Effect of Chitosan Oligosaccharide Solution Against *Candida Albicans* and Color Stability of Heat-cured Polymethylmethacrylate

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Abstract

To investigate the effect of various concentrations of chitosan oligosaccharide solution (COS) and different immersion times on the antifungal activity against *Candida albicans (C. albicans)* on heat-cured polymethylmethacrylate (PMMA) and examine the color stability after 28 days of application.

The specimens were immersed in distilled water (DW), 0.2% Chlorhexidine gluconate (CHX) and various concentrations of COS. A number of fungal colonies were recorded after 1 and 8 h of immersion. The most effective method was further examined for color stability using a colorimeter and the Commission Internationale de l'Eclairage (CIE) L*a*b* system. The color alterations were determined at day 0, 1, 7, 14, 21, and 28 of immersion. Data were analyzed using Two-way ANOVA ($p<0.05$).

The efficiency in reducing the viability of *C. albicans* biofilm was better in COS than DW but lower than CHX. There was no significant difference among various COS concentrations. 8-h immersion showed greater inhibition compared to 1 h. Moreover, the color of all specimens changed significantly within clinically acceptable limits after 28 days of immersion. The greatest discoloration was found with CHX.

Immersing dentures in 0.5 MFC (350 mg/ml) COS for 8 h can effectively reduce *C. albicans* viability without compromising the color stability of heat-cured PMMA.

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Introduction

Nowadays, the demographic transition towards aging societies and the associated health issues has triggered a global focus. Even though global edentulism levels have declined in recent decades, a significant number of older adults still require prosthetic treatment.[1] One of the materials widely used for fabricating prostheses is PMMA. Despite their excellent properties, several properties of PMMA lead to more microbial adhesion and the ease of discoloration.[2] The use of intraoral devices and improper care of oral hygiene are the common causes that induce the contribution of denture stomatitis which is often associated with *C. albicans* infection.[3] However, the treatment of oral candidiasis by antifungal agents can cause adverse effects and lead to increased drug resistance.[4] Thus, adequate hygienic care is necessary to maintain a healthy oral mucosa. Guidelines recommend using a combination of mechanical and chemical cleansing techniques particularly in the elderly population with poor dexterity.[5,6]

Although various commercial cleansing agents are available, most of these cleansers can negatively affect the characteristics of the denture.[7-9] Whitening effect and staining on the denture base material has been reported which may be an esthetic problem.[10,11] Therefore, several attempts have been made to develop a novel denture cleansing agent that provide antimicrobial activity without damaging the prosthetic materials.

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Recently, chitosan, a polymer from crustaceans and insects, has been used in multiple applications in dentistry. [12] However, wide range of chitosan derivatives exists, varying in molecular weight and modifications. Each exhibits distinct properties and antimicrobial capabilities. [13-15] COS is a derivative of chitosan that has shorter chain lengths and free amino groups in the molecule. It is water soluble and possesses advantageous properties, such as broad antimicrobial activity, biodegradability, and biocompatibility. [16] Although various studies have demonstrated the antifungal activity of chitosan, there are limited studies that have evaluated the effect of COS dissolved in DW as a denture cleanser on both antifungal activity and color stability of heat-cured PMMA denture base.

Therefore, this study aimed to determine the antifungal activity against *C. albicans* and the color change of heat-cured PMMA after immersion in various concentrations of COS over different immersion time. The null hypotheses of this study encompassed several aspects. First, COS has no antifungal effect against *C. albicans*. Second, various concentrations and different immersion times of COS demonstrate no different antifungal effect against *C. albicans* on heat-cured PMMA. Finally, the color of heat-cured PMMA is not different after being treated with COS for 28 days.

Materials and methods

The minimum fungicidal concentration (MFC) test examines the lowest concentration of antifungal agents sufficient to kill certain fungi where no visible colony growth was observed, thus indicating the complete inhibition of fungal growth. *C.albicans* ATCC10231 (Department of Medical Science, Ministry of Public Health, Thailand) was inoculated into 2% Sabouraud dextrose broth (SDB) and incubated overnight at 37°C. After that, Candidal cells were adjusted at the optical density of 530 nm to be equal to McFarland No. 0.5 (10^6 CFU/ml). Each Eppendorf contained 1 ml of the suspension, which included 50 µl of *C. albicans* in planktonic form, 950 µl of sterile DW, and various amounts of COS (Marine Bio Resources Co., Ltd., Thailand). The suspension was mixed until homogeneous and then incubated for 24 h at 37°C. Due to the yellowish-brown color of the COS, the streak plate technique was continued to examine the MFC. A 1 µl inoculating loop was used to streak the solution onto 4% Sabouraud dextrose agar (SDA). Then, the plates were incubated for 24 h at 37°C.

Specimen preparation

A specimen was fabricated using heat-cured PMMA (Vertex Rapid Simplified, Vertex-Dental B.V., Netherlands) according to the manufacturer's instructions in the conventional fabrication method. [15,17] A Disk shape specimen = 10 mm in diameter and 2 mm in thickness, was fabricated for the antifungal activity test and 15 mm in diameter and 2 mm in thickness for color stability test. It was then placed in DW at 37°C for 48 h to eliminate residual monomer. [9,15,18] The resins were polished sequentially with waterproof silicon carbide abrasive paper up to 1000-grits (Mirka Ltd., Finland), and standardized using a digital vernier caliper (Mitutoyo, Japan) and a profilometer (Mitutoyo Surftest SJ-310, Japan). Finally, the specimens were sterilized with ethylene gas before testing.

Antifungal activity test

The artificial saliva (Chiang Mai University, Thailand) was dropped onto specimens and incubated at 37°C for 2 h to form an acquired pellicle. After that, the discs were washed with PBS to eliminate excess saliva. Subsequently, a diluted suspension of isolated *C. albicans* (0.5 McFarland) was dropped into each well and incubated for 90 min. The samples were washed with PBS to remove non-adherent cells. Then, SDB was dropped in to each well and replace every 24 h throughout the 48-hour incubation. At the end of the incubation period, the whole supernatant in each well was completely drained and the specimens were washed with PBS. The growth of *C. albicans* biofilm was considered before proceeding with further experiments. After biofilm formation, the contaminated specimens were randomly divided into 10 groups for different immersion methods as following.

Group 1: DW, 1 h (negative control for 1 h)
Group 2: DW, 8 h (negative control for 8 h)
Group 3: 0.5 MFC of COS in DW (COS 0.5), 1 h
Group 4: COS 0.5, 8 h
Group 5: 1 MFC of COS in DW (COS 1), 1 h
Group 6: COS 1, 8 h
Group 7: 1.5 MFC of COS in DW (COS 1.5), 1 h
Group 8: COS 1.5, 8 h
Group 9: CHX, 1 h
Group 10: CHX, 8 h

During immersion, each specimen was faced upward and completely covered with the solution at room temperature (Fig 1).

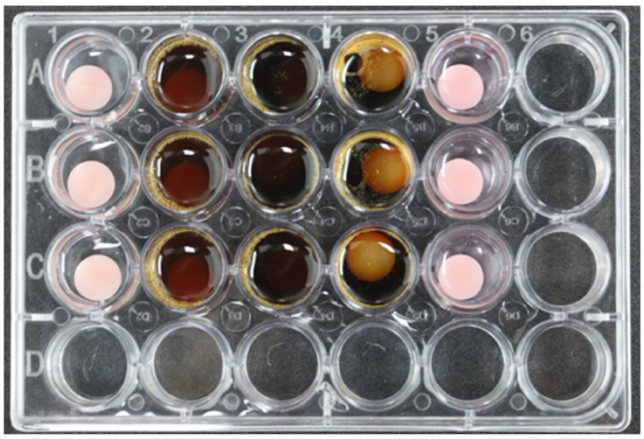

Figure 1. Specimens in different cleansing solution as assigned (distilled water, 0.5 Minimum Fungicidal Concentration of chitosan oligosaccharide solution (0.5 MFC), 1 MFC, 1.5 MFC and 0.2% Chlorehexidine gluconate).

The experiment was performed with three replicates per group and was repeated twice. After immersion, the direct culture technique was conducted. The specimens were rinsed with PBS to eliminate non-adherent fungal cells and transferred to a new vial containing SDB. Then, the vials were placed in an ultrasonic cleaner and vortexed to detach the biofilm on the specimen. The solution was serially diluted before spreading onto SDA followed by incubation for 24 h at 37°C. Finally, the number of *C. albicans* colonies was counted, and the percentage of inhibition was calculated using the following formula: Percentage of inhibition = [(Numbers of colony $_{control}$ – Numbers of colony $_{sample}$) / Numbers of colony $_{control}$] x 100%.

Color analysis

Finally, the concentration and immersion time of COS that provides the most effective antifungal effect were further examined for color stability. Ten specimens per group were immersed using the selected cleansing solutions method followed by a rinse with DW and then stored in DW at 37°C. [9,17] This procedure was repeated daily for 28 days, with denture cleansing agents replaced after each cycle.

The baseline color of heat-cured PMMA was measured at day 0, with subsequent color analyses on day 1, 7, 14, 21, and 28 of immersion using a colorimeter (Minolta CR-5, Konica Minolta, Japan) and the CIE L*a*b* color scale. At each time interval, the data were recorded including 3 parameters: L*, a*, and b*.

- L* represents lightness ranging from 0 (black) to 100 (white),
- a* represents the red-green axis which red corresponds to a positive value and green corresponds to a negative value
- b* represents yellow-blue which yellow corresponds to a positive value, and blue corresponds to a negative value. [19,20]

Color change (ΔE) was calculated between baseline and after immersion using the formula: $\Delta E = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$. [21,22] The changes were evaluated per the National Bureau of Standards (NBS) formula: NBS unit = ΔE × 0.92, correlated with clinical implications (Table 1). [9,23] At the day of measurement, a photograph of specimens was taken with an X-rite Color Checker passport and was further calibrated in Adobe Photoshop to correct the error of the camera.

	NBS
Indicial	0.0-0.5
Slight	0.5-1.5
Noticeable	1.5-3.0
Considerable	3.0-6.0
Large	6.0-12.0
Excessive	+12.0

Table 1. The assessment of color by the National Bureau of Standards.[9,23]

The statistical analysis was performed using statistical program SPSS 26.0 (SPSS Inc., Chicago, IL, USA) at a 95% confidence level. The percentages of inhibition and the ΔE values were presented as means and standard deviations. The data was analyzed using Two-Way ANOVA for antifungal activity test and repeated Two-Way ANOVA for color stability test.

Results

The inhibition percentage of *C. albicans* was influenced by the concentration of COS. A concentration of 700 mg/ml was the lowest concentration that completely inhibited *C. albicans* growth, designated as the MFC (Fig 2). Two-way ANOVA data showed that the immersion time and immersing solution significantly interacted with the mean percentage of the inhibition of *C. albicans.*

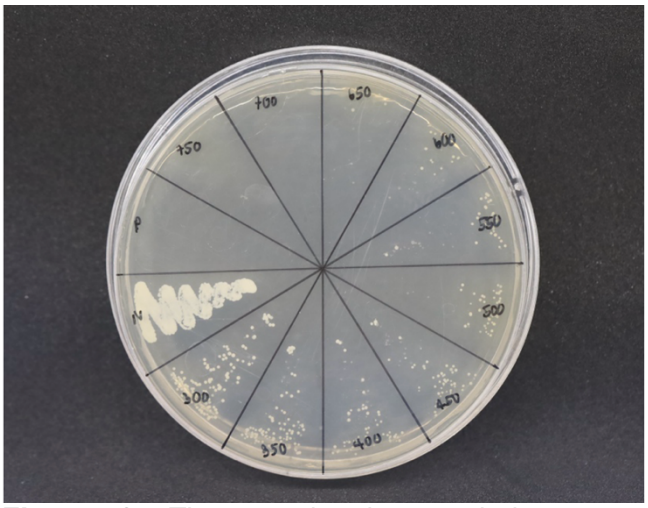

Figure 2. The streak plate technique was performed to evaluate the Minimum Fungicidal Concentration of chitosan oligosaccharide solution (COS). The complete inhibition of *Candida albicans* started at the concentration of 700 mg/ml of COS.

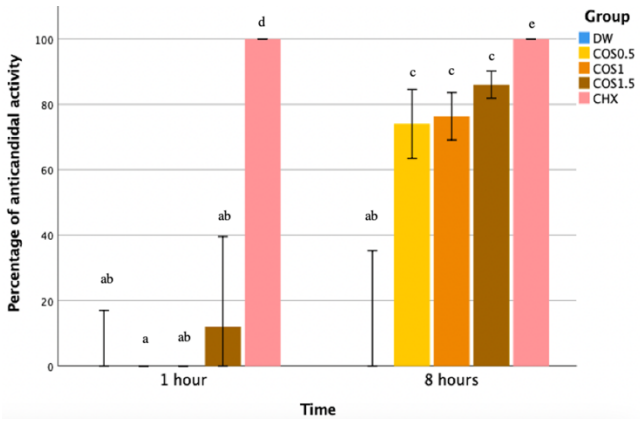

Figure 3. The mean percentage of the inhibition of *Candida albicans* and standard deviations of each group at 1 h and 8 h is shown as a bar graph. Different letters represent significant differences ($p<0.05$).

Further analysis with the Post hoc test indicated significant differences in the mean percentage of inhibition ± standard deviations between groups (Fig 3). An 8 h immersion time showed better efficiency in reducing the viability of *C. albicans* biofilm on heat-cured PMMA than 1 h. However, CHX exhibited a 100% inhibition in both durations. After a 1-hour immersion period, there were no significant differences in the percentage of inhibition among all groups, except in CHX group. After 8 h of immersion, DW had the lowest level of inhibition. Immersing in COS provided a higher level of inhibition, though there were no significant differences in the percentage of inhibition across various concentrations of COS groups. Thus, the method of immersing in COS 0.5 for 8 h was further tested.

For the color stability test, the result indicated that the color of all specimens differed significantly over the test period. According to the ANOVA test, the color change in heat-cured PMMA was significantly affected by the combination of cleansing solution (group) and immersion time (day). Significant differences were found in the mean calculated ΔE among the different denture cleansers at every duration time ($p<0.05$) (Fig 4).

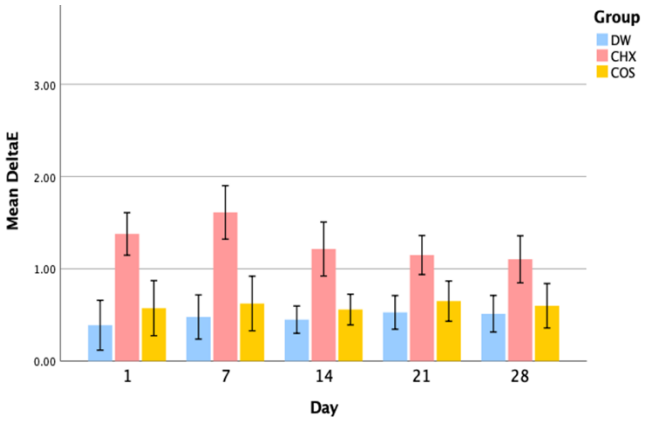

Figure 4. The color alteration between different cleansing solution at each immersion duration. The color change (ΔE) values of heat-cured Polymethylmethacrylate ranged from 0.07-1.03 in the distilled water group, 0.21-1.17 in the chitosan oligosaccharide solution group, and 0.72-1.76 in the 0.2% Chlorhexidine gluconate group.

The ΔE values of CHX group were found to be statistically significantly higher than other groups and the least discoloration was found in DW group ($p<0.05$). However, there was no difference in ΔE between DW and COS groups ($p<0.05$). The image of the specimens at each duration time is shown in Fig 5. After 28 days of immersion, PMMA specimens demonstrated “indical change” in DW group, while specimens of COS and CHX group demonstrated “slight change”. However, all color changes were considered clinically acceptable by current standards.

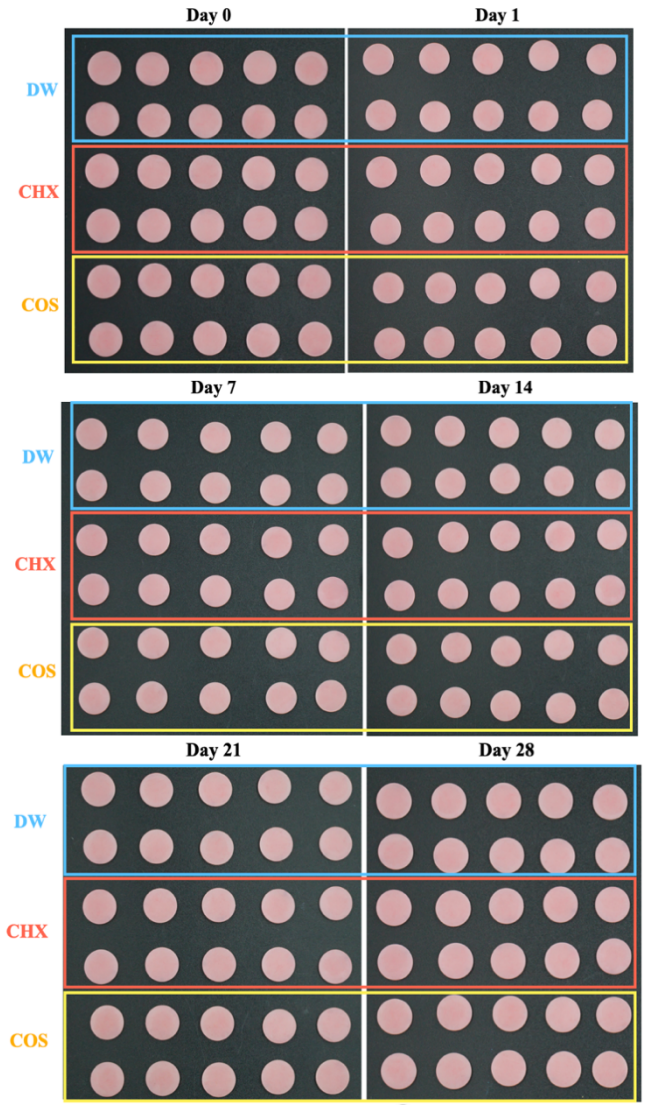

Figure 5. Specimens color at baseline (Day 0), after 1, 7, 14, 21 and 28 day of immersion.

Discussion

According to the result, the null hypothesis that COS has no antifungal effect against *C. albicans* was rejected. In addition, interactions were observed between various concentrations and different immersion times in relation to the antifungal effect against *C. albicans* on heat-cured PMMA. Furthermore, the results revealed a significant alteration of color. Consequently, the null hypothesis which stated that the color of heat-cured PMMA would not be different after being treated with COS for 28 days was also rejected.

The results were consistent with prior research that showed promising results regarding the antifungal activity of chitosan and its derivatives. [15,24-26] There are different hypotheses on the antifungal activities of chitosan:

- Cationic chitosan molecules interact with negatively charged phospholipids on fungi's cell membrane resulting in membrane leakage and cell apoptosis. [13]
- After entering chitosan molecules into the nucleus of microbial cells, the creation of DNA/mRNA and protein is disrupted. [13]
- As a chelating agent, chitosan can bind to calcium and iron, causing impairment of the nutrient transduction process. [27]

The antifungal activity of additional chitosan derivatives was investigated against *C. albicans* biofilm compared to CHX, effervescent tablet, and acetic acid. The study concluded that low-molecular weight chitosan dissolved in acetic acid effectively diminished the viability of *C. albicans* biofilm on PMMA after 12 h of immersion. [15] However, according to Evelyna et al, chitosan solution in acetic acid had potential cytotoxic effects on normal skin cells. Thus, further research was recommended using solvents other than acetic acid. [28]

Chlorhexidine gluconate was used to compare both the antifungal activity and color stability. The effectiveness of CHX as an antifungal agent has been substantiated through various clinical trials. CHX has been widely used as a mouthrinse for managing denture stomatitis and has been regarded as the antiseptic of choice for disinfecting the prostheses that *C. albicans* infect. [29,30] Following the exposure to CHX, several effects are observed, including the coagulation of nucleoproteins, inhibition of budding and alteration of cell wall which may lead to cell lysis and detachment from the underlying cytoplasm. [30] These changes can indicate both the fungicidal and fungistatic effects of the antiseptic.

Most prosthetic polymers are made from PMMA, which can be used in two forms: a linear chain polymer or a light cross-linking achieved by incorporating a bifunctional methacrylate. [31] The water sorption, hygroscopic expansion, and the molecular polarity of PMMA enhances the susceptibility of resin to absorb substances into the resin matrix. [31] When a liquid is absorbed, it diffuses into the polymer network, leading to hydrolysis and the formation of acrylic regions with varying optical characteristics. Moreover, the soluble components and plasticizers can leach out from acrylic resin, resulting in a color change.

These processes might explain color changes occur after using denture cleansers or even when immersed in DW. [17] Similar findings were found in other researches. [7,17] Nevertheless, due to the lack of studies focused on assessing the influence of COS on the color stability of heat-cured PMMA, there is no study comparable to ours. The primary staining mechanism observed in COS group is probably related to the absorption of liquids, with COS exhibiting a yellow color.

Additionally, several studies have reported brown discoloration on denture bases soaked in CHX which could be associated with the denaturation, the precipitation of salivary mucinous proteins and the formation of pigmented metal sulfide. [10,24,30,32] A Previous study demonstrated an increased discoloration index after the use of CHX mouthrinses for 4 weeks. [24] Therefore, discoloration along with other side effects such as disruption of the taste perceptions can pose a significant barrier when considering CHX as a daily decontamination product.

As the capacity of the human eye to perceive variances in color varies from person to person due to a combination of eye characteristics and the individual's skill, three distinct ranges were used to distinguish the color differences.

- $\Delta E < 1$ undetectable to the human eye
- $1 < \Delta E < 3.3$ noticeable by experienced operators
- $\Delta E \geq 3.3$ noticeable to untrained individuals (clinically unacceptable) [11]

However, detecting a color change can be challenging because the entire color of the denture base changes. Consequently, the threshold considered clinically unacceptable would probably exceed $\Delta E=3.7$. [9]

This research was conducted under a controlled environment that could not replicate the oral cavity environment. Further studies are required to investigate the effect of COS on multispecies biofilm, evaluate the cytotoxicity and examine other properties of denture base materials.

Conclusions

The current findings imply that COS could be an alternative option for a denture cleanser. The recommended instruction for using COS is to immerse denture in COS 0.5 (350 mg/ml) for 8 h. This approach effectively reduces *C. albicans* viability without compromising the color stability of heat-cured PMMA dentures.

Acknowledgments

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Declaration of Interest

The authors declare no conflicts of interest relevant to this article.

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