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A Study on combined effect of Methylene blue and Sodium anthraquinone-2- sulphonate on inactivation efficiency of Escherichia coli and Enterococcus hirae

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Abstract : In this study, the effect on photoinactivation efficiency of photosensitizers MB and SAQS is studied when applied together for inactivating *Enterococcus hirae* and *Escherichia coli* employing statistically valid full factorial design. Photo inactivation efficiency of MB and SAQS in combination and at pH 9.0 varied in the range of 32.87% - 49.50% for *E. hirae* and in the range of 27.02% - 37.06% for *E. coli*. Statistical analysis of the photo-inactivation results in the form of analysis of variance (ANOVA) and student't' test revealed significant individual effect of MB on *E. hirae* inactivation but no significant effect on *E. coli* whereas SAQS had no significant effect on both *E. hirae* and *E. coli* inactivation.
Keywords: Photo inactivation, photo sensitizer, methylene blue (MB) and sodium-2-anthraquinone sulphonate.

1. Introduction

Tertiary treatment of wastewater effluents and disinfection of drinking water is the prerequisite in developing nations because contamination with microorganisms leads to several water borne diseases like diarrhoea, typhoid, cholera etc. [6]. Several disinfection techniques like chlorination, ozonation and UV treatment are currently in use but they have their own set of drawbacks. Chlorination is associated with formation of trihalomethane's (THM's) and haloaceticacids (HAA) as disinfection by products with carcinogenic and mutagenic effects on mammals [8] [9] whereas UV and ozone forms a different set of toxic disinfection by products [7].

Among the processes currently under development, photosensitization can be a promising technology for disinfection of wastewater. It requires a chemical compound which in presence of light and oxygen produces highly reactive oxygen species, such as superoxide ion, hydroxyl radical or singlet oxygen. Studies on photodynamic inactivation of bacteria and viruses have been reported in early 1960's [12].

Other research groups have also studied different aspects related to photosensitization process in detail for better understanding of inactivation of microorganisms such as the effect of concentration of photosensitizer [1], effect of cell density [5], pH of the photo sensitizer [3], photo sensitizer charge [2] and type of light and irradiation parameters [4][10]. From these reports it can be concluded that there is strong relation between these parameters and inactivation of microorganisms.

The present work is focussed on combined effect of photo sensitizer's methyleneblue (MB) and sodium-2- anthraquinone sulphonate (SAQS) on photo dynamic inactivation of *E. coli* and *E. hirae*.

2. Materials and method

2.1 Chemicals and reagents

Photo sensitizers (PS) MB, SAQS and analytical reagent dihydrochlorofluorescin diacetate (DCFDA) were purchased from Sigma Aldrich India. The other chemicals NaCl, KCl, Na_2HPO_4, KH_2PO_4 and growth medium nutrient broth, brain heart infusion broth and agar were all purchased from Merck India.

2.2. Microorganisms and culture conditions

Bacterial strains of *Escherichia coli* (MTCC 1610) and *Enterococcus hirae* (MTCC 3612) were procured from IMTECH, Chandigarh, India. *E. coli* and *E. hirae* were cultured using nutrient broth and brain heart infusion broth respectively at 37°C, 180 rpm for 24 hours.

2.3. Inactivation experiments

Batch experiments were conducted in order to study the combined effect of PS on inactivation of microorganisms; experiments were planned as per full factorial design for the concentration of PS MB and SAQS. The low and high levels of the PS are taken as 0.73 µmol/l and 1.25 µmol/l respectively from the previous batch study conducted in our laboratory. The study had revealed positive effect of alkaline pH and higher dilution on the inactivation efficiency of microorganisms. Hence, in our present study we have taken pH = 9.0 and 1000 times dilution of the bacterial suspension.

Inactivation experiments were carried out by transferring 1ml, 24 hour grown culture in 1.5 ml eppendorf tube and centrifuging the biomass at 10,000 g for 10 minutes. The pellets obtained were washed twice with phosphate buffer saline (PBS) of pH 9.0 and re- suspended in itfollowed by serial dilution up to 1000 times. One set of PBS suspended 1000 times diluted bacterial suspension added with a combination of MB and SAQS from their respective 1mM stock solution as per the design (Table 1)were kept in dark as dark control. The other set added with the dyes were kept under dark condition on a gel rocker for 30 minutes with constant shaking and later exposed to a light of intensity 1500 lux (measured by a digital luxmeter) for 10 minutes using two 6 W tube light and two 6 W UV-A tube lights in a closed chamber.Photo sensitized bacterial suspension (10µl) was then spread on brain heart infusion agar plates and nutrient agar plates for *E. hirae* and *E. coli* respectively and incubated at 37°C for 24 hours. The experiments were carried out in triplicates and viable cells in the culture plates were enumerated by colony counting method.

Table 1 Combination of photo sensitizers and their levels used in the photo-inactivation experiments

Experimental run no.	Coded levels of the variables	
	MB	SAQS
1	-1	-1
2	-1	+1
3	0	0
4	+1	-1
5	+1	+1

Note: -1 corresponds to 0.73µmol/l concentration, 0 corresponds to 0.99µmol/l and +1 corresponds to 1.25µmol/l.

The percentage inactivation of microorganisms from each duplicate runs in the study was calculated as per the following equation and the results shown are average of two values:

$$\%\ inactivation = \frac{C_i - C_f}{C_i} \times 100 \qquad (1)$$

where, C_i and C_f are the initial and final viable cell counts.

Statistical analysis in the form of analysis of variance (ANOVA) and student 't' test was carried out to validate the roles played by different parameters and their interactions on the bacterial photo-inactivation. All these statistical analyses were performed using the software MINITAB (version 16, PA, USA).

2.4 Measurement of Reactive Oxygen Species (ROS)

The bacterial cell suspension were prepared in pH 9.0 phosphate buffered saline and diluted 1000 times and were added with 20µl DCFDA (dihydrochlorofluorescin diacetate) of 20µM concentration and incubated for 30 minutes at 37°C, then the suspension was added with both the photosensitizer as described in Table 1 and kept in dark on a gel rocker for 30 minutes. Later, it was shined by visible and UV-Alight using two 6W tube light each.

10 µl of the suspension after light period was spread on agar plates for viable cell count and 1 ml of the suspension was checked for DCF (2, 7 dichlorofluorescin) fluorescence by exciting at 488nm and emission spectra studied over 510 to 540 nm using Fluoromax 4 [11].

3. Results and Discussion

3.1. Inactivation of *E. hirae* and *E. coli*

Photo inactivation efficiency of MB and SAQS in combination and at pH 9.0 varied in the range of 32.87% - 49.50% for *E. hirae* and in the range of 27.02% - 37.06% for *E. coli*(Fig. 1). An increase in inactivation efficiencies of PS (MB and SAQS) is observed in case of both the microorganisms when compared with individual inactivation efficiencies of the PS [11].

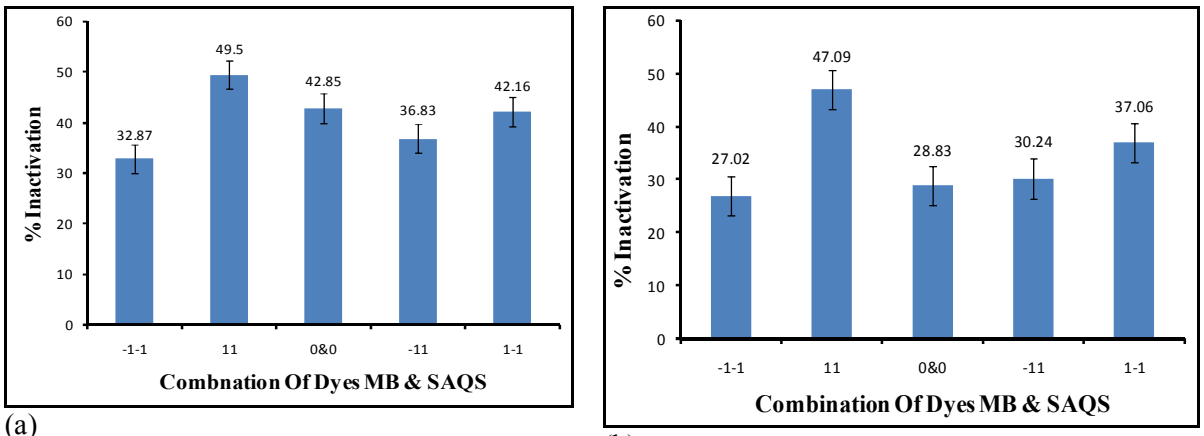


Fig. 1.Photo-inactivation of *E. hirae* and *E. coli* obtained in the different experimental runs using MB and SAQS together at pH = 9.0, 1000 dilutions and 30 minutes dark incubation period (a) *E. hirae* (b) *E. coli.*

This increase in inactivation efficiency can be accounted due to the increase in reactive oxygen species (ROS) production. MB and SAQS are known to produce ROS when illuminated with light and inactivate microorganisms as also deduced from our previous study but when both the PS are mixed together it was expected that the inactivation efficiency will increase significantly as compared to individual PS and will be near to the cumulative efficiency by individual PS whereas the observed results were only slightly higher (1.5% - 2%) than with individual PS.The low increase in inactivation may be due to hindrance or interaction between the PS.

Both the PS were added to the same 15 ml bacterial suspension so the total concentration of PS in each sample was double the concentration when added individually in previous study.The duration of light is kept constant for 10 minutes but the intensity has decreased from 2700 lux to 1500 lux, so insufficient light may be a cause for less increase in inactivation efficiency.

3.2 Statistical analysis

For a better understanding of the combined PS effect on the inactivation of these two bacteria statistical analysis of the results in the form of analysis of variance (ANOVA) and student 't' test was performed.

In Table 2, which presents ANOVA of photo-inactivation results obtained for combined effect of PS, the high Fischer's 'F' value and a low probability 'P' value of the regression model indicates its validity in explaining the variations in the results. Further, the results suggest that individually MB has a significant effect. Accuracy and precision of the models, in the form of determination coefficient (R^2), adjusted R^2, standard deviation (SD) and predicted residual error sum of squares (PRESS) shown in Table 2a & 2b, suggest that the models were average in predicting the experimental photo-inactivation results.

Table 2 ANOVA of viable cell count at the end of the inactivation at 30 minutes dark incubation period with MB and SAQS (a) *E. hirae* (b) *E. coli*

(a)

E. hirae						
	F	**P**	**R^2**	**R^2 Adj**	**SD**	**PRESS**
Main effects	9.86	0.004	67.69	54.76	24.901	13578.4
Two-way interaction effects	0.09	0.769				

(b)

E. coli						
	F	**P**	**R^2**	**R^2 Adj**	**SD**	**PRESS**
Main effects	1.68	0.236	29.58	1.41	37.424	29511.6
Two-way interaction effects	0.24	0.637				

Table 3 Student '*t*' test of the regression coefficients of photo-inactivation of *E. hirae* and *E. coli* using MB and SAQS together.

(a)

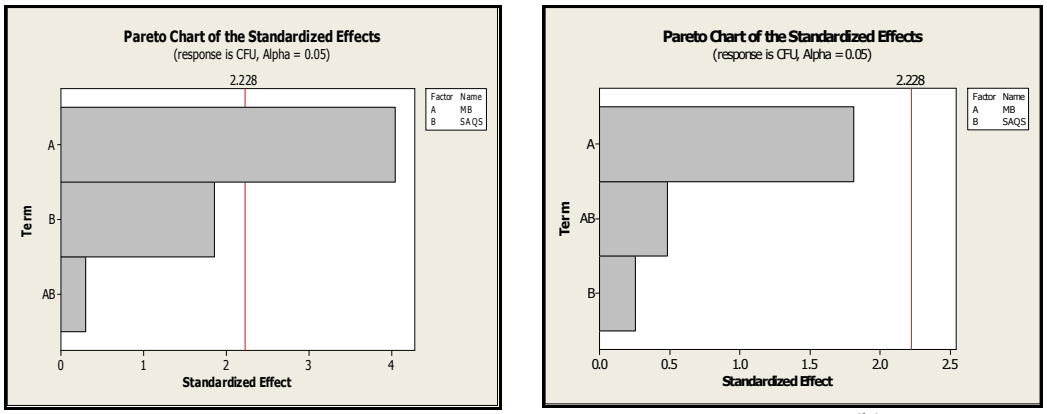
E. hirae			
Term	**Coeff.**	**T**	**P**
Constant	7.188	48.67	0.000
MB	7.188	-4.03	0.002
SAQS	7.188	-1.85	0.093
MB & SAQS	7.188	-0.30	0.769

(b)

E. coli			
Term	**Coeff.**	**T**	**P**
Constant	10.80	30.99	0.000
MB	10.80	-1.81	0.100
SAQS	10.80	-0.25	0.804
MB & SAQS	10.80	0.49	0.637

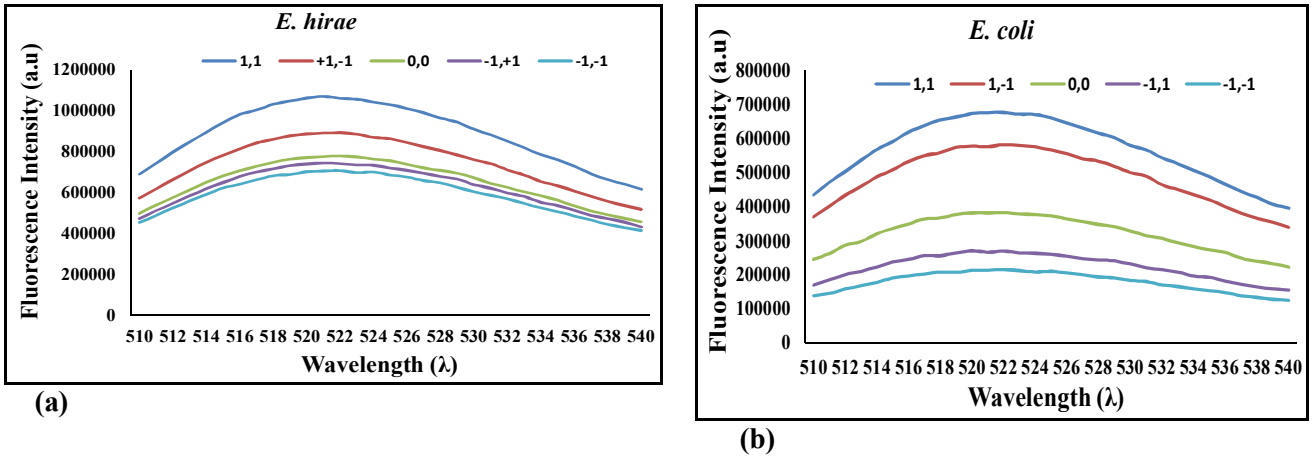
The estimated coefficients of individual and interaction effects between the variables, presented in Table 3, as well confirmed these results. Table 3a indicates a highly significant effect of MB concentration (P<0.05)for *E. hirae* but no significant effect for*E. coli* (Table 3b) inactivation however, the other individual and interaction effects were found insignificant.

All these results of effect of PS on the photo-inactivation of *E. hirae* and *E.coli* are depicted in a better way in the form of pareto charts and are illustrated in) Fig. 2. Horizontal bars in these charts represent effects (i.e. individual and interaction terms) of the parameters and the effects which extend past the reference line (vertical line on the chart) denote the significant ones (α = 0.05).



(a) (b)

Fig. 2.Pareto chart showing the effect of MB and SAQS on photo-inactivation of (a) *E. hirae* (b) *E. coli.*



(a) **(b)**

Fig. 3. Fluorescence curves for different experimental run with both the dyes (a)*E. hirae* (b) *E. coli*

3.3 Reactive oxygen species (ROS) production.

ROS is measured as explained in section 2.4 and the results revealed that experimental run with maximum concentration of both the dyes gives (Fig.3a and 3b) the maximum fluorescence in bacterial suspension and hence it can be concluded that maximum ROS is generated in this experimental run. ROS data

when (fig.3) compared to viable cell count data (fig.1) shows direct relationship between inactivation and ROS production.

4. Conclusion

The results obtained in this study revealed that there is an increase in inactivation efficiency of both *E. hirae* and *E. coli* when MB and SAQS are used together as compared to when used individually. Statistical analysis of the results revealed that MB has significant effect in the case of Gram positive *E. hirae* whereas the effect is insignificant for gram negative *E. coli* (due to the presence of external lipopolysaccharide coat). It also revealed that other individual and interaction effect were insignificant.

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