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EVALUATING THE EFFICACY OF EDTA, GRAPEFRUIT SEED EXTRACT, LYSOZYME AND SODIUM BENZOATE INCORPORATED IN STARCH-GLYCEROL BASED ANTIMICROBIAL FOOD PACKAGING FILM

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ABSTRACT

The consortia of micro organisms obtained from contaminated food (Milkfed Verka Kheer) was effectively tested against antibacterial agents, i.e. Sodium benzoate, grinded grape fruit seed powder GSP, Lysozyme and EDTA by agar diffusion method. Bacterial inhibition by combinations using two levels from each of the three factors (EDTA, Sodium benzoate and Lysozyme) without AM films was evaluated using liquid incubation method. The levels of the agents were selected according to their permissibility standards. Statistical analysis of experimental data for their antimicrobial spectrum was carried out by multi regression analysis and framed poly-quadratic equation using coded factors and percentage contribution of antimicrobial agents was determined using Design-Expert software. Properties such as thickness, opacity, transparency, UV absorbance and efficient working pH of the film were also determined. The best result was observed with EDTA: Sodium benzoate: Lysozyme at 100 mM: 100 mM: 1000 IU respectively at pH 9, where maximum zone of inhibition was observed that is 21mm. No zone of inhibition was observed using GSP as antibacterial factor limiting its widely supported usage. The work screened was imperative in performing optimization studies for the combination treatments to incorporate in starch-glycerol based active packaging film.

Keywords: Active Packaging, Antimicrobial Activity, Food Preservation, Response Surface Methodology

INTRODUCTION

Active packaging has been defined as a type of packaging that molds the packaging condition to extend shelf life and improve safety or sensory properties while maintaining the quality of the food (**Jin *et al.*, 2008**). There can be many categories for active packaging techniques in order to preserve and improve quality and safety of food. These are absorbers or scavengers for O_2, CO_2, moisture, ethylene and odor; releasing systems for CO_2, antioxidants, flavors, ethylene, antimicrobial agents and preservatives. Antimicrobial (AM) packaging materials are designed to extend the lag phase and reduce the growth rate of microorganisms to prolong the shelf life and maintain food quality and safety (**Han, 2000**). Diffusion between the packaging material and the food and partitioning at the interface are the main migration phenomena. AM food packaging is one of the special applications of active food packaging that controls inside food and atmospheric conditions actively and responsively (**Muhamad, 2005**).

Ethylenediaminetetraacetate (EDTA) acts as a hexadentate ligand and chelating agent. It has been widely used in cell lysis protocol as EDTA facilitate the detachment of cells from biofilm and enhances the killing of biofilm-producing microorganisms by depriving Mg^{2+} associated with lipo-polysaccharides and depriving Ca^{2+}, and Fe^{2+}, which are essential factors for microbial growth (**Banin *et al.*, 2006**). Chelation of the cell membrane results in the release of the LPS (lipopolycassharide) from the Gram negative bacteria (**Ko *et al.*, 2008**). EDTA can act as a food additive as it is generally recognized as safe. The average usage concentration is 100 to 300 parts per million. EDTA is being widely used in food industry to perform listed functions: sequestering metals, preventing discoloration of potato products, stabilizing vitamins, preventing discoloration of fish and shellfish and preventing flavor changes in milk (**Aamoth *et al.*, 1960; Furia, 1964**). Chelating agents are observed to be nontoxic to many forms of life on acute exposure (***Lanigan et al., 2002***).

Grapefruit Seed extract (GSE) is a commercial product derived from the seeds of grapefruit (*Citrus paradisi* Macf, *Rutaceae*). GSE is commonly reported to have powerful antimicrobial, antifungal, antiviral and antiparasitic properties. Its capability to treat various diseases like eczema, acne, thrush, cold sore, gastric infection and ulcers is been reported (**Ionescu *et al.*, 1991; Tirillini, 2000; Heggers *et al.*, 2002; Reagor *et al.*, 2002**). It contains large quantities of polyphenolic compounds and antioxidants (**Saito *et al.*, 1998; Shoko *et al.*, 1999; Armando C *et al.*, 1998**). It has been found that the extract can disrupts the bacterial membrane within 15minutes. The in vitro investigation by **Krajewska-Kulak *et al.* 2003** showed that the commercial 33% grapefruit- water glycerol solution exerted potent antifungal activity against the yeast-like fungi strains. GSE products, 33% water-glycerol solutions are also widely used as naturopathic remedies, natural foodstuff supplements, disinfectant and sanitizing agents as well as preservatives in food and cosmetic industry. Efficiency of the ethanolic extract of GSE was also checked against 20 bacterial and 10 yeast strains (**Cvetnic *et al.*, 2004**). In recent reports, artificial agents, such as benzethonium chloride, triclosan and methyl parabene, were also identified to be incorporated in commercially available products (**Takeoka G *et al.*, 2001; Sakamoto S *et al.*, 1996**).

Antimicrobial activity of lysozyme is primarily described by the lysis of the enzymes present in the cell wall of the microorganisms (**Hughey *et al.*, 1987**). Lysozyme functions by attacking peptidoglycans (found in the cell walls of bacteria, especially Gram-positive bacteria) and hydrolyzing the 1,4 glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine. Lysozyme has been widely used to preserve fresh fruits and vegetables, tofu bean curd, sea foods, meats and sausages, potato salads and varieties of semihard cheeses. The application of lysozyme has been implemented in clinical usages in the treatment of periodontitis to prevent tooth decay and also been administered in chewing gums **(Proctor and Cunningham 1988)**. It has also been administered to patients suffering from cancer for its analgesic effect and has been used as a potentiating agent in antibiotic therapy (**Verhamme *et al.*, 1988; Le Moli *et al.*, 1986**). Their usage in AM based starch film has also been reported (**Khairuddin *et al.*, 2009**).

Sodium benzoate is bacterio-static and fungi-static under acidic conditions. At higher concentrations (2-10 mM) it enters the yeast cell in the undissociated form, and its neutralization within the cell cause a shift of the pH of the intracellular water by more than 1 pH unit. Sodium benzoate also inhibited the production of phosphofructokinase in glycolysis resulting in downregulation of ATP and thus restricting growth (**Krebs *et al.*, 1983**). It is most widely used as preservative in acidic foods such as salad dressings (vinegar), carbonated drinks, jams and fruit juices, pickles, and condiments. Sodium benzoate is also used in antiseptics, tobacco and as lubricant. In industry sodium benzoate is acting as corrosion inhibitor for antifreeze products. Permissible limit as food preservative is in the range of 2000 mg/kg of food (**Chipley, 1983**).

Glycerol is hygroscopic by nature which has ability to absorb the moisture from atmosphere. Glycerol is used in pharmaceutical industry as plasticizer, humectants, solvent and lubricant. It was observed that its use in the food packaging and in intimate contact with food beverages cannot be a source of contamination. Glycerol is nontoxic and sweet in nature. It aids in increasing the mechanical properties of the starch based antimicrobial film (**Miner, 1953**).

Statistical methods had proven a promising aspect in optimizing the particular processes by considering the mutual interactions among the variables chosen and give an estimate of combined effect of these variables on final interpretation. Factorial designs are also used primarily for screening significant factors, but can also be used sequentially to model and refine a process (**Chauhan *et al.*, 2006**). Response Surface Methodology (RSM) is a collection of statistical and mathematical techniques useful for developing, improving, and optimizing processes. The field of response surface methodology consists of the experimental strategy for exploring the space of the process or independent variables, empirical statistical modeling to develop an appropriate approximating relationship between the yield and the process variables, and optimization methods for finding the values of the process variables that produce desirable values of the response (**Axerio *et al.*, 2010**).

In the present context, MIC of chosen antimicrobial agents were determined experimentally and application of factorial designs and RSM is envisaged using user-friendly software package Design-Expert® 8.0.7.1 software. The system response is antimicrobial activity and the system variables are antimicrobial agent's concentration of EDTA, Lysozyme and Sodium Benzoate at different liquid incubation period. The optimization method involves estimating coefficients in a mathematical model, predicting the percentage contribution of each factor to the response and checking the adequacy of the model. The keen objective of the present study was also to determine starch-glycerol based AM packaging film properties such as thickness, opacity and to screen the efficient working of the film by incorporating antimicrobial agents with different combinations of EDTA, Grapefruit Seed Extract, Lysozyme and Sodium Benzoate by Agar diffusion bioassay.

MATERIALS AND METHODS

Microbial source

The commercial food sample, Milkfed Verka Kheer was allowed to get contaminated from the open environmental sources for2 days. After serial dilution, spread plate technique was employed and confluent layer of cells were grown on petriplates containing Starch media and an aliquot of consortia was screened by gram staining (**Gram, 1884**), for Methyl Red (**Clarke, 1941**), Indole (**MacFaddin, 1980**) and Catalase (**Clarke *et al.*, 1952**) test. Culture was maintained in Starch broth (0.5 % peptone, 0.3 % beef extract, and 0.2 % starch) at 4 °C.

Antimicrobials agents

Disodium EDTA was obtained from Remeck, India. Lysozyme was obtained from Medox, India, Sodium Benzoate was purchased from Loba Chemie and Grinded Grape seed powder was dissolved in sterile water according to the concentration required. Standard stock solutions of 5000 IU/ml lysozyme, 1 M Sodium benzoate and 100 mM EDTA were prepared in 1 X Citrate Buffer.

Starch/antimicrobial agents film preparation

For antimicrobial film preparation, 10.4 % Starch (Sisco Research Lab, India) was dissolved in 20 % ethanol as solvent and heated at 40ºC in water bath. The combination of antimicrobial agents as listed in Table 1 were added to it and desired pH values of 6-9 were set and then 4 % glycerol (Quailkems) was dispersed into it. 250 µl of the film solution prepared from each of the set was casted onto a thin layer plate overlaid with a parafilm® M and allowed to air dry overnight at room temperature. The films were peeled off and placed on agar plates having confluent culture density.

Film thickness and optical measurements

Thickness of the antimicrobial films was determined using a dial thickness screw gauge (micrometer) at five random positions on the casted circular film and the mean thickness was calculated. For optical measurement, each antimicrobial film sample of different pH was placed into spectrophotometer (Shimadzu) and measurements were performed using air as the reference. The light transmittance of the films was scanned from wavelength of 190 to 800 nm. The measurement was done in triplicate and the average of three spectra was calculated. The transparency at 600 nm (T600) was obtained by using this formula (**Han *et al.*, 1997**). **T600=Log %T/ Average Thickness.** The opacity of the films was calculated by the following equation according to the method described (**Gontard and Guilbert, 1994**). **Opacity = absorbance at 500*thickness.**

Bacterial inhibition testing

Bacterial inhibition by combination of different levels of different factors (listed in Table 1) without antimicrobial films was evaluated using a liquid incubation method as described by (**Appendini and Hotchkiss, 2002**) and with antimicrobial films was done by agar diffusion method (**Chung *et al.*, 2003**). In the agar diffusion test, each film sample was placed on the surface of a starch agar plate overlaid with the 1.5 %w/v of agar. The seed density of overlay was approximately 10^6 CFU/mL. The agar plates were incubated at 37 °C for 24 h. Diameters of zone of inhibition around film specimen determines the antimicrobial activity and the diffusivity of agents in each film sample.

Table 1 Level wise distribution of antimicrobial factors incorporated in starch based antimicrobial film and in Starch Broth media for the determination of the inhibitory activities

Factors	Levels	Experimental values	Values for AM Film
EDTA	3	0, 10, 20 mM	20 mM
Lysozyme	3	0, 1000, 5000 IU/ml	2000 IU/ml
Sodium Benzoate	3	0, 100, 1000 mM	20 mM

Microtitre plate assay

For the liquid incubation test, working cultures were prepared by keeping the initial cell concentration at 10^6 CFU/ml and incubating with different combinations of antibacterial agents at 37 °C, 180 rpm and turbidity was evaluated after 1, 2, 4 and 24 h. A Biorad iMark™ 96 well microplate absorbance reader was used to monitor optical density of culture at 600 nm following a 10-s shake cycle to determine MIC. 2^3sets were prepared from stock of 5000 IU Lysozyme, 100 mM EDTA and 1 M Sodium benzoate to evaluate the effects of the three antimicrobials combined in starch broth as total of 300 µl and culture was added to the wells to achieve a final concentration of 2.5 *10^5 CFU/mL with a positive control in which no antimicrobial agent was added and keeping starch broth as blank. The sampling was done in evaluation mode where three plate readings per one of measurement were performed and the average data of their plate readings is generated as a plate data.

Statistical analysis

Antimicrobial experiments on food spoilage causing microbes with different combinations of three factors using liquid incubation method were conducted in triplicate on different days. A general factorial design for three independent variables was used to obtain the combination of values that optimizes the response within the region of three dimensional observation spaces, which allows one to design a minimal number of experiments. The experiments were designed using the software, Design Expert Version 6.0.10 trial version (State Ease, Minneapolis, MN). The medium components (independent variables) selected for the optimization were EDTA, Sodium benzoate and Lysozyme. Regression analysis was performed on the data obtained from the design experiments. The polynomial model equation generated was determined by Fisher's test value and the proportion of variance explained by the model was given by the multiple coefficient of determination. The data was also represented graphically by surface responses showing interactions between two factors, while the other factors were fixed at values at the centre of the domain. Replicates at the centre of the domain in three blocks permit the checking of the absence of bias between several sets of experiments (**Shrivastava *et al.*, 2008**). Values at a significance level of 0.05 was used for experimental design, regression and graphical analysis of the data obtained.

RESULTS AND DISCUSSION

Biochemical characterization of the microbial source and AM packaging film

Consortia of the microorganisms isolated from food spoilage source on screening were found to be positive for Methyl red test, indole test and negative for catalase test. Both gram negative and gram positive microorganisms were observed as rod and cocci shaped in compound microscope. Thicknesses observed by the micrometer screw gauge of the starch-glycerol AM films prepared with EDTA: Sodium benzoate: Lysozyme at 100 mM: 100 mM: 1000 IU respectively at pH 6-9 values were in the range 0.42-0.58 mm (Table 2). The AM films prepared were easy to dispatch from the paraffin wax and it neither roll over nor break even from the edges. All the antimicrobial film blocked the Ultra violet wavelength at 190-300nm as checked under UV Spectrophotometer. Its absorbance was observed in the range of 1-3. The result were in agreement that starch based antimicrobial film have the potential to prevent the food spoilage and retard the lipid oxidation induced by UV in food system **(Bekbölet, 1990)** and has tendency

to keep the food products more perishable. Antimicrobial films developed were colorless at different pH ranges. AM films were also checked for opacity and transparency. With the increase in pH of the films prepared, opacity was found to decrease. The minimum opacity of the antimicrobial film was observed at pH 9 i.e. 0.192. At higher pH values, solubility of the antimicrobial agents is high thus, enabling the formation of a homogeneous film. Therefore, more light penetrates through the film giving lower opacity values **(Hewage and Vithanarachchi, 2009)**. Opacity is inversely proportional to the transparency so the maximum transparency was also observed of the antimicrobial film prepared with pH 9 i.e. 2.97 (Table 2). More the transparency of the AM film better will be the appearance of the actual food texture coated with the AM film. No variation in color of the antimicrobial film was observed even when the pH and different antimicrobial composites were varied.

Table 2 Transparency of the antimicrobial film

pH	Average Thickness (mm)	Log %T	T 600	Absorbance at 500 nm	Opacity
6	0.58 ± 0.016	1.5315	2.6405	0.563	0.325
7	0.588 ± 0.025	0.7634	1.2983	0.568	0.334
8	0.473 ± 0.059	1.3424	2.8381	0.575	0.272
9	0.421 ± 0.019	1.2529	2.9759	0.456	0.192

GSP polar metabolites are not antimicrobial agents

Our results showed that grinded grape fruit seed powder casted in polar solvent for AM film development didn't show any antimicrobial activity with 5%, 10% and 20% concentration (w/v) when performed in triplicates. Other references had proved that 20% grinded grapefruit seed powder extracted using non-polar solvents like methanol and ethanol show the antimicrobial activity on gram positive and negative bacteria. The antibacterial activity had also been observed even in 2.1% (w/v) concentration from the grinded grapefruit seed extracted using non polar solvents. The method of extraction influence the chemical composition of grinded grapefruit seed powder and Benzethonium Chloride component extracted from commercial GSE using chloroform had been proved as the potential antimicrobial agent. **(Wentao *et al.*, 2007; Takeoka *et al.*, 2001; Cvetnic *et al.*, 2004)** The antimicrobial activity checked for GSP in our laboratory environmental conditions with polar solvents were not in agreement with the published results **(Al- Ani *et al.*, 2011; Dike and William, 2004)**. This inferred that food spoilage causing microbes may not be sensitive to the metabolites present in grinded grapefruit seed powder dissolved in polar solvent. Profound results also been stated by researchers that commercial GSE products not containing any preservatives and several self-made preparations failed to show antimicrobial efficacy and concluded that antimicrobial activity being attributed to GSE is merely due to the synthetic preservative agents it contains **(Woedtke *et al*., 1999)**.

Coating of the food samples (bread, apple) with antimicrobial film containing EDTA, Lysozyme and Sodium benzoate

As one of the simplest visualization assay to check for food deterioration, slices of apple without coating gets oxidized within 2 days and show signs of depletion by 6th day but in case of the sliced apple coated with antimicrobial film, firmness retained and oxidation started from 4th day of incubation. This reflects that antimicrobial film increases the shelf life of apple almost double the time period. In second case, the bread piece showed signs of fungal contamination at 3rd day and had appeared brownish in comparison with the coated AM film packaging where bread texture did not change and showed signs of a consumable piece (properties are reflected in tabular form as Table 3; Supplementary Figure 1).

Table 3 Properties reflecting the coating of food slice samples (bread, apple) with antimicrobial film containing EDTA, Lysozyme and Sodium benzoate at 20 mM: 2000 IU: 20 mM respectively

Incubation duration (in days)	Coating of apple with starch and glycerol solution	Coating of apple with AM film containing antimicrobial agents	Coating of bread with starch and glycerol solution	Coating of bread with AM film containing antimicrobial agents
Day 1	Firm Texture	Firm Texture	Firm Texture	Firm Texture
Day 2	Oxidation started	Firm Texture	Firm Texture	Firm Texture
Day 3	Fiber content is oxidized	Firm Texture	Color Change and start of contamination	Firm Texture
Day 4	Lost Fiber content	Oxidation started	Fungal contamination observed	Firm Texture
Day 5	Slice is Deteriorated	Fiber content is oxidized	Fungal contamination outspread	Firm Texture
Day 6	Only Apple Peel remained	Fiber content is further oxidized	Fungal contamination outspread	Firm Texture

Inhibition spectrum of the antimicrobial film of at different pH range by using Agar diffusion method

The agar diffusion test was performed to simulate the test for solid food packaging. Antimicrobial activity of the film was measured in the form of zone of inhibition. The polymer films containing only starch and glycerol as plasticizer act as negative control. The pH affects the viscosity of the solution, changes the degree of ionization of the most active chemicals, thereby changing the activity of the AM agents and had influential impact on the growth rate of target microorganisms **(Han, 2000; Cuq *et al.*, 1995)**. To measure the effect of pH variation on the diffusivity of three combinatorial agents chosen: EDTA, Sodium benzoate and lysozyme at concentration of 100mM: 100mM: 1000 IU, films were prepared with the desired pH range 6-9. All the pH variation studies showed effective zone of inhibition (Table 4, Figure 1) and the maximum antimicrobial inhibition was observed at pH 9. Maximum zone of inhibition observed was 21mm and the ratio of the diameter of inhibition zone to the diameter of the film specimen (11 mm) was 2.90 indicating that antimicrobial agents were easily diffused from starch based film and plasticizer used as negative control didn't exhibit any antimicrobial activity alone (not shown).

Table 4 The ratio of the diameter of inhibition zone to the diameter of the film specimen (11 mm) at different pH range (6-9)

pH of the film	Diameter of zone of inhibition (mm)	Film antimicrobial efficiency
6	27-11=16	2.45
7	26-11=15	2.36
8	28-11= 17	2.54
9	32-11= 21	2.9

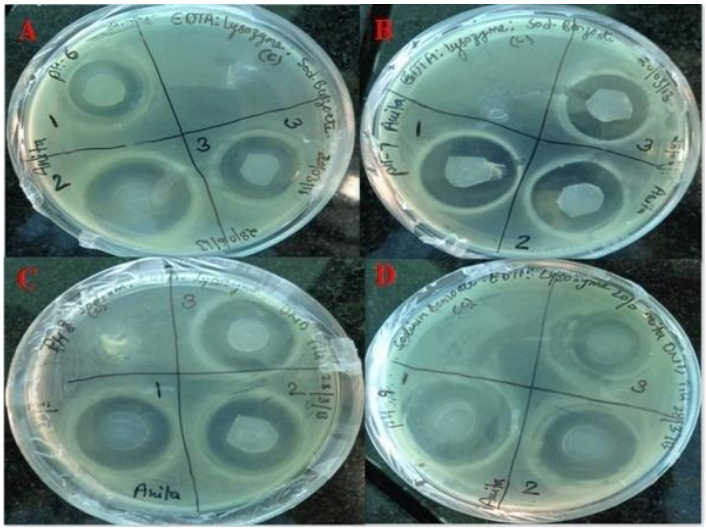

Figure 1 Results of bacterial growth inhibition by agar diffusion method. Inhibition effectiveness of the antimicrobial film of EDTA, Sodium benzoate and lysozyme at 20 mM: 20 mM: 2000 IU at different pH range 6-9, Average diameter observed for A: pH 6, 16 mm. B: pH 7, 15 mm. C: pH 8, 17 mm. D: pH 9, 21 mm.

Statistical analysis of combined effects of EDTA, Lysozyme and Sodium Benzoate on spoilage micro organism in starch media

The spectroscopic results obtained at 600nm were evaluated using Design Expert 8.0.7.1. Antimicrobial responses of combinatorial antimicrobial agents in 2^3 sets (i.e. 2 levels for three factors) (Table 1) were measured at 1, 2, 4 and 24 h of incubation and was represented by a full quadratic model with 7 coefficients for three variables (Table 5) describing interaction terms and relationships between responses and experimental factors to represent the polynomial quadratic equation:

$$\eta j = \beta j_0 + \sum_{i=1}^{n} \beta j_i X_i + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \beta j_{ij} X_i X_j$$

where ηj is the dependent variable; βj_0 is the constant coefficient; X_i are the coded independent variables; βj_i are the linear coefficients and βj_{ii} are the quadratic coefficients.

The percentage contributions of the antimicrobial agents changed with the increase in incubation period (Table 6). At 1 hr, maximum contribution as antimicrobial agent was observed by C (lysozyme) component i.e. 45.41%, but at 2nd hour, the maximum percentage contribution were observed by AC component which were EDTA-Lysozyme association i.e.41.27%. It can be inferred that in 1st hour, lysozyme showed its activity depending on its higher molar concentration and in 2nd hour, its show synergistic effect with EDTA. In 4th and 24th hour incubation the maximum percentage contribution as antimicrobial agent was observed by AB (EDTA and Sodium benzoate) component that was 42.51% and 36.34% respectively reflecting sodium benzoate-EDTA association along with lysozyme-EDTA association (which places as second highest in their percent contribution to antibacterial activity) act in concordance upto 24 hour. Its regression value is 0.96. This means that this model is fully significant at 24 hr.

Table 5 Coefficients of the quadratic model for three variables: sodium benzoate (100mM and 1000mM), lysozyme (1000 and 5000 IU/ml) and EDTA concentrations (100 and 1000 mM) as determined by inhibition assays after incubation at 37 °C for 1, 2, 4 and 24 h

Factors	1 h	2 h	4 h	24 h
Intercept	-0.111	0.382	-0.186	-0.252
A-EDTA	-0.06	0.001	0.058	-0.09
B-Sodium benzoate	0.038	0.012	0.064	-0.046
C-Lysozyme	0.228	0.175	0.098	0.137
AB	0.161	0.007	-0.118	0.205
AC	-0.057	0.186	-0.038	-0.196
BC	0.108	0.002	0.009	-0.035
R^2	0.85	0.85	0.98	0.96

Table 6 Percentage contribution of three antimicrobial agents: sodium benzoate (100mM to 1000mM), lysozyme (1000 and 5000 IU/ml) and EDTA concentrations (100 and 1000 mM) is determined by inhibition spectrum after incubation at 37 °C for 1, 2, 4 and 24 h

Term	% Contribution			
	1 h	2 h	4 h	24 h
A-EDTA	3.11	0.15	10.31	7.02
B-Sodium benzoate	1.27	2.66	12.41	1.81
C-Lysozyme	45.41	38.66	28.99	16.22
AB	22.74	1.51	42.51	36.64
AC	2.85	41.27	4.44	33.5
BC	10.14	0.36	0.26	1.04
ABC	14.49	15.36	1.08	3.77

The three-dimensional (3-D) response surfaces were plotted on the basis of the model equation to investigate the interaction among two antimicrobial variables and to determine the optimum concentration of each factor for maximum antimicrobial activity by liquid culturing. The response surfaces shown in Figure 2 were based on the final model, holding one variable constant at its optimum level, while the other two within their experimental range. The shape of the response surface implies synergistic interaction between EDTA and Sodium Benzoate was stronger than between EDTA and Lysozyme.

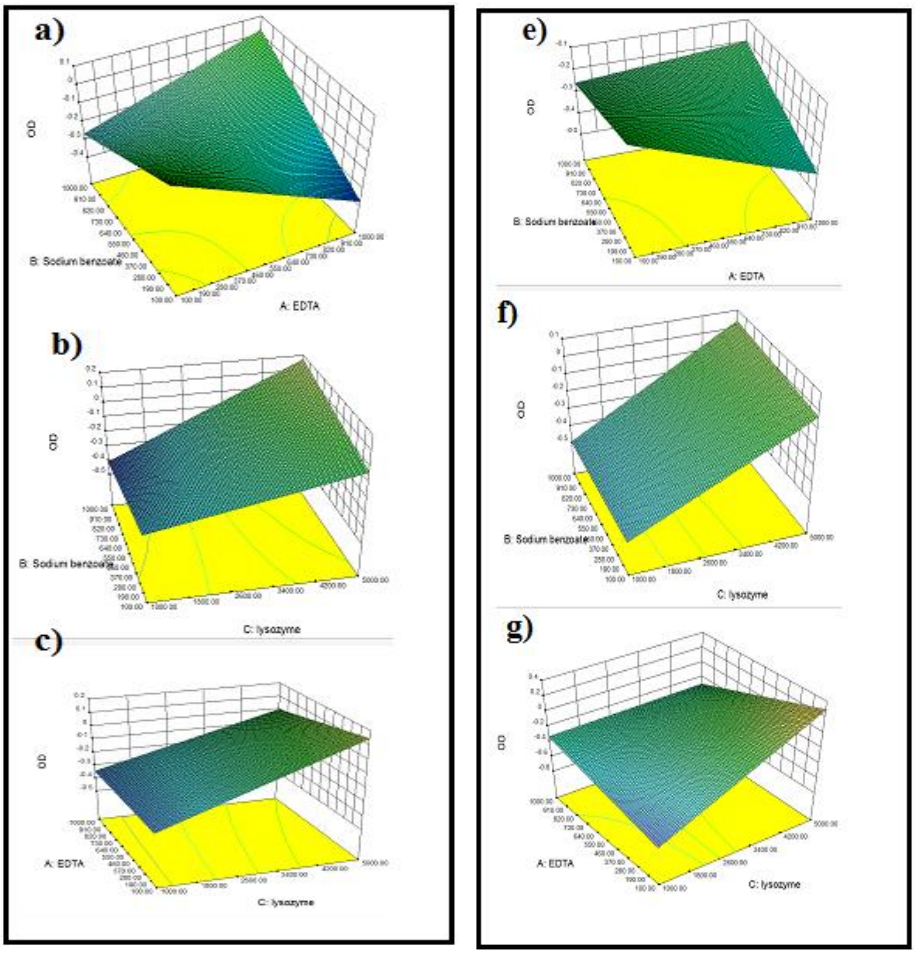

Figure 2.1 Response surface plot at 1 hr and 2 hr respectively: - a, e) effect of antimicrobial agents EDTA and sodium benzoate when lysozyme was at zero level. B, f) Effect of antimicrobial agents sodium benzoate and lysozyme when EDTA was at zero level. C, g) Effect of antimicrobial agents EDTA and lysozyme when sodium benzoate was at zero level.

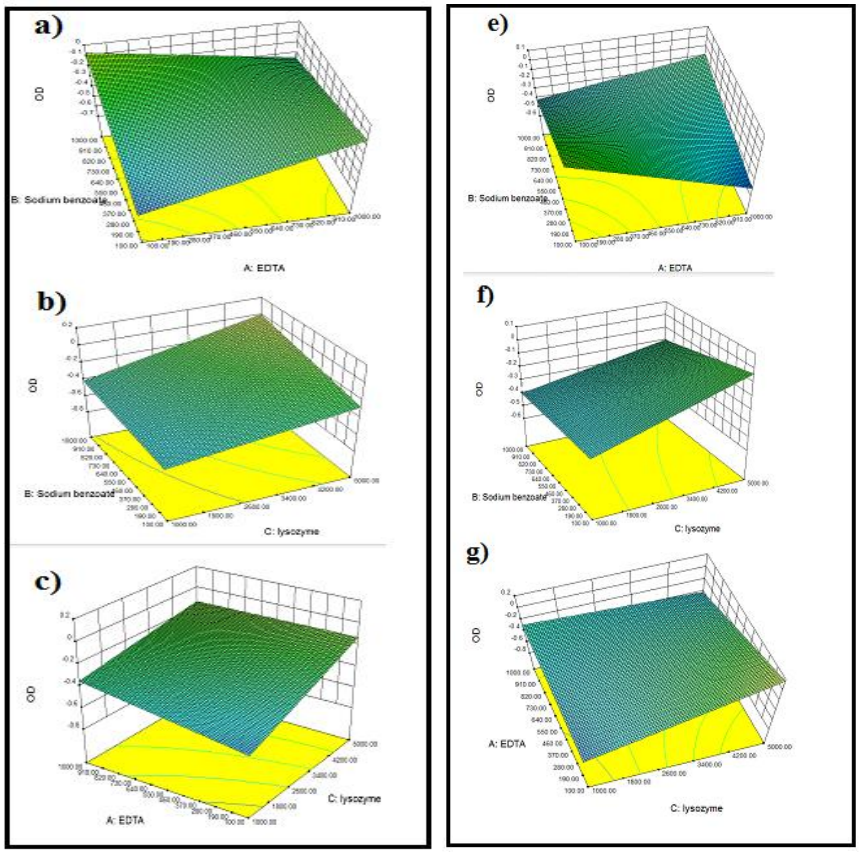

Figure 2.2 Response surface plot at 4 hr and 24 hr respectively: - a, e) effect of antimicrobial agents EDTA and sodium benzoate when lysozyme was at zero level. b, f) Effect of antimicrobial agents sodium benzoate and lysozyme when EDTA was at zero level. c, g) Effect of antimicrobial agents EDTA and lysozyme when sodium benzoate was at zero level.

CONCLUSION

The use of starch in combination with sodium benzoate, EDTA and Lysozyme had a great potential in antimicrobial food packaging to reduce post-process growth of food pathogens and spoilage causing micro-organisms. The purpose of this research is to develop an antimicrobial consumable starch-based film with glycerol as plasticizers to reduce brittleness and to improve the flexibility of active packaging which ensures to prolong the shelf life of food products maintaining its quality and integrity. It was found that the film developed with GRAS AM agents within their permissible limits is effective towards inhibition of food spoilage causing microbes doubling its shelf life. Statistical analysis using Design-Expert software showed that the percentage contribution of the AM agents changed with the increase in incubation period in which lysozyme acts as proactive component and the coefficients obtained can be fit for drafting poly-quadratic equation to optimize the effective combination of the three ingredients used for making biofilm against food spoilage causing micro organisms.

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