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Effect of ozonisation against the microorganisms of lime fruits

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Abstract
The effectiveness of ozone treatment was tested on the lime fruits and packed in the two different size of the packaging (25 µ and 50 µ) and the packed samples were stored with and without holes to check the effectiveness of packaging film in a refrigerated space at 10 °C temperature for 150 days. The microbial population of total plate count, yeast and mould count, *E. coli* and *Salmonella* of lime fruits was also assessed at a fifteen days interval during the storage period. The ozone treated lime fruits with 3 min of ozone gas application at flow rate of 400 mg/h and packed in 25 µ with holes (1 pinpoint hole per 2 × 2 square inch area of plastic bag) was found the most effective in preserving the lime fruits up to 120 days.

Keywords: Ozone treatment, microorganism, lime fruit, packaging, storage

1. Introduction
Post-harvest losses of fruits & vegetables are about the great concern for the society. There are many factors responsible for post-harvest losses of the fruits & vegetables; they are able to develop food diseases. Microbes are responsible for the predominant post-harvest diseases that cause severe post-harvest losses and affect food quality during the supply chain. Microorganisms do not only responsible for post-harvest losses but also responsible for human diseases. Raw fruits & vegetables have been known to serve as vehicles of human disease for at least a century. The centre for Disease Control and Prevention (CDC), Atlanta, USA estimates that there are 76 million cases of food borne illness each year [1]. The microbial floras availed on the fresh fruits & vegetables are from several external sources such as soil, air, water, fertilizers, animals and humans.
The increasing demand for sanitation as a means for controlling infection and diseases in food and the need for reducing the emission of polluting substances have made researchers search for safe and new sanitizing methods. Ozone has proved to be suitable for this purpose. Ozone is a highly reactive form of oxygen where three molecules are bonded together. Generated electrically on-site where needed, it has potent antimicrobial activity and other characteristics. Interest in ozone applications for agriculture and food processing has increased in recent years (EPRI Expert Panel, 1997). In 2001, ozone was declared a GRAS (generally recognized as safe) substance by the FDA [2].
Because ozone is a safe, powerful disinfectant as well as the strongest commercially available oxidant, it can be used to control biological growth of unwanted organisms in products and equipment used in the food processing industries. Ozone is particularly suited to the food industry because of its ability to disinfect microorganisms & pests without adding chemical by-products to the food. Again, ozone is 52% stronger than chlorine, thus when bubbled through water, it provides a more effective sterilization than chlorine without any residual taste or smell [3].
Ozone has been reported to not only successfully deactivate pests as well as microflora and mycotoxin [4, 5, 6]. The ozonisation process has been categorized into phase-I and phase-II. During phase-I, ozone reacts with the active sites of microorganisms, and once all sites are saturated, ozone concentration gradually increases (phase-II) to level lethal for target organisms [7]. The length of these two phases is affected by the number of microbes, quantity of fruits / vegetables / grains, ozone flow rate, initial concentration of ozone, temperature and moisture content [8].
Hence, to control the microbial load at the farm as well as APMC such an experiment is considered worthy for farmers as well as consumers. The objectives of this study were to find out the strength of microorganisms before and after the ozonisation treatment, to find out

effective packaging materials for ozonised lime fruits and evaluate the effect of ozone treatments with different time exposure & packaging materials against the microbes on lime fruits.

2. Materials and Methods
2.1 Materials
In this experiment, the lime fruit samples were obtained from the local farms of Junagadh district. These limes were placed in LDPE plastic bag (25 µ and 50 µ) treatments as per the treatment combinations after ozone treatment and the microbial analysis was carried out at periodical time interval.

2.2 Experimental procedure
Lime fruits were subjected to the ozone treatment at three levels of exposure time (O_1-1 min, O_2-2 min and O_3-3 min, respectively) with the ozone flow rate of 400 mg/h. The treated fruits were pace inside two different types of packaging films P_1 (25 µ) and P_2 (50 µ), respectively and this packaging were subjected to have 1 pinpoint hole on 2 × 2 square inch area of plastic bag (treatment H_1). In another treatment H_2, packed fruits were stored without any holes in packaging. All the packed fruits were stored at 10 °C temperature. A control treatment included the lime fruits to be stored at 10 °C without any ozone treatment in loose condition without placing in any packaging film. All the samples were analysed for the different microbial populations at 15 days of intervals.

2.3 Microbial analysis of lime fruits
Standard procedure for analysis of microbial flora on the surface of lime fruits were carried out at 15 days of interval [9]. The homogenate from sample preparation in distilled water were used for the following procedures: Total plate counts on N-agar plate, yeast and mould counts on Potato Dextrose Agar (PDA) medium, growth of E. coli on EMB agar plate growth of Salmonella on Salmonella-Shigella agar (SS) medium was recorded and calculated from 15 to 150 days using the following equation:

$$\text{Total Colony Count (CFU/g)} = \frac{\text{Colony count on agar plate}}{\text{Total dilution of tube} \times \text{amount plated on a plate (0.1 ml)}}$$

2.4 Statistical analysis
Experimental design consists of three independent variables i.e., ozone treatment (min) at 3 levels, packaging film (µ) at 2 levels, Holes in packaging at 2 levels each with total 12 treatment combinations. A Factorial Completely Randomized Design (FCRD) will be used for designing the experiment trials. All the treatments will be replicated twice for two different years for the experimental analysis.

2.5 FSSAI standards
Microbial limit for minimally processed Fruits and vegetables as per the Food Safety & Standards Authority of India (FSSAI) were kept in a consideration for the assessment of microbial limits and optimum shelf-life of lime fruits. The maximum load for the total plate count (TPC) and Yeast and Mould Count (YMC) are 1×10^6 cfu/g and 1×10^3 cfu/g, respectively. *E. coli* and *Salmonella* should be absent in samples [10].

3. Results and Discussion
3.1 Total plate count (TPC)
The total plate count of treated lime fruits was measured from 15 days to 150 days. The safe limit of total plate count for fruits as per the FSSAI guidance were remained till 120 days of storage. Total plate count was under the prescribed limit while at 150 days of storage, the bacterial count was observed over the prescribed limit as per the FSSAI. In lime fruits, average TPC data in treatment no. T_2, T_3 & T_{10} were found to be 14.92×10^4 cfu/g, 14.72×10^4 cfu/g and 11.15×10^5cfu/g respectively at 120 days (Table 1). During the storage, total plate count was decreased as the time for the ozone treatment was increased. The ozone treatment had positive correlation with the total plate count. The ozone treated sample packed in 25 µ bags had comparatively lower microbial load than the fruits stored in 50 µ bags. The total plate count was also lower in the lime stored in the packaging films with the square holes. After this, the bacterial load was found more than the prescribed limit in all the treatments.

Total plate count in lime fruits, after 120 days of storage, were revealed that individual parameters like ozone treatments, plastic packaging materials as well as no. of holes on plastic packaging materials are statistically significant and the minimum bacterial count was found in O_3, P_1 and H_1. In two factors interaction effect between ozone exposure time and type of packaging material, ozone exposure time and no. of holes on plastic packaging materials, type of packaging material and no. of holes on plastic packaging materials were also found statistically significant. In three factors interaction effect, ozone exposure time, type of packaging material and no. of holes on plastic packaging materials were also found statistically significant. The minimum bacterial count was found in $O_3P_1H_1$ treatment. The second next good treatment is $O_2P_1H_1$.

3.2 Yeast and mould count (YMC)
The analysis of yeast and mould counts were analyzed from 15 days to 150 days (Table 2). It was found that the lime fruit remained good up to 120 days of storage. No fungal count was observed in treatment T_2, T_3, T_8 and T_9 till 150 days. The fungal count was over the prescribed limit given by the FSSAI in treatment no. T_{10} which is poor treatments amongst all treatments.

The yeast & mould count in lime fruits revealed that individual parameters like ozone treatments, plastic packaging materials as well as no. of holes on plastic packaging materials were statistically significant and the minimum fungal count was found in O_3, P_1 & H_1. In two factors interaction effect between ozone exposure time and type of packaging material, ozone exposure time and no. of holes on plastic packaging materials, type of packaging material and no. of holes on plastic packaging materials were also found statistically significant. In three factors interaction effect, ozone exposure time, type of packaging material and no. of holes on plastic packaging materials were also found statistically significant. No fungal count was found in $O_2P_1H_1$, $O_3P_1H_1$, $O_2P_2H_1$, $O_3P_2H_1$ treatment, respectively.

3.3 *E. coli* and *Salmonella*
The lime fruits in all the treatments were found to have absence of *E. coli* and *Salmonella* even after 150 days of cold storage. No sight of both *E. coli* and *Salmonella* were found, which is satisfactory as per the FSSAI standards in all the treated samples.

3.4 Economic analysis of lime fruits
Economic analysis of storage of lime fruits was carried out considering the cost of packaging materials, treatment cost

and storage cost. The treatment no. T_3 ($O_3P_1H_1$) & T_2 ($O_2P_1H_1$) were found similar in terms of reducing microbial load in lime fruits. Cost economics was analysed between T_3 & T_2 in lime fruits (Table 3). Good Incremental Cost Benefit Ratio (ICBR) of lime fruits was obtained 1:4.61 at 60 days of storage for treatment T_3. However, the healthy lime fruits were obtained up to 120 days of storage (75-80 kg).

Table 1: Average total plate count ($\times 10^4$ cfu/g) of surface microbial flora of lime fruits from 0 days to 150 days

Sr. No.	Treatments	15 days			30 days			45 days			60 days			75 days		
		2017	2018	Mean	2017	2018	Mean	2017	2018	Mean	2017	2018	Mean	2017	2018	Mean
T_1	$O_1P_1H_1$	0	0	0	0	0	0	0	0	0	0.069	0.01	0.085	0.01	1.4	1.21
T_2	$O_2P_1H_1$	0	0	0	0	0	0	0	0	0	0	0	0	0.23	0.5	0.365
T_3	$O_3P_1H_1$	0	0	0	0	0	0	0	0	0	0	0	0	0.3	0.35	0.325
T_4	$O_1P_1H_2$	0	0	0	0	0.015	0.075	0.035	0.05	0.043	0.091	0.133	0.113	1.875	2.25	2.062
T_5	$O_2P_1H_2$	0	0	0	0.029	0.065	0.047	0.05	0.065	0.0575	0.098	0.125	1.11	1.59	1.95	1.77
T_6	$O_3P_1H_2$	0	0	0	0.019	0	0.095	0.035	0	0.0175	0.079	0.09	0.0845	1.285	1.65	1.467
T_7	$O_1P_2H_1$	0	0	0	0	0.025	0.013	0	0.04	0.02	0.073	0.09	0.087	1.42	1.60	1.51
T_8	$O_2P_2H_1$	0	0	0	0	0	0	0	0	0	0.029	0.05	0.039	0.585	0.95	0.767
T_9	$O_3P_2H_1$	0	0	0	0	0	0	0	0	0	0.013	0.025	0.0193	0.325	0.5	0.412
T_{10}	$O_1P_2H_2$	0	0	0	4.05	11.5	7.775	7.95	9.5	8.725	14.6	19	16.8	2.75	2.4	2.575
T_{11}	$O_2P_2H_2$	0	0	0	3.7	11	7.35	7.6	11	9.3	15.9	17.5	16.7	3.125	3.05	3.087
T_{12}	$O_3P_2H_2$	0	0	0	0.0315	0.02	0.02575	0.07	0.03	0.05	0.0108	0.0125	0.0117	1.715	2.9	2.307
T_{13}	Control	8	12	10	18	16.5	17.25	28.9	34.5	31.7	36.65	61	48.82	550	605	577.5

Sr. No.	Treatments	90 days			105 days			120 days			135 days			150 days		
		2017	2018	Mean	2017	2018	Mean	2017	2018	Mean	2017	2018	Mean	2017	2018	Mean
T_1	$O_1P_1H_1$	18.3	21	19.67	25.15	30.5	27.82	32.6	36.5	34.55	3650	4650	4150	6040	7000	6520
T_2	$O_2P_1H_1$	6.1	7.5	6.8	9.9	12	10.95	13.85	16	14.92	1700	2450	2075	3435	3550	3492
T_3	$O_3P_1H_1$	6.1	6.5	6.3	10.45	8.5	9.47	13.95	15.5	14.72	1100	1700	1400	31206	3800	3460
T_4	$O_1P_1H_2$	31	25.5	28.25	47.15	51	49.07	63.5	57	60.25	8900	9150	9025	10840	9400	10120
T_5	$O_2P_1H_2$	24.8	28	26.4	40.95	43.5	42.22	51	53	52	7550	7950	7750	10140	9700	9920
T_6	$O_3P_1H_2$	22.35	28	25.17	34.8	41	37.9	45.65	59	52.32	6900	6550	6725	8970	7200	8085
T_7	$O_1P_2H_1$	26.95	23.5	25.22	38.95	34	36.47	52.8	45	48.9	6050	6500	6275	7145	7650	7397
T_8	$O_2P_2H_1$	10	8.5	9.25	15.2	19.5	17.35	24.05	23.5	23.77	3800	3650	3725	6155	6200	6100
T_9	$O_3P_2H_1$	6.75	11	8.87	10.15	13.5	11.82	17.4	20	18.7	2300	2600	2450	3700	3650	36820
T_{10}	$O_1P_2H_2$	49.9	54	51.95	71.25	77.5	74.37	98	125	111.5	14000	13000	13500	17400	16500	16950
T_{11}	$O_2P_2H_2$	43.85	46	44.92	57.55	64.5	61.02	81.45	83.5	82.47	9950	9150	9550	12525	13300	12912
T_{12}	$O_3P_2H_2$	26.25	33.5	29.87	45.2	53.5	49.35	70.45	75.5	72.97	9100	10050	9575	12775	14400	13587
T_{13}	Control	Fruits were deteriorated and destroyed.														

Table 2: Average Yeast & Mould Count ($\times 10^2$ cfu/g) of the surface microbial flora of lime fruits from 0 days to 150 days

Sr. No.	Treatments	15 days			30 days			45 days			60 days			75 days		
		2017	2018	Mean	2017	2018	Mean	2017	2018	Mean	2017	2018	Mean	2017	2018	Mean
T_1	$O_1P_1H_1$	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T_2	$O_2P_1H_1$	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T_3	$O_3P_1H_1$	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T_4	$O_1P_1H_2$	0.135	0.2	0.167	0.21	0.3	0.255	0.34	0.6	0.47	0.59	0.9	0.75	8.3	13	10.65
T_5	$O_2P_1H_2$	0	0	0	0	0	0	0.125	0.15	0.137	0.215	0.35	0.282	4.15	7	5.57
T_6	$O_3P_1H_2$	0	0	0	0	0	0	0	0	0	0	0	0	1.65	2.5	2.07
T_7	$O_1P_2H_1$	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T_8	$O_2P_2H_1$	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T_9	$O_3P_2H_1$	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T_{10}	$O_1P_2H_2$	0.34	0.65	0.495	0.62	0.9	0.76	0.98	1.4	1.19	1.561	2.05	1.807	21	24.5	22.75
T_{11}	$O_2P_2H_2$	0.235	0.35	0.292	0.42	0.65	0.535	0.635	1.05	0.842	0.785	1.3	1.042	12.95	13	12.97
T_{12}	$O_3P_2H_2$	0.19	0.4	0.295	0.315	0.4	0.357	0.59	0.85	0.72	0.79	1.15	0.97	10.3	16	13.15
T_{13}	Control	6.1	8	7.05	0.995	1.6	1.3	1.49	2.1	1.792	1.92	2.95	2.432	210	225	217.5

Sr. No.	Treatments	90 days			105 days			120 days			135 days			150 days		
		2017	2018	Mean	2017	2018	Mean	2017	2018	Mean	2017	2018	Mean	2017	2018	Mean
T_1	$O_1P_1H_1$	0	0	0	1.25	0	0.62	1.35	1	1.17	20	30	25	30	55	42.5
T_2	$O_2P_1H_1$	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T_3	$O_3P_1H_1$	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T_4	$O_1P_1H_2$	12.8	14.5	13.65	18	22	20	27.3	29.5	28.4	50	40	45	45	105	75
T_5	$O_2P_1H_2$	6.85	9	7.925	9.45	13.5	11.475	11	14.5	12.75	25	35	30	60	95	77.5
T_6	$O_3P_1H_2$	6.05	6	6.02	10	9.5	9.75	12.3	12.5	12.4	25	20	22.5	40	155	97.5
T_7	$O_1P_2H_1$	0	0	0	0	0	0	2.15	4	3.07	35	65	50	45	90	67.5
T_8	$O_2P_2H_1$	0	0	0	0	0	0	0	0	0	0	0	0	0	10	5
T_9	$O_3P_2H_1$	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

T_{10}	$O_1P_2H_2$	34.6	9	21.8	44.05	42.5	43.27	55.3	60	57.65	70	85	77.5	115	90	102.5
T_{11}	$O_2P_2H_2$	19.4	22.5	20.95	23.05	27	25.02	31.4	39.5	35.45	55	50	52.5	55	80	67.5
T_{12}	$O_3P_2H_2$	14.1	20	17.05	22.5	25	23.75	28.3	32.5	30.4	45	60	52.5	75	125	100
T_{13}	Control	Fruits were deteriorated and destroyed.														

Table 3: Economics of best treatment (T_3- $O_3P_1H_1$) for lime fruits

Sr. No.	Treatments	Cost in Rs. / 100 Kg			Total Cost of Treatments (Rs.)	Healthy Limes were obtained after Storage Period	Price of Healthy Limes (Rs.)	Net Gain over Control (Rs.)	ICBR	Remarks
		Packaging Materials Cost (Rs.)	Treatment Cost (Rs.) (Labour charges + Electricity charges etc.)	Storage Cost (Rent) (Rs.)						
T_2	$O_2P_1H_1$	50/-	300/-	300/- (60 days)	650 /-	80 kg (60 days)	4,000 /-	2,500/-	1: 3.85	-
T_3	$O_3P_1H_1$	50/-	300/-	300 /- (60 days)	650 /-	90 kg (60 days)	4,500 /-	3,000/-	1: 4.61	ICBR was calculated on 60 days of storage. However, the healthy lime fruits were obtained up to 120 days of storage (75-80 kg).
T_{13}	Control	-	-	300 /- (60 days)	300 /-	30 kg (60 days)	1,500 /-	-	-	-

Note:
1. Packaging Material Cos t= Rs. 10 / 20 kg Plastic bag
2. Storage Cost = Rs. 1.50 / kg / month
3. Price of Limes after 60 Days = Rs. 50 /kg

4. Conclusion

The lime fruits were treated with the ozone gas for different time and packed in different types of packaging film with or without holes to assess the microbial population during the storage. The microbial limits of total plate count, yeast and mould count, *E. coli* and *Salmonella* were in the safe limit till 120 days of storage. The treatment $O_3P_1H_1$ in which the ozone treatment for 3 min at flow rate 400 mg/h and packed in 25 µ plastic bag having 1 pinpoint hole per 2 x 2 square inch area and kept for storage at 10 °C temperature remain safe against the microbial load up to 120 days and obtained 75-80% healthy lime fruits after storage. The economic analysis of two best ozone treated lime fruits ($O_3P_1H_1$ and $O_2P_1H_1$) was carried out for the treatment. It was found that treatment $O_3P_1H_1$ had good cost benefit ratio which showed better economic return for the storage up to 60 days.

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