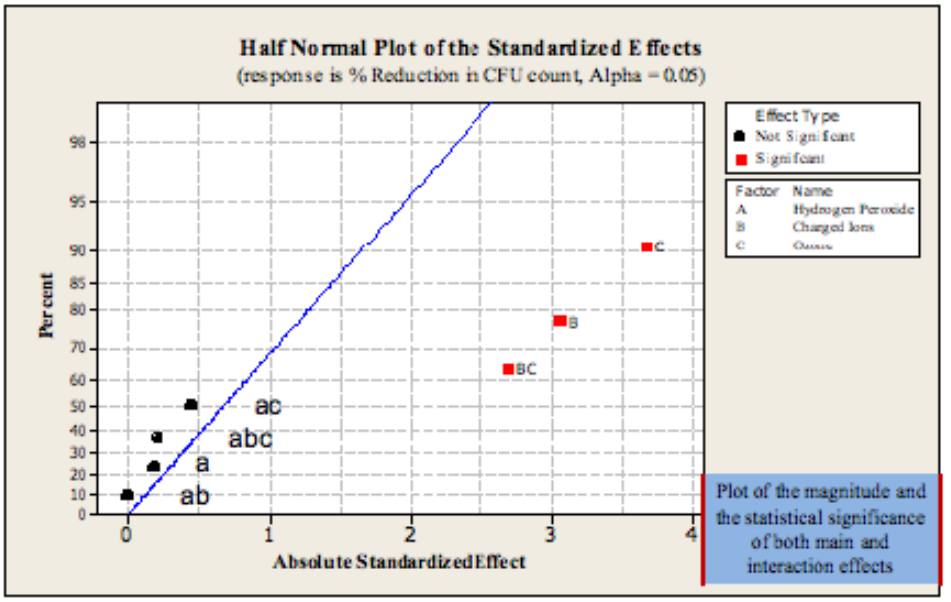


**FIGURE 4.** Half normal effects plot.

in the model, air is the most significant, closely followed by charged ions. The measured percentage kill rate corresponding to the combination of the factors and the levels are plotted in Fig. 5. The standard deviation of the kill rate in relation to the factors is recorded in Fig. 6. Both the graphs are consistent and clearly denote a high level of kill rate with low variability only in the case of significant factors. Thus, the results are consistent with the interpretations from the ANOVA. The summary of nature of model fit is addressed in Table 4. The $R^2$, which represents the proportion of variation in data accounted by the model, is approximately 66%. Another notable feature is a closely fol-

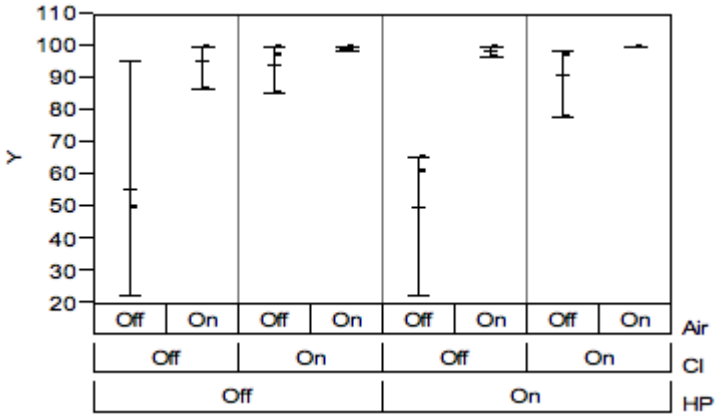


**FIGURE 5.** Variability gauge chart for percentage kill rate. CI, charged ions; HP, hydrogen peroxide.

**TABLE 4.** Summary of Model Fit

| | |
|---|---|
| $R^2$ | 0.663362 |
| Adjusted $R^2$ | 0.612866 |
| Root mean square error | 14.92197 |
| Mean of response | 85.80042 |
| Observations (summed weights) | 24 |

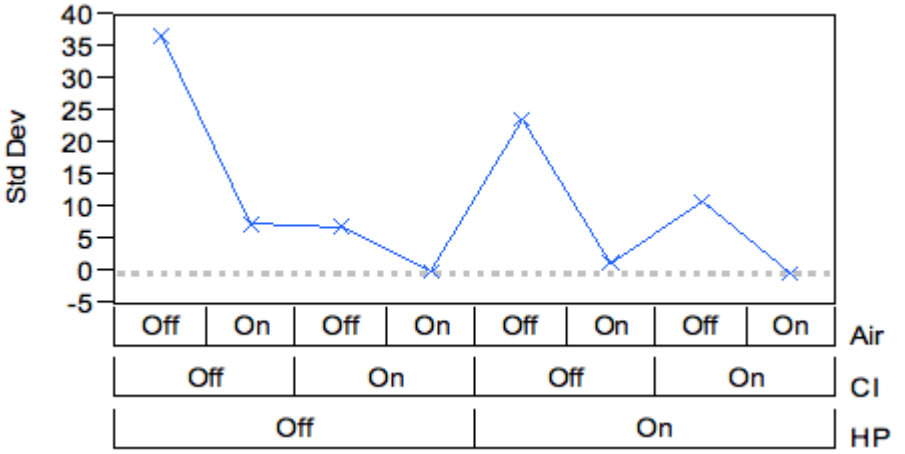


**FIGURE 6.** Standard deviation plot for inactivation factors. CI, charged ions; HP, hydrogen peroxide.

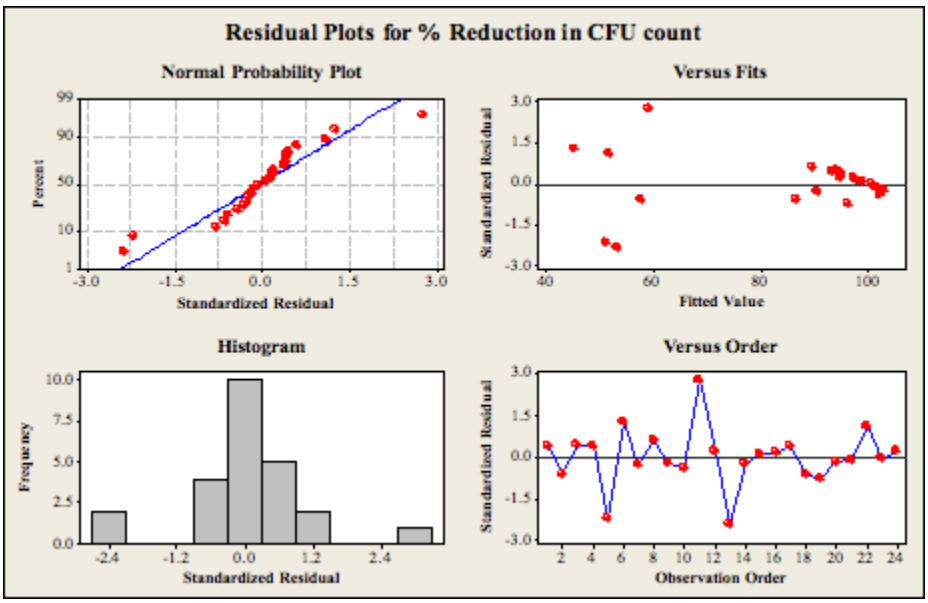


**FIGURE 7.** Measure of residuals fit. CFU, colony-forming unit.

lowed adjusted $R^2$ value of 61%. Lack of large separation between the 2 values indicates a good model fit. The normal probability plot, the histogram of the residuals in Fig. 7, shows no evidence of nonnormality. Considering the relatively small dataset, there is no evidence of skewness in the normal probability; however, there are a couple of outliers on the tail of the distribution. The histogram of the distribution of residuals resembles a normal distribution, thus indicating normality in the data. The normality tests of the residuals are not essential in the context of the experimental design, but the normality of the residuals becomes absolutely imperative when the model is being used to predict or optimize the levels of factors. In this case, the residuals have been analyzed to obtain an enhanced understanding of the model.

## V. SUMMARY AND CONCLUSIONS

Plasma discharges have been used increasingly in a wide array of applications, ranging from surface modification to biological decontamination. It has proven particularly effective in the sterilization of bacteria, lambda phages, and spores within few minutes of treatment of the plasma surface. An enhanced understanding of the nature of the inactivation agents would help to optimize discharge systems to meet increasing sterilization demands in the medical arena. Analytical experimentation with a strong statistical base could be the key to unlocking crucial interaction effects that could otherwise go unnoticed. This article attempts to establish the level of significance of inactivation agents such as charged ions or reactive species, air (oxygen-based compounds), and hydrogen peroxide in an atmospheric pressure RBD through a structured experimental design based on Design of Experiments. To this effect, a 2-factorial ($2^3$) screening design was used to estimate the effects of the inactivation agents. Results of ANOVA and regression analysis of surface plasma treatment confirmed the statistical significance of charged ions, air, and the interaction between the 2 factors to be statistically significant in atmospheric pressure plasma sterilization. The ANOVA shows that hydrogen peroxide seem to be only as effective as water, possibly because the discharge converts both water and hydrogen peroxide into some more effective agent.

Statistical techniques such as Design of Experiments, ANOVA, and regression analysis have proven to be useful tools to extract meaningful information from experimental data. In the case of the atmospheric pressure RBD, as shown in Fig. 4, there is evidence to support the claim that charged ions and air are statistically significant factors. Comparatively, hydrogen peroxide seems to play a much lesser or indirect role in the sterilization process. Interestingly, in addition to the main effects, results suggest the presence of an interaction effect between charged ingredients and oxygen-based compounds

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