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Antimicrobial Activity of Natural Agents against *Saccharomyces cerevisiae*

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Abstract

The antimicrobial (AM) activity of starch-based films coated with linalool, carvacrol or thymol against *Saccharomyces cerevisiae in vitro* and/or inoculated on the surface of Cheddar cheese was investigated. In solid medium using the agar diffusion method and in experiments involving the inoculation of the microorganism on the surface of Cheddar cheese, all the films containing these AM agents in coatings demonstrated an inhibitory effect against *S. cerevisiae*. The results suggest that the overall inhibitory effect of linalool, carvacrol or thymol increased significantly ($p \leq 0.05$) with the concentration of each of the AM agents in the film coating and that the response is linear in the concentration range of 1% to 5% (w/w) of the AM agent. Thymol had the highest AM efficacy followed by carvacrol whereas linalool had the lowest efficacy amongst the three systems. The zones of inhibition in the agar diffusion test method at 25˚C for *S. cerevisiae* were found to be 7.6, 7.1 and 6.1 mm for thymol, carvacrol and linalool at 1% (w/w) loading and 13.2, 12.2 and 11.2 mm at 5% (w/w) loading of the AM agents respectively. The death rates of *S. cerevisiae* on cheddar cheese wrapped in films coated with thymol, carvacrol and linalool and stored for up to 28 days at 15°C were found to be 0.044, 0.043 and 0.038 day^{-1} at 1% (w/w) loading and 0.077, 0.073 and 0.063 day^{-1} at 5% (w/w) loading of the AM agents respectively.

Keywords: biodegradable packaging, antimicrobial packaging, antimicrobial agents, *Saccharomyces cerevisiae*

1 Introduction

To prevent the growth of spoilage and pathogenic microorganisms in food products, various food preservation techniques such as heat treatment, salting, acidification and drying have been used in the food industry [1-4]. In recent years, a rise in the consumer demand for the provision of fresh and natural foods of high quality and extended shelf-life, as well as renewable packaging materials has led to the development of active packaging (AP) technologies [4-11]. An AP system is one in which the product, the package and the environment interact in a positive way to extend shelf-life and improve the microbial safety or sensory properties while maintaining the quality of food products [1, 12-15].

Active packaging technologies can be achieved by the incorporation or coating of antimicrobial (AM) agents into either synthetic or biodegradable polymers [16]. Synthetic polymers derived from crude oil have been extensively used for many years in the food packaging industry. However, most of these materials decompose very slowly in the environment. Recently, interest has increased in the potential use of biodegradable films and coatings manufactured from renewable biopolymers as food packaging materials. A polymeric film mixed with an AM agent may be effective in controlling microbial growth on the surfaces of foods and hence lead to an extension of the shelf-life and/or improved microbial safety of food products [17]. Various biodegradable materials that incorporate natural and/or synthetic AM agents have demonstrated significant inhibitory activity on the growth of various microorganisms such as *S. enteritidis, L. plantarum, B. thermosphaceta B2, and L. monocytogenes, E. coli O157:H7, E. coli, S. aureus, S. typhimurium* [18-20]. Examples of biodegradable materials that have been evaluated for AM packaging systems include yam starch impregnated with chitosan [20], pea starch containing lysozyme [19], corn starch impregnated with potassium sorbate and lactic acid [21], wheat starch-based films incorporated with lauric acid and chitosan [22], whey protein films incorporated with oregano, rosemary and garlic essential oil [23] and calcium alginate and chitosan films immobilised with organic acids [24-26]. The findings of these studies showed that biodegradable polymers incorporated and/or coated with AM agents have a potential for expanding active food packaging applications.

In the present study the effectiveness of the natural AM agents thymol, carvacrol or linalool coated on starch-based films was examined against *S. cerevisiae in vitro*. The study also

evaluated the inhibitory effect of these films against *S. cerevisiae* inoculated on the surface of Cheddar cheese samples. The AM activity was evaluated at three concentrations for each of the AM agents coated onto the polymer films in order to determine the optimum concentration of the AM agent that can be used practically in the preservation of food products.

2 Materials and Methods

2.1 Polymer and Coating Agents

A commercial starch-based film (Biograde-F) supplied by Biograde Ltd., Australia was used in the present study. Biograde-F is a biodegradable material based on a blend of thermoplastic starch, aliphatic polyesters and natural plasticisers. Methylcellulose (MC, 18,804-2); hydroxypropyl methylcellulose (HPMC, 42,321-1) and polyethylene glycol (PEG, 20,236-3) were purchased from Sigma-Aldrich Pty. Ltd., Australia.

2.2 AM Agents

The AM agents were thymol (TO501); linalool (L2602) and carvacrol (W224502) with a purity of 99.5%, 97% and 98% respectively. All of these AM agents were purchased from Sigma-Aldrich Pty. Ltd., Australia.

2.3 Media and Microorganism

The media were malt extract agar (AM 109), malt extract broth (AM 110) and potato dextrose agar (AM 149) all of which were purchased from Amyl Ltd., Australia. The 3M Petrifilm^{TM} yeast and mould count plates (6417), were purchased from 3M Microbiology Products, USA. The microorganism *Saccharomyces cerevisiae* (UNSW 703100) was obtained from the culture collection of the University of New South Wales, Australia. Bacteriological peptone (LP0037) was purchased from Oxoid Ltd., Hampshire, England.

2.4 Film Preparation

A coating solution was prepared from MC and HPMC using the method described elsewhere [27]. Methylcellulose and HPMC were added to absolute ethanol and heated with magnetic stirring on a hotplate. The heating was discontinued when the temperature reached 65°C. During continuous agitation, a mixture of PEG and distilled water was slowly added as a plasticiser to the MC-HPMC dispersion whilst the dispersion cooled. This process resulted in the formation of a uniformly clear coating solution or gel. The AM agent (carvacrol, linalool or thymol) was

then added to the coating solution at one of three different concentrations of 1, 3 and 5% (w/w). The coating medium was applied to the starch-based material using a roller and the film was then dried under ambient conditions. Similarly, a coating solution without any AM agent was prepared and coated onto the substrate for use as the control material.

2.5 Antimicrobial Activity on Solid Medium

The effectiveness of the AM starch-based films on a solid medium was determined using an agar disc diffusion assay in accordance with the method described by Suppakul *et al.* [28]. The yeast culture was maintained at -80°C in malt extract broth (AM 110) containing 30% (w/v) glycerol and was sub-cultured in broth twice before being used. The *S. cerevisiae* suspension of 2.41×10^6 CFU mL^{-1} was prepared in 0.1% (w/v) sterile peptone solution. The AM starch-based and control films were cut into circular discs (6 mm in diameter) and sterilised for 2 min using UV light [29]. The cut pieces were aseptically placed on malt extract agar plates seeded with 0.1 mL of *S. cerevisiae* suspension. The inoculated plates were incubated at 25°C for 48 h. After the incubation, the diameters of the clear zones formed around the film samples were measured using a Vernier calliper and reported as the zone of inhibition. All the experiments were performed in triplicate and data were represented by the mean.

2.6 Preparation and Inoculation of *S. cerevisiae* on Cheese

Cheddar cheese was purchased from a local supermarket and stored at 4°C prior to being used. For the experiments, samples of the cheese weighing 20 ± 1 g each measuring approximately 8 × 6 × 0.5 cm were cut [30, 31]. The samples were divided into four sets in order to study the control film and the three AM coated films containing linalool, carvacrol or thymol. The cheese samples were then sterilised on all sides for 1 h using UV light. The control and the AM films were also sterilised with UV light prior to use. Each of the cheese samples was inoculated on the surface and at the bottom with the *S. cerevisiae* suspension at the level of 10^4 CFU g^{-1} [30]. The inoculated suspension was spread using a sterile glass rod prior to wrapping with the control or AM film. The packaged cheese samples were prepared in duplicate and stored at 15°C for 28 days. Analyses of the cheese samples were carried out immediately after inoculation and periodically during the storage time.

2.7 Analysis and Enumeration

Two packages from each film treatment were opened aseptically on the sampling day. Cheese samples were grated and an 11 g sample of cheese was aseptically transferred to a sterile stomacher bag. A 99 mL aliquot of sterile peptone saline diluent was added to the sample which was then homogenised using a laboratory blender (Seward Stomach® 400, Seward Medical, UK) for 3 min [31]. To obtain a quantifiable colony count for *S. cerevisiae*, 1 mL of each serially diluted sample was plated, in duplicate, on a 3M Petrifilm^{TM} count plate and then incubated for 5 days at 25°C. The colonies were counted and the results were expressed as colony-forming units per gram (CFU g^{-1}). The death curves of *S. cerevisiae* were plotted as a function of storage time.

2.8 Data Analysis

Experiments for each of the AM agents (carvacrol, linalool or thymol) present in the coatings of starch-based films were performed separately. Experiments on solid media were performed in triplicate for each film treatment. For the determination of AM activity of an AM agent on cheddar cheese, each experiment was replicated twice on different days with two observations per film treatment (n = 4). The general linear model procedure and analysis of variance (ANOVA) were used to determine any significant effect of film treatment and AM agent level. Differences amongst the treatments were examined by a least significant differences test at the 0.05 confidence level using SAS (SAS version 9.5, SAS Institute, Cary, NC).

3 Results and Discussion

3.1 Antimicrobial Activity of AM Starch-Based Films on Solid Media

The AM activity of the starch-based films containing the three different AM agents in their coatings was examined by the agar disc diffusion test. Figure 1 shows the AM activity of these films in terms of the clear zones that appear around the samples after 48 h incubation at 25°C. Each clear zone indicates the inhibition caused by the release of the AM agent from the film sample. Numerical values for the zones of inhibition were determined and average values are presented in Table 1. As expected, no inhibitory effect against *S. cerevisiae* growth on agar disc diffusion test was observed in the case of the control film.

The results in Table 1 indicate that the starch-based films coated with thymol were the most effective against *S. cerevisiae* at each of the concentrations tested. The greatest clear zone of inhibition among all samples was achieved in the films coated with 5% (w/w) thymol. The

starch-based films coated with carvacrol also demonstrated a positive AM activity against *S. cerevisiae* on the solid media. The AM activity of carvacrol against *S. cerevisiae* increased significantly ($p \leq 0.05$) with the increase in concentration in the film coating. These observations are consistent with the study of Rupika *et al.* [31] who found that polyethylene-based films containing carvacrol and/or thymol demonstrated significant inhibitory activity against *S. cerevisiae* using the agar disc diffusion assay. The AM activity of starch-based films coated with linalool at 1, 3 and 5% (w/w) were also found to be effective against the growth of *S. cerevisiae* on solid media (see Table 1). In these systems the zone of inhibition increased with the increase in linalool concentration from 1% to 5% (w/w). The data indicate that the inhibitory effect of the films containing linalool against *S. cerevisiae* on the solid medium is significantly less than that of the films containing thymol in all cases.

In order to assess the relative effectiveness of the three natural AM agents, the zone of inhibition data in Table 1 were plotted against the concentration of AM agent as shown in Figure 2. The effectiveness response of each of these systems to an increase in concentration was found to be linear over the tested concentration range. However, it also appears that the response is non-linear in the concentration range between zero and 1% (w/w) of AM agent in each case as demonstrated by the non-zero axial intercept of each plot. The gradients of these plots reflect the sensitivity of *S. cerevisiae* to changes in the concentration of the AM agents. The latter results suggest that this microorganism is equally sensitive to concentration changes in the linalool and carvacrol systems with a slightly higher sensitivity (*ca*. 10%) in the thymol system. A statistical analysis of the results showed that at the 1% (w/w) level of AM agent, there were significant differences in the effectiveness between the linalool and carvacrol or thymol but no significant differences between the carvacrol and thymol systems. In contrast, significant differences were found amongst all three systems at the 5% (w/w) level of AM agent.

3.2 Antimicrobial Activity on Cheddar Cheese

The coated starch-based films were evaluated for their AM activity on Cheddar cheese inoculated with *S. cerevisiae*. The challenge test on the cheese was performed at 15°C in order to determine the activity of the three AM agents against *S. cerevisiae* under temperature abuse conditions. The AM efficacy of the films was compared quantitatively in accordance with the method described by Bachrouri *et al.* [32]. Figure 3 is a plot of the decadic logarithm of the population counts of *S. cerevisiae* on the surface of the cheese as a function of storage time in the starch-based AM films

incorporating linalool in their coatings. Similar curves were obtained for the death curves of the films that incorporated carvacrol and thymol in their coatings (not shown). Data for the control film are also plotted for comparison.

These results suggest that all the starch-based films containing linalool in their coating had a significant ($p \leq 0.05$) inhibitory effect on the growth of *S. cerevisiae* on Cheddar cheese at all tested concentrations compared to the control film. The results also suggest a decline of the *S. cerevisiae* population count on the cheese during the 28 days of storage at 15°C. The starch-based film containing linalool in its coating reduced the *S. cerevisiae* population count on the cheese samples by 1.19, 1.41 and 1.93 log(CFU g^{-1}) units for the films containing 1%, 3% and 5% (w/w) of linalool respectively during the 28 days of storage at 15°C. In comparison, the population count of *S. cerevisiae* for the cheese samples wrapped in the control film decreased by 0.8 log(CFU g^{-1}) units only when stored under identical conditions.

Further to the AM activity of linalool, the population of *S. cerevisiae* on the Cheddar cheese samples was found to be significantly ($p \leq 0.05$) lower when these were packed in starch-based films containing carvacrol in their coating at each of the three concentration levels tested. The population of the *S. cerevisiae* samples wrapped with films containing 1, 3 and 5% (w/w) carvacrol in their coating decreased by 24.2%, 32.9% and 43.8% respectively after 28 days of storage at 15°C compared with the control film where the population count decreased by 11% over the same period. This corresponds to a reduction of 1.2, 1.6 and 2.4 log(CFU g^{-1}) units for the systems containing 1, 3 and 5% (w/w) of carvacrol in their coatings compared with the control film (i.e. 0.8 log(CFU g^{-1})). Moreover, the starch-based films containing thymol at 1, 3 and 5% (w/w) in their coatings also significantly reduced the population of *S. cerevisiae* inoculated on the cheese by 1.4, 1.8 and 2.7 log(CFU g^{-1}) units respectively compared to the control film (i.e. 0.8 log(CFU g^{-1})). In accordance with the results in the agar diffusion assay, thymol demonstrated the highest AM inhibitory effect, followed by carvacrol and then linalool that demonstrated the least inhibitory effect against *S. cerevisiae* on Cheddar cheese. These observations are also in agreement with the results of Kuorwel *et al.* [33] who reported the AM activity of starch-based films containing linalool, carvacrol or thymol in the film coatings against *S. aureus* to be dependent on the concentration of the AM agent in the coating.

Table 2 lists the linear regression parameters from the analysis of the specific death rate data obtained for *S. cerevisiae* on the surface of Cheddar cheese that was wrapped in starch-based films containing 1, 3 or 5% (w/w) linalool, carvacrol or thymol in their coatings and stored under temperature abuse conditions of 15°C for up to 28 days. The data show a high degree of reproducibility within the experiments as indicated by the consistent values of the vertical axis intercepts. Specifically, the consistency of these data confirms the consistency in the initial inoculation procedure.

In order to explore in greater detail the effect of changes in the concentration of AM agent in the film coating on the death rate in these systems, the specific death rate data given in Table 2 were plotted as a function of the AM concentration in the film coating. Figure 4 indicates that the sensitivity of *S. cerevisiae* to changes in the concentration of AM agent in the film coatings remains constant over the concentration range investigated in this study. The figure also reveals the *ca*. 7% higher sensitivity of this organism to changes in the concentration of thymol compared with linalool. A statistical analysis of the results suggests that although a significant difference exists between the sensitivity of *S. cerevisiae* to thymol and linalool no such significant difference exists between its sensitivity to carvacrol and linalool. Furthermore, the data suggest the overall relative order of the sensitivities determined in these storage experiments at 15°C is the same as that found in the case of the solid media experiments conducted at 25°C suggesting that the relative order remains unaffected within this temperature range.

4 Conclusions

The activity of AM agents linalool, carvacrol or thymol in the coating of starch-based packaging films determined by the agar diffusion method, demonstrated an inhibitory effect against *S. cerevisiae*. Each of the starch-based systems was also found to successfully inhibit the growth of *S. cerevisiae* on the surface of Cheddar cheese. The inhibitory effect of linalool, carvacrol and thymol against *S. cerevisiae* was found to be dependent on the concentration of the AM agent in the surface coating. Thymol was found to be the most effective AM agent when used with a starch-based material as the substrate. This is followed by carvacrol and linalool in order of decreasing efficacy. It is suggested that such AM starch-based films containing any of the three AM agents have a potential for applications in AM packaging and may help to reduce the risk of food-borne illness associated with microbial contamination in Cheddar cheese. Further research

is continuing to investigate the AM effect of these agents coated onto starch-based substrates at low concentrations.

5 References

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Table 1. Zone of inhibition data in solid media for *S. cerevisiae* at 25°C in the presence of starch-based film coated with the AM agents linalool, carvacrol or thymol at 1, 3 and 5% (w/w) in the coating.

AM agent	Zone of inhibition/mm for *S. cerevisiae*			Gradient dz/dc	Intercept	Correlation coefficient (R^2)
	1% (w/w)	3% (w/w)	5% (w/w)			
Linalool	6.1 ± 0.3	8.2 ± 0.9	11.2 ± 0.3	1.26	4.71	0.988
Carvacrol	7.1 ± 0.4	9.3 ± 0.6	12.2 ± 0.4	1.28	5.69	0.993
Thymol	7.6 ± 0.6	10.6 ± 0.9	13.2 ± 0.3	1.4	6.30	0.999

Table 2. Linear regression parameters from the analysis of death rate data for *S. cerevisiae* on the surface of Cheddar cheese wrapped in starch-based films containing 1, 3 or 5% (w/w) linalool, carvacrol or thymol in their coatings and stored under temperature abuse conditions of 15°C for up to 28 days.

Treatment	Formulation % (w/w)	Specific death rate μ/day^{-1}	Intercept	Correlation coefficient (R^2)
Control	0	0.030	5.02	0.971
Linalool	1	0.038	4.94	0.986
	3	0.047	4.81	0.991
	5	0.063	4.73	0.987
Carvacrol	1	0.043	4.94	0.996
	3	0.055	4.87	0.991
	5	0.073	4.78	0.996
Thymol	1	0.044	4.97	0.978
	3	0.058	4.85	0.996
	5	0.077	4.73	0.992

Figures

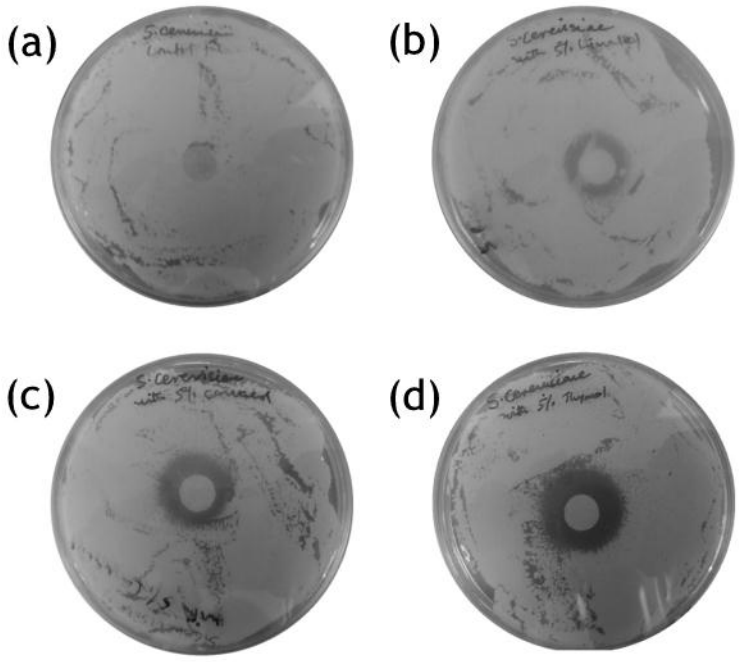


Figure 1. Inhibition of *S. cerevisiae* on solid media by starch-based AM films coated with: (a) nil AM agent (control), (b) 5% (w/w) linalool, (c) 5% (w/w) carvacrol, and (d) 5% (w/w) thymol, after incubation for 48 h at 25°C.

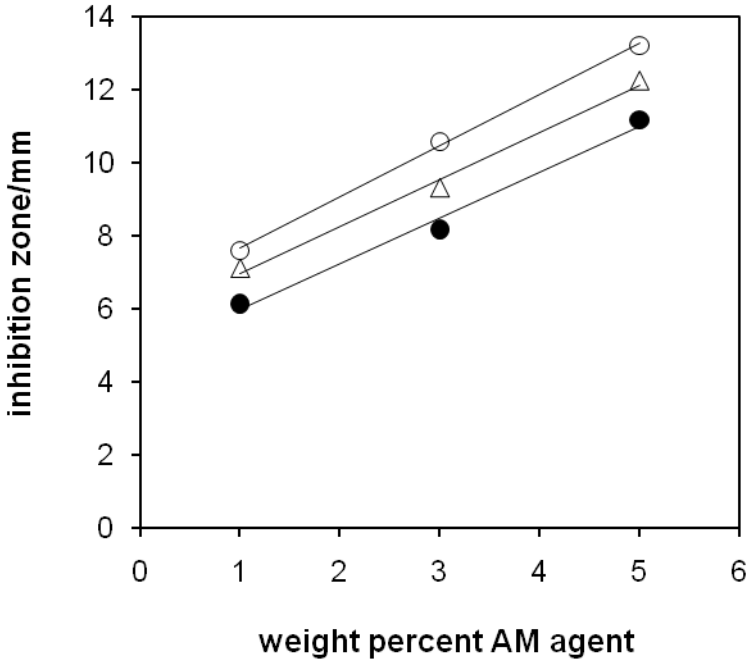


Figure 2. Plot of the zone of inhibition of *S. cerevisiae* versus concentration of AM agents for starch-based films containing: (●) linalool, (Δ) carvacrol and (□) thymol in their coating. The zones of inhibition were created on solid media after incubation for 48 h at 25°C.

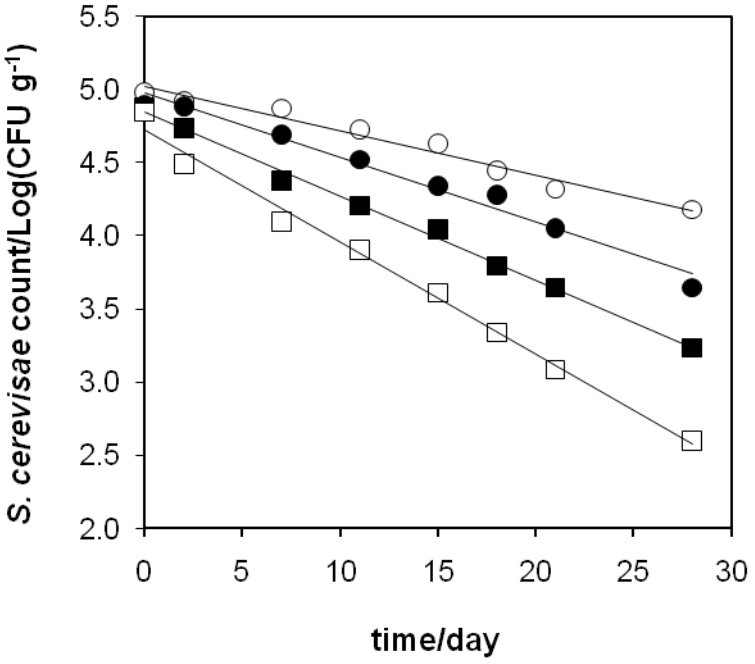


Figure 3. Inhibition of *S. cerevisiae* on the surface of Cheddar cheese packaged and stored under temperature abuse conditions of 15°C in starch-based films with a coating containing thymol at: (●) 1% (w/w), (■) 3% (w/w) and (□) 5% (w/w). The control film (○) containing no AM agent is shown for the comparison.

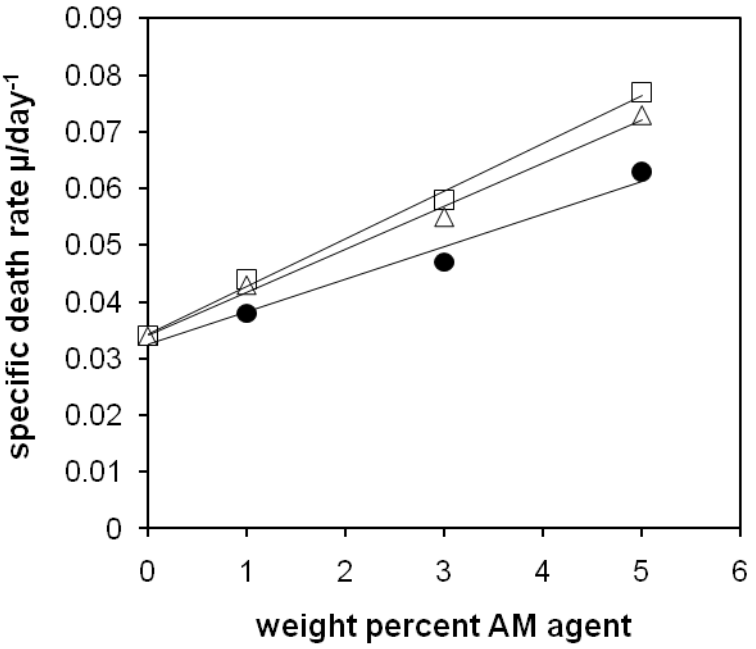
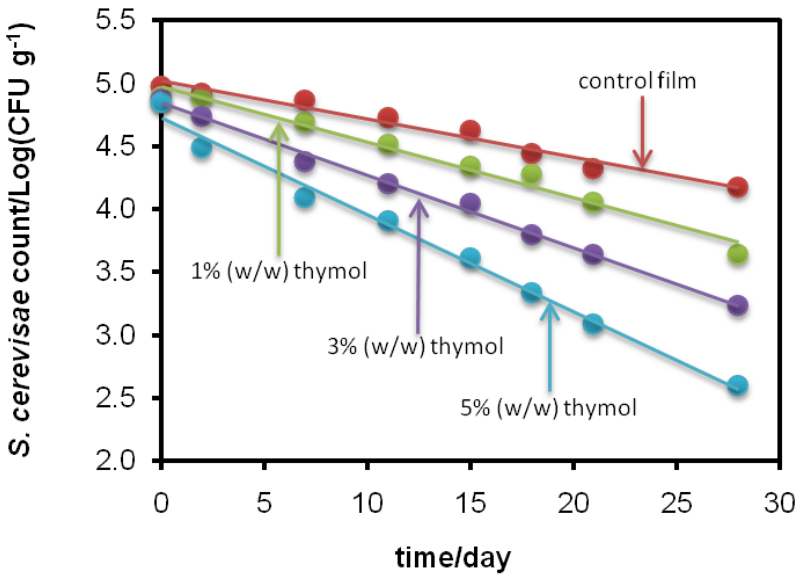


Figure 4. Plot of the death rate of *S. cerevisiae* on the surface of Cheddar cheese as a function of the concentration of: (●) linalool, (Δ) carvacrol and (□) thymol present in the surface coatings of starch-based films that were used to wrap the cheese samples under temperature abuse conditions of 15°C.

Graphical Table of Contents Figure & Text

Colour is acceptable for web only content and text is 80 words maximum (to be submitted when manuscript is accepted).



The activity of naturally derived antimicrobial (AM) agents' linalool, carvacrol or thymol coated on starch-based packaging films demonstrated an inhibitory effect against *S. cerevisiae*. Each of the starch-based systems was found to successfully inhibit the growth of *S. cerevisiae* on the surface of Cheddar cheese and on agar media. The inhibitory effect was found to be dependent on the concentration of the AM agent in the surface coating with thymol the most effective AM agent followed by carvacrol and linalool.