

Effect of Temperature on Antimicrobial Activity and Mode of Action of Thymol against *Staphylococcus aureus*

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Abstract

Objective: *Staphylococcus aureus* is one of the important food borne pathogens. The use of chemical food preservations to prevent the contamination of such bacterial strain in foods has potential to pose harm to consumers. Therefore, currently there have been attempts to find safe natural products such as thymol to use as alternatives. **Methods:** This study was to examine the effect of temperature on antimicrobial activity and mode of action of thymol against *S. aureus*. As studied at 37°C and 20°C, thymol had ability to inhibit the bacterium with the Minimal Inhibitory Concentration (MIC) of 1.25 mM and had bacteriocidal mode of action. **Findings:** These results suggest that temperature has no effect on antimicrobial activity and mode of action of thymol against *S. aureus* that is a gram positive bacterium. The findings are different from those of previous research works reporting that temperature had an effect on antimicrobial activity of thymol against gram negative bacteria. **Application:** Thus, this report is the first to propose the possible explanation on why temperature affects antimicrobial activity of thymol against gram positive and gram negative bacteria differently.

Keywords: Antimicrobial Activity, Mode of Action, *Staphylococcus aureus*, Thymol

1. Introduction

Food borne pathogens are groups of disease causing microorganisms that are associated with foods. They can be bacteria, fungi and viruses. Symptoms resulting from these pathogens range from mild to severe depending on infected persons' health and amount of pathogens that they receive. Among food borne pathogens, *Staphylococcus aureus* is considered to be the most common causative agents of food borne diseases in humans. Therefore, it is often used as representatives of bacterial food borne pathogens in several experiments. *S.*

aureus when grow in foods make toxins that cause nausea, stomach cramps, vomiting, and diarrhea¹. Currently, chemical food preservatives such as benzoic acid, sorbic acid, sulfur dioxide, nitrites and nitrates have been the most common used agents to prevent the growth of pathogens in foods. However, their potential harms to consumers' health have been continuously reported² and recently driven consumer demand for natural and safe alternatives.

Essential oils and their components are natural products obtained from many parts of plants including

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flowers, seeds, leaves, twigs and roots. They have been known for their antimicrobial activity since ancient times. The safe use of essential oils and their components has led to their current status of generally recognized as safe (GRAS) food ingredients. Thymol is a major component of several plants' essential oils including thyme and oregano^{3,4}. It has been found to exhibit antimicrobial activity against a variety of food borne pathogenic bacteria, including *S. aureus*⁵⁻⁸. However, effect of temperature on antimicrobial activity and mode of action of thymol against the bacterium has not been performed. Therefore, in the present study, we focused on how temperature affects the MIC and mode of action of thymol against *S. aureus*.

2. Materials and Methods

2.1 Bacterial Strain and Culture Conditions

The bacterial strain used in this study was *S. aureus* ATCC 25923 that was obtained from the American Type Culture Collection (ATCC). Its identity was confirmed using the API Stap-Ident system (bioMerieux Industry, Hazelwood, MO, USA), respectively. The bacteria strain was grown at 37°C in BHI (Brain Heart Infusion) broth. Its stock culture was stored as a frozen culture at -80°C in BHI broth containing 20% glycerol (v/v).

2.2 Chemicals

Thymol was obtained from Himedia, Mumbai, India. The stock solution of thymol (100 mM) was made in 95% ethanol and kept at 4°C in a tightly sealed container in the dark.

2.3 Antimicrobial Activity of Thymol against *S. aureus*

In this study, the swab-paper disc technique was used to detect the antimicrobial activity of thymol against *S. aureus*. An exponential phase culture of the tested bacterial strain was spread with a sterile swab on a BHI agar plate. A sterile 6-mm-diameter filter paper disc

(Schleicher & Schuell, Inc., Keene, N.H.) was placed on the agar plate containing the tested bacterial strain. Ten mL of thymol were spotted onto the paper disc. For a control, 95% ethanol was used instead of thymol. The plates were incubated overnight at 37°C and then checked for an inhibition zone around the paper disc. This experiment was performed in triplicate.

2.4 Determination of Minimal Inhibitory Concentration

Thymol stock solution was subjected to twofold serial dilution using 95% ethanol as a diluent. One hundred µL of each dilution were added to 1.9 mL of *S. aureus* culture (approximately 10³ CFU/mL) to obtain the final concentrations of thymol as follows: 5.00, 2.50, 1.25, 0.63, 0.31, 0.16 and 0.08 mM. For control, 95% ethanol (100 µL) was used instead of thymol solution. The mixture was incubated at 37°C for 24 h before observing bacterial growth. The minimal inhibitory concentration (MIC) value of thymol for the tested bacterial strain represented the lowest concentration of thymol causing no bacterial growth. The MIC value was determined from three experiments. The same experiment was also performed at 20°C to examine the effect of temperature on the MIC value of thymol against *S. aureus*.

2.5 Examination of Mode of Action

To examine whether thymol exhibited bacteriostatic or bactericidal effect on *S. aureus*, 100 µL of thymol solution (at a final concentration equal to the MIC value) were added to a 1.9 mL culture of the tested bacterial strain (approximately 10³ CFU/mL). For control, 95% ethanol was used to replace thymol solution. After incubation for 24 h (at 20°C or 37°C), a 100 µL aliquot of the mixture was spread onto a (thymol free) BHI agar plate. The appearance of bacterial colony on the agar plate was observed after incubation the agar plate at 37°C for 24 h. The presence and absence of (thymol treated) bacterial colony on thymol free agar indicated the bacteriostatic and bactericidal effects of thymol, respectively.

3. Results and Discussion

3.1 Antimicrobial Activity of Thymol against *S. aureus*

By using swab paper disc method, it was found that thymol had antimicrobial activity against *S. aureus*, indicating by an inhibition zone on the lawn of the bacterial strain. The diameter of the inhibition zone was about 18 mm (Figure 1). On the other hand, no inhibition zone was observed in the control experiment using 95% ethanol instead of

thymol (Figure 1). Our results support reports of previous research works demonstrating the broad antimicrobial spectrum of thymol against both gram positive and gram negative bacteria⁵⁻⁸.

3.2 Determination of Minimal Inhibitory Concentration

The MIC value of thymol for *S. aureus* was determined by observing bacterial growth at 20°C and 37°C in the BHI broth having thymol with concentrations increased two-fold ranging from 0.08 mM to 5.00 mM. No difference in

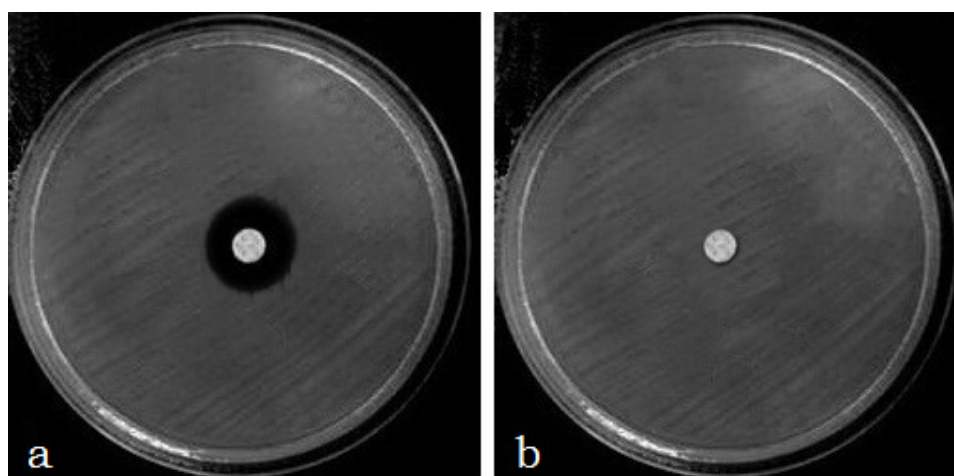


Figure 1. Antimicrobial activity of (a) thymol (b) 95% ethanol against *S. aureus* as studied by the swab-paper disc technique.

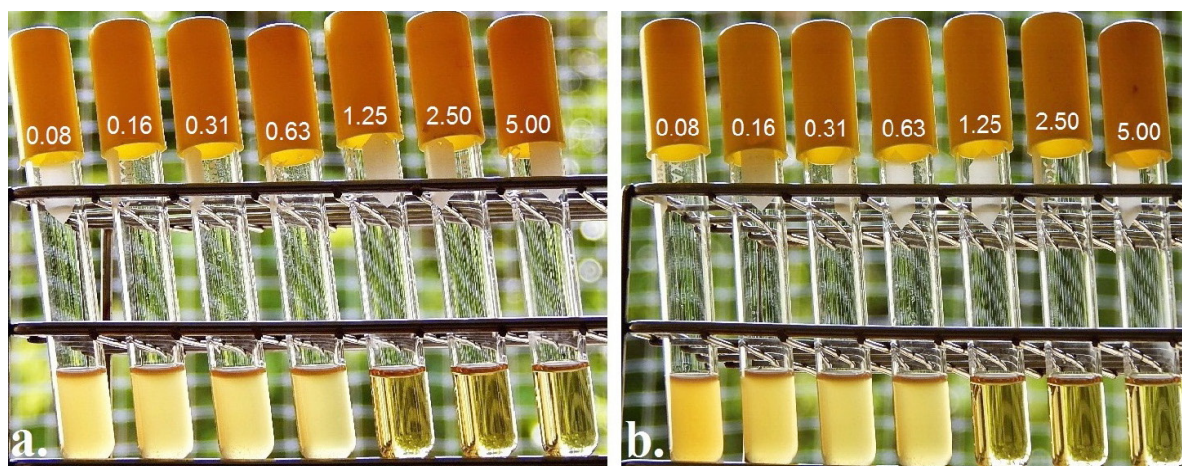


Figure 2. Growth of *S. aureus* at different thymol concentrations at (a) 20°C and (b) 37°C. Numbers on the test tube caps are concentrations of thymol in mM.

bacterial growth in BHI broth containing different concentrations of thymol was found at 20°C and 37°C. At both temperatures, no growth of bacteria was observed in BHI broth having thymol with concentrations of 1.25, 2.50 and 5.00 mM (Figure 2). These results suggested that temperature might have no effect on the antimicrobial activity of thymol against *S. aureus* because at both temperatures the MIC of thymol was 1.25 mM.

Temperature has been shown to be a major factor influencing antimicrobial activity of several essential oil

components⁹⁻¹². Reported that thymol had higher antimicrobial activity against gram negative *Shigella sonnei* at 37°C than at 4°C. Similarly, our previous study reported that thymol had higher antimicrobial activity against gram negative *E. coli* O157:H7 at 37°C than at 20°C. However, this study found that temperature had no effect on antimicrobial activity of thymol against gram positive *S. aureus*. This difference might be resulted from high lipid solubility of thymol and high lipid contents of bacterial outer membrane (OM), a structure that exists only in

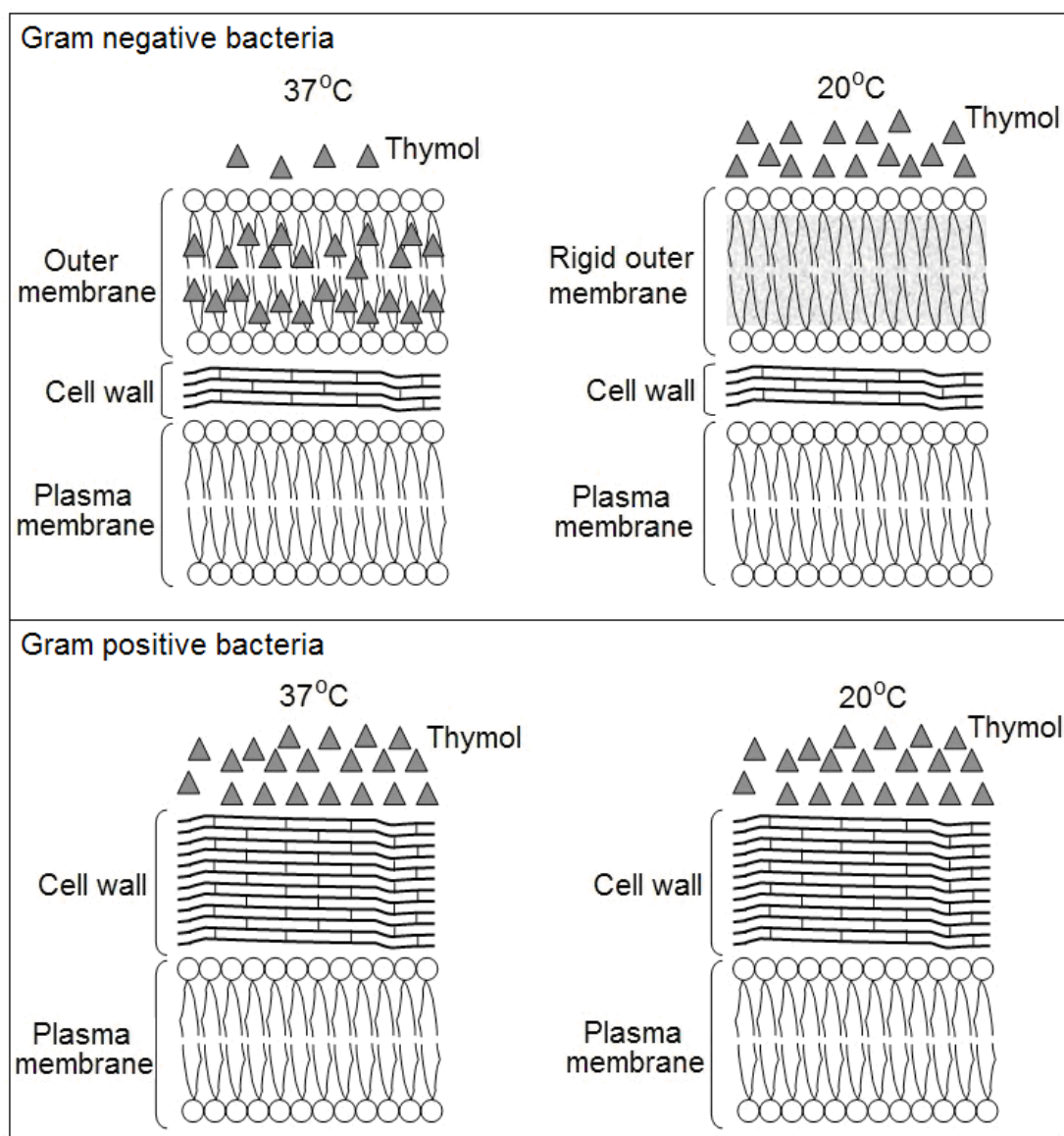


Figure 3. Hypothetical explanation of effect of temperature on antimicrobial activity of thymol against gram negative and gram positive bacteria.

gram negative bacteria. At low temperature (20°C), the fluidity of bacterial OM may lessen and become more rigid, which would lower the number of thymol dissolving in the OM and sensitivity of the bacterium to the compound. This situation may not occur in gram positive bacteria, including *S. aureus*, because it has no OM and the temperature may not affect the structure of its cell wall (Figure 3). However, further investigation is required to elucidate the actual cause of this difference.

3.3 Examination of Mode of Action

Mode of action of thymol was examined by observing the ability of bacterial cells pre-treated with thymol (at MIC) for 24 h to grow on thymol free BHI agar.

S. aureus cells pre-treated with thymol (at MIC) for 24 h at both 20°C and 37°C could not resume their growth on thymol free BHI agar indicating that thymol had a temperature independent bacteriocidal effect against *S. aureus*. These results are in agreement with previous reports showing that thymol had bactericidal effect on several strain of bacteria including *Bacillus cereus*, *Campylobacter jejuni*, *E. coli*, *Listeria monocytogenes* and *Salmonella enterica*^{13,14}. Our previous work also found that thymol had a temperature independent bactericidal effect against *E. coli* O157:H7. The temperature independent bactericidal mode of action of thymol may broaden its applications in a wide range of temperature.

4. Conclusion

Thymol has been shown by many research works including this work to have antimicrobial activity against a variety of pathogenic bacteria. However, in order to use it effectively as a biological control substance, information about its properties and factors influencing its properties is required. This study is the first report presenting the difference in effect of temperature on antimicrobial activity of thymol against gram negative and gram positive bacteria and proposing the possible cause of the difference.

5. References

1. Buzby JC, Roberts T. The economics of enteric infections: Human food borne disease costs. *Gastroenterology*. 2009; 136(6):1851–62. <https://doi.org/10.1053/j.gastro.2009.01.074>.
2. Sharma S. Food preservatives and their harmful effects. *International Journal of Scientific Research Publications*. 2015; 5(4):1–2.
3. Burt S. Essential oils: Their antibacterial properties and potential applications in foods - A review. *International Journal of Food Microbiology*. 2004; 94(3):223–53. <https://doi.org/10.1016/j.ijfoodmicro.2004.03.022>.
4. Cowan MM. Plant products as antimicrobial agents. *Clinical Microbiology Reviews*. 1999; 12(2):564–82. <https://doi.org/10.1128/CMR.12.4.564>.
5. Cosentino S, Tuberioso CIG, Pisano B, Satta M, Mascia V, Arzedi E, Palmas F. In vitro antimicrobial activity and chemical composition of Sardinian Thymus essential oils. *Letters in Applied Microbiology*. 1999; 29(2):130–5. <https://doi.org/10.1046/j.1472-765X.1999.00605.x>.
6. Lambert RJW, Skandamis PN, Coote P, Nychas GJE. A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. *Journal of Applied Microbiology*. 2001; 91(3):453–62. <https://doi.org/10.1046/j.1365-2672.2001.01428.x>.
7. Nazzaro F, Fratianni F, De Martino L, Coppola R, De Feo V. Effect of essential oils on pathogenic bacteria. *Pharmaceuticals (Basel)*. 2013; 6(12):1451–74. <https://doi.org/10.3390/ph6121451>.
8. Punyauappa-Path S, Phumkhachorn P, Rattanachaikunsopon P. Factors influencing synergistic antimicrobial activity of thymol and nisin against *Shigella* spp. in sugarcane juice. *Biologia*. 2015; 7(8):1003–10. <https://doi.org/10.1515/biolog-2015-0128>.
9. Periago PM, Moezelaar R. Combined effect of nisin and carvacrol at different pH and temperature levels on the viability of different strains of *Bacillus cereus*. *International Journal of Food Microbiology*. 2001; 68(1–2):141–8. [https://doi.org/10.1016/S0168-1605\(01\)00461-5](https://doi.org/10.1016/S0168-1605(01)00461-5).
10. Periago PM, Conesa R, Delgado B, Fernandez PS, Palop A. *Bacillus megaterium* spore germination and growth inhibition by a treatment combining heat with natural antimicrobials. *Food Technology and Biotechnology*. 2006; 44(1):17–23.
11. Tajikarimi MM, Ibrahim SA, Cliver DO. Antimicrobial herb and spice compounds in food. *Food Control*. 2010; 21(9):1199–218. <https://doi.org/10.1016/j.foodcont.2010.02.003>.

12. Macwan SR, Dabhi BK, Aparnathi KD, Prajapati JB. Essential oils of herbs and spices: their antimicrobial activity and application in preservation of food. *International Journal of Current Microbiology and Applied Sciences*. 2016; 5(5):885–901. <https://doi.org/10.20546/ijcmas.2016.505.092>.
13. Friedman M, Henika PR, Mandrell RE. Bactericidal activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica*. *Journal of Food Protection*. 2002; 65(10):1545–60. <https://doi.org/10.4315/0362-028X-65.10.1545>.
14. Valero M, Frances E. Synergistic bactericidal effect of carvacrol, cinnamaldehyde or thymol and refrigeration to inhibit *Bacillus cereus* in carrot broth. *Food Microbiology*. 2006; 23(1):68–73. <https://doi.org/10.1016/j.fm.2005.01.016>.