COMPARISON OF THE EFFICACY OF CHEMICAL CLEANING METHODS IN REMOVING *CANDIDA ALBICANS* FROM POLYMETHYL METHACRYLATE

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Prosthodontics
Department of Prosthodontics
Faculty of Dentistry
Chulalongkorn University
Academic Year 2018

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การเปรียบเทียบประสิทธิภาพของวิธีการทำความสะอาดด้วยสารเคมีในการกำจัดเชื้อแคนดิดา อัลบิแคนส์ออกจากโพลีเมทิล เมทาคริเลท

น.ส.ธนาภรณ์ ธนามี

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาทันตกรรมประดิษฐ์ ภาควิชาทันตกรรมประดิษฐ์
คณะทันตแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2561

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-สุขอนามัยที่ดีของฟันเทียมสำคัญในการลดความเสี่ยงการเกิดปากอักเสบเหตุฟันเทียม วิธีการทำความสะอาดฟันเทียมมีทั้งวิธีทำความสะอาดเชิงกลและวิธีการทำความสะอาดด้วยสารเคมี ซึ่งวิธีทำความสะอาดเชิงกลเพียงอย่างเดียวนั้นไม่เพียงพอที่จะกำจัดคราบจุลินทรีย์ออกจากฟันเทียมได้หมด จึงจำเป็นที่จะต้องใช้วิธีการทำความสะอาดด้วยสารเคมีร่วมด้วย วัตถุประสงค์ของการศึกษาคือเปรียบเทียบประสิทธิภาพของวิธีการทำความสะอาดฟันเทียมด้วยสารเคมีในการกำจัดเชื้อแคนดิดา อัลบิแคนส์ ออกจากโพลีเมทิล เมทาคริเลท เปรียบเทียบเซลล์มีชีวิตที่เหลืออยู่หลังการทำความสะอาด วิธีทดสอบ เตรียมชิ้นงานโพลีเมทิล เมทาคริเลท 120 ชิ้น นำไปเพาะเชื้อแคนดิดา อัลบิแคนส์ ที่อยู่ในรูปของสารละลาย 1 มิลลิลิตร ในถาดหลุมเพาะเลี้ยง 24 หลุม ที่อุณหภูมิ 37 องศาเซลเซียส 24 ชั่วโมง เพื่อให้เกิดไบโอฟิล์มบนชิ้นงาน จากนั้นนำชิ้นงานแช่ในกลุ่มการทดลองทั้งหมด 20 กลุ่ม ได้แก่ น้ำเปล่า 1 และ 12 ชั่วโมงเป็นกลุ่มควบคุม, 0.1% กรดแอซีติก 1 และ 12 ชั่วโมง, 0.2% กรดแอซีติก 1 และ 12 ชั่วโมง, ไคโตซานโอลิโกเมอร์ ความเข้มข้น 3 มิลลิกรัม/มิลลิลิตร 1 และ 12 ชั่วโมง , ไคโตซานโอลิโกเมอร์ ความเข้มข้น 6 มิลลิกรัม/มิลลิลิตร 1 และ 12 ชั่วโมง, ไคโตซาน 30 กิโลดาลตัน ความเข้มข้น 3 มิลลิกรัม/มิลลิลิตร 1 และ 12 ชั่วโมง, ไคโตซาน 30 กิโลดาลตัน ความเข้มข้น 6 มิลลิกรัม/มิลลิลิตร 1 และ 12 ชั่วโมง โพลิเดนท์ 15 นาที, 1 และ 12 ชั่วโมง และ 0.2% คลอร์เฮ็กซิดีน 15 นาที, 1 และ 12 ชั่วโมง วัดผลเชื้อมีชีวิตที่เหลืออยู่ด้วยวิธี MTT colorimetric assay โดยวัดด้วยค่าการดูดกลืนแสงและคำนวณเป็นร้อยละเพื่อวิเคราะห์ทางสถิติ ผลการทดลองพบว่า ไคโตซานโอลิโกเมอร์ ความเข้มข้น 3 มิลลิกรัม/มิลลิลิตร ที่แช่เป็นเวลา 12 ชั่วโมง มีประสิทธิภาพดีที่สุดอย่างมีนัยสำคัญทางสถิติเมื่อเปรียบเทียบกับทุกกลุ่มการทดลอง ยกเว้นกลุ่มการทดลองที่ใช้ไคโตซานทุกกลุ่มที่แช่เป็นเวลา 12 ชั่วโมง ร้อยละของเซลล์ที่เหลืออยู่หลังการทำความสะอาด คือ 6.22±4.30% ($p < 0.05$) โดยใช้ One-Way ANOVA ในการวิเคราะห์ทางสถิติ จากผลการทดลองสรุปว่า ไคโตซานโอลิโกเมอร์สามารถใช้เป็นสารต้านเชื้อราและใช้ทำความสะอาดฟันเทียมเพื่อลดจำนวนเชื้อแคนดิดา อัลบิแคนส์

สาขาวิชา	ทันตกรรมประดิษฐ์	ลายมือชื่อนิสิต ....
ปีการศึกษา	2561	ลายมือชื่อ อ.ที่ปรึกษาหลัก ....
		ลายมือชื่อ อ.ที่ปรึกษาร่วม ....

5975815832 : MAJOR PROSTHODONTICS
KEYWORD: polymethyl methacrylate, Candida albicans, denture stomatitis, denture cleaning methods

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COMPARISON OF THE EFFICACY OF CHEMICAL CLEANING METHODS IN REMOVING *CANDIDA ALBICANS* FROM POLYMETHYL METHACRYLATE. Advisor: Assoc. Prof. VIRITPON SRIMANEEPONG, D.D.S., M.S., Ph.D. Co-advisor: Assoc. Prof. ORANART MATANGKASOMBUT, D.D.S., Ph.D.

-The proper denture hygiene is important to reduce the risk of denture stomatitis. Mechanical cleaning is not sufficient to remove the dental plaque from acrylic denture base, accordingly chemical cleaning is needed. The purpose of this study was to compare the efficacy of chemical cleaning methods in removing *Candida albicans* from polymethyl methacrylate (PMMA) by comparing the remaining viable cells after cleaning. The total of 120 specimens were prepared. *Candida albicans* was cultured in broth to log phase. All specimens were randomly placed in 24-well tissue culture plate with 1 ml of *Candida albicans* cultured for biofilm formation at 37 degree Celsius for 24 hours. After that, all specimens were randomly immersed in 20 experimental groups of cleaning methods including distilled water as the negative controls for 1 hour and 12, 0.1% acetic acid for 1 hour and 12 hours, 0.2% acetic acid for 1 hour and 12 hours, 3 mg/ml oligomer chitosan for 1 hour and 12 hours, 6 mg/ml oligomer chitosan for 1 hour and 12 hours, 3 mg/ml 30 kDa chitosan for 1 hour and 12 hours, 6 mg/ml 30 kDa chitosan for 1 hour and 12 hours, Polident® for 5 minutes, 1 hour and 12 hours and 0.2% chlorhexidine for 15 minutes, 1 hour and 12 hours. The viable cells of *Candida albicans* after cleaning were determined by MTT assay as optical density and calculated into the percentage for statistically analysis. The results showed that the cleaning method which had the highest efficacy to remove *Candida albicans* from PMMA was using 3 mg/ml oligomer chitosan with 12-hours immersion time compared with other experimental groups except all of chitosan groups with 12-hours immersion, the percentage of viable cells after this cleaning method was 6.22±4.30% ($p < 0.05$) by using One-Way ANOVA for statistically analysis. The results of this study concluded that 3 mg/ml oligomer chitosan can used as

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Academic Year:	2018	Advisor's Signature ....
		Co-advisor's Signature ....

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ACKNOWLEDGEMENTS

First, I am grateful to Prosthodontics department, Faculty of Dentistry, Chulalongkorn University for making it possible for me to study here.

I would like to express my sincere gratitude to my advisor and co-advisor, Assoc. Prof. Viritpon Srimaneepong and Assoc. Prof. Oranart Matangkasombut for their patient guidance, encouragement and advise throughout the course of this reserch. I am extremely lucky to have a supervisor who cared about my work and thank you for the kindness of their personal time to meet me. This thesis would not have been completed without all the support that I have always received from them. I am very fortunate to be their student.

Besides my advisors, I would like to thank you to the thesis committee members; Asst. Prof. Prarom Salimee, Asst. Prof. Wacharasak Tumrasvin and Clinical Prof. Issarawan Boonsiri for their encouragement, insight comments and professional guidance.

I am thankful to Asst. Prof. Soranun Chantarangsu for their advice and suggestions for the statistical analysis.

Lastly, I am also deeply thankful to my family for always giving me love, support and motivation with their best wishes for me through my study and my life.

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CHAPTER I
INTRODUCTION

BACKGROUND AND RATIONALE

The elderly is becoming a major part of the Thai population [1]. The dental condition that is usually found in geriatric patients is tooth loss and they will turn to be partially or completely edentulous. There were fixed prosthesis including dental implant and removable prosthesis as the treatment options for the patients who had edentulous. Removable prosthesis is the treatments of choice to replace the missing teeth in edentulous patients, it also improves their chewing, phonetics, esthetics and facial appearance. Under the dental health insurance system in Thailand, acrylic resin-based denture which made from polymethyl methacrylate is the commonly selected treatment options more than metal-based denture because of the lower service charge with acceptable function and appearance.

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However, acrylic resin is not an ideal material for fabricating denture base because of the disadvantages from its properties, especially the adverse biological effects to oral soft tissues. The porosities and surface roughness are the disadvantages of acrylic resins that allow microbial accumulation, especially on tissue surface of denture base. The involvement of *Candida albicans* in the accumulation and colonization of microorganisms on the denture base is the cause of “denture stomatitis” [2, 3].

Denture stomatitis is arising from multifactorial etiologies. The etiologies have been reported include ill-fitting denture causing oral mucosal trauma, increasing age of dentures and denture wearers, bacterial and fungal infection, and low oral hygiene maintenance [4]. The various of denture cleaning methods have been recommended to patients, however it not had obviously evidences which stated the appropriate denture-cleansing regimen.

To clean plaque and debris from the dentures for proper denture hygiene, there are mechanical and chemical cleaning methods which usually advised to the denture wearers. Several studies suggested denture cleaning methods to remove *Candida albicans* from acrylic resin denture base, the combined method such as cleaning with running water and brush daily, soaking in alkaline peroxides denture cleaning solution such as Polident® or Efferdent®, or soaking in chlorhexidine which classed in disinfectant denture cleansing. There are evidences showing that only mechanical cleaning is insufficient to remove denture plaque, so chemical cleaning is needed [5]. Moreover, there were the studies showed that chitosan had antifungal effect against *Candida albicans* by its antimicrobial activity. Therefore, the application of chitosan can be used as the one of chemical denture cleaning. However, it cannot be summarized which one is the best method to clean the dentures and there is insufficient of evidence about comparative effectiveness of cleaning in acrylic denture base. Accordingly, the aim of this study is to compare the

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efficacy of chemical cleaning methods in removing *Candida albicans* from polymethyl methacrylate.

RESEARCH QUESTIONS

Which chemical cleaning methods have the highest efficacy in removing *Candida albicans* from polymethyl methacrylate?

RESEARCH OBJECTIVES

To compare the efficacy of chemical cleaning methods in removing *Candida albicans* from polymethyl methacrylate by comparing the remaining viable cells after cleaning

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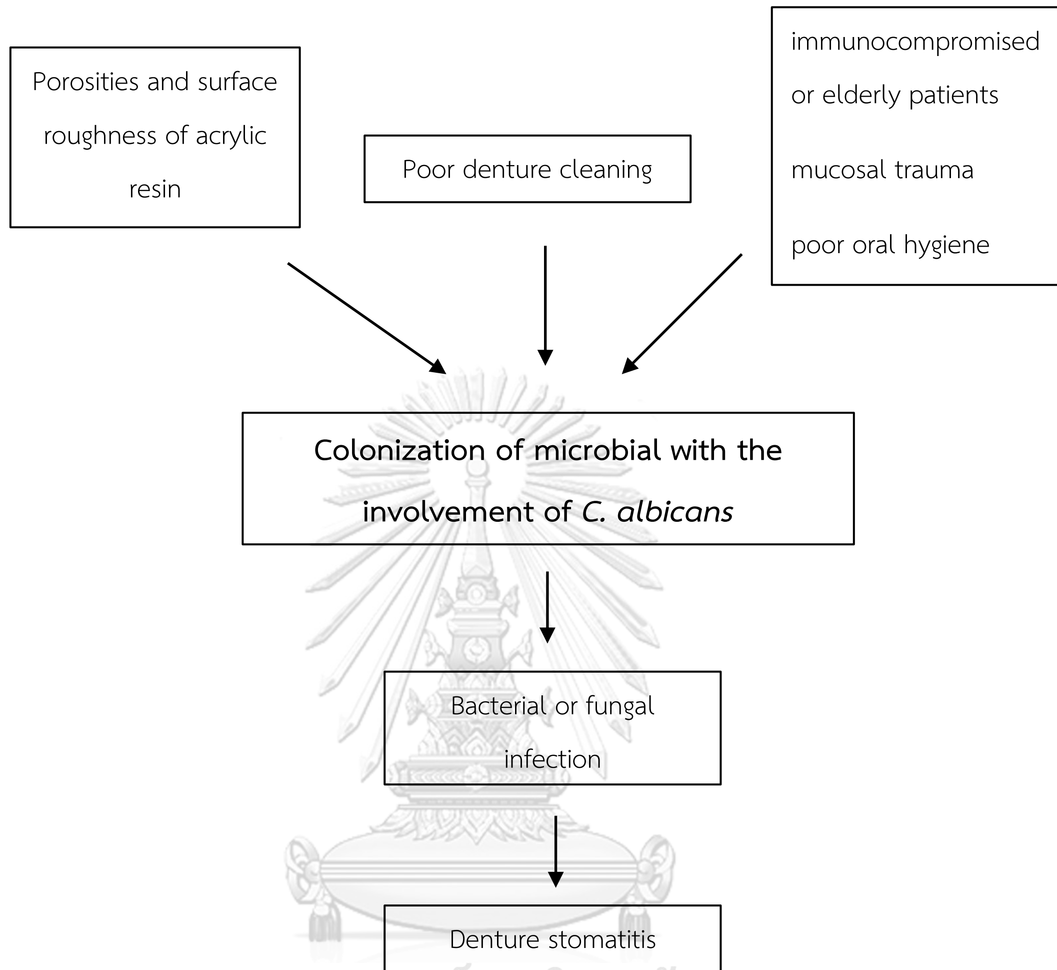
RESEARCH HYPOTHESIS

H_0: The amount of remaining viable *C. albicans* cells after cleaning with different methods are not different.

H_a: The amount of remaining viable *C. albicans* cells after cleaning with different methods are different.

CONCEPTUAL FRAMEWORK



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KEYWORDS

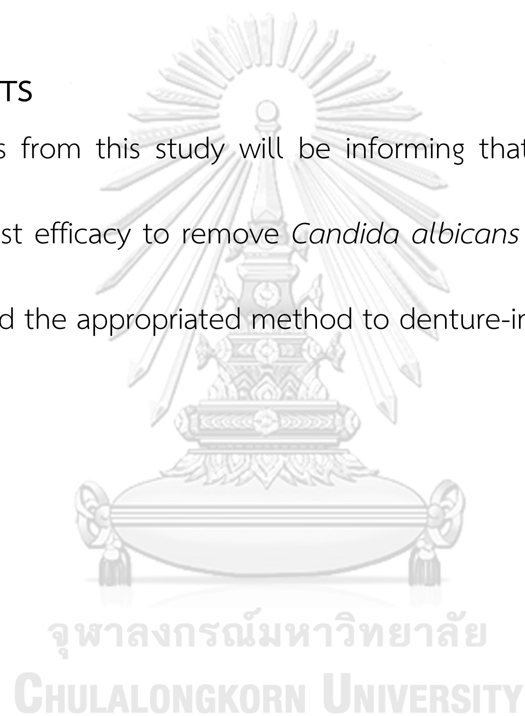
Polymethyl methacrylate, *Candida albicans*, denture stomatitis, denture cleaning methods

RESEARCH DESIGN

Experimental study

EXPECTED BENEFITS

The results from this study will be informing that which chemical cleaning method has highest efficacy to remove *Candida albicans* from acrylic denture base and can be advised the appropriated method to denture-induced stomatitis patients.



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CHAPTER II
REVIEW OF RELATED LITERATURES

Polymethyl methacrylate

Polymethyl methacrylate (PMMA) resin has been used for fabricating denture base since the mid-1940s. Colorless transparent solid is a characteristic of pure polymethyl methacrylate. The tinted polymer can have any color, shade and translucency for use in dental works. Its color, optical characteristic and dimensional stability are stable in oral cavity,

and its physical properties are suitable for dental uses [6, 7].

For easily manipulation, polymethyl methacrylate provided as a powder-liquid system. Liquid contains nonpolymerized methyl methacrylate and modified with additional kinds of monomers. An inhibitor is used to prevent these monomers from polymerization by heat, light, or traces oxygen. The powder of most commercial brands contains prepolymerized polymethyl methacrylate resin as microbeads or spheres with small amount of alkyl methacrylate, such as ethyl or butyl, to produce a polymer which is resistant to fracture. Benzoyol peroxide is an initiator of polymerization. The mixing of powder and liquid in appropriate proportions will form a workable mass and continue the polymerization process [6, 7].

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Denture base acrylic resin has been divided into 3 types based on activation methods

1. Heat-activated denture base resin

This type of acrylic resin materials is used for fabricating denture base. It is available in a powder-liquid system. The powder contains polymethyl methacrylate beads with benzoyl peroxide for initiator, dibutyl phthalate for plasticizer, pigments and opacifiers. The liquid contains methyl methacrylate monomer with hydroquinone for inhibitor, glycol dimethacrylate for cross-linking agent and plasticizers. Compression molding technique is the procedure to shape these materials. Moreover, injection-molding technique can fabricate this type of denture base by using specially designed flasks. The polymerization process is exothermic. When heating temperature is above 60°c, benzoyl peroxide decomposes to form a free radical which reacts to monomer, then it initiates polymerization. Due to a poor thermal conductor characteristic of resin, it causes an undiminished heat in a thick segment of resins. So, poorly controlled heating occurs, peak temperature of resins will rise above the boiling point of monomer, then it produces porosities in denture base from unreacted monomer [6, 7].

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2. Chemically-activated denture base resins

Denture base polymerization occurs at room temperature by chemical activators and does not need a thermal energy. This type of denture base resin is referred to as self-curing, cold-curing, or auto-polymerized resins.

The chemical activators are amines, it causes benzoyl peroxide decomposes then free radicals initiate polymerization. This type of denture base resin does not completely polymerize compared with heat-activated denture base resin. This polymerization generates 3% to 5% free monomer; it is the cause of oral tissue irritation [6-8].

3. Light-activated denture base resins

This type of denture base resins has been described as resin-based composites having matrices of urethane dimethacrylate, microfine silica, and high molecular weight acrylic resin monomers. The activator is visible light. The initiator for polymerization is a photosensitizing agent such as camphoquinone. It is a single-component denture base resins provide in sheet and rope forms packed in lightproof boxes to prevent inadvertent polymerization. The denture base begins polymerization when exposed to a high-intensity visible light source for an appropriate time [7, 9].

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From polymerization process, residual monomer is the cause of porosities in acrylic resin.[10] The porosities and surface roughness on unpolished or tissue surface

can allow microbial accumulation on acrylic denture base [2, 3, 11]. The important role of accumulation and colonization of microbial with the involvement of *Candida albicans* is the cause of fungal infection and "denture stomatitis" [2, 3].

Candida albicans

Candida albicans (*C. albicans*) is an asexual diploid fungus and is dimorphic [12]. It forms soft creamy colonies with yeast-like odor and growth on medium that has a pH at 2.5-7.5 under aerobic conditions and the range of temperature is 20-38°c. Microscopically, *C. albicans* can transition from ovoid yeast cells to hyphae; this is called dimorphism. The size of yeast cells vary from 2.9-7.2 μm [13]. It is the most common of *Candida* species found in oral cavity. The primary source of *C. albicans* in oral cavity is at dorsum of the tongue. It is usually found as harmless commensals. However, when the host defense mechanism is impaired due to any alterations, such as immune-compromised condition especially in elderly with poor oral hygiene, the virulence of *C. albicans* leads to an infection and it is the cause of candidiasis [14-16].

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In 1936, Cahn first described *Candida* as a potential causative agent in denture-induced stomatitis [17]. Erythematous type of oral candidiasis is one of common types; it has association with patients who wear dental prosthesis such as dentures and often leads to denture-induced stomatitis. *C. albicans* prefer to adhere and form biofilms on acrylic resin (PMMA), compared with other materials [18]. *C.*

albicans frequently detected at the dorsal of tongue of denture wearers compared with non-denture wearers, suggesting that denture is its reservoir [19].

C. albicans biofilms most consist of complex networks of yeast cells and hyphae embedded deeply into imperfections and cracks of denture base materials. The cracks and surface roughness of acrylic resins are significant for colonization and attachment of *C. albicans* to greater extent than smooth surface. Therefore, if the surface of materials is unpolished, *C. albicans* can deeply penetrate and may be strongly protected from any cleaning. The hyphae increased mass of biofilm, increased retention of *C. albicans* on acrylic denture base and resistance to remove [12, 20-23].

Adherence theory of dental and denture plaque

Radford and colleagues have described the mechanism of microorganism adhesion to epithelial and hard surfaces in 4 phases [12].

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Phase 1: Transport to the surface

It is associated with either diffusion (Brownian motion) or active movement such as chemotaxis.

Phase 2: Initial adhesion

Microorganisms are attached to the surfaces by van der Walls force within the distances less than 50 nm.

Phase 3: Attachment

Mannan is the major antigen of *C. albicans* cell wall, which covalently bonded to proteins to form mannoproteins. These proteins act as a receptor to bind with endothelial cells and promote adherence to plastic surfaces.

Phase 4: Colonization

The growth and plaque formation of microorganisms, called biofilm, occurred in this phase. This phase also has inter-connection of microorganisms.

These 4 phases depend on surface roughness and surface free energy.

Denture stomatitis

Denture stomatitis can be referred to as denture sore mouth, denture-induced stomatitis or chronic atrophic candidiasis. It is the most common type of oropharyngeal candidosis associated with *C. albicans* infection in elderly patients. The characteristic of denture stomatitis is a localized or generalized erythema and edema of oral soft tissues in the area covered by removable prosthesis, commonly found at palatal surface of maxillary alveolar ridge. The lesions are either asymptomatic, burning, or itching [4, 15-17].

The etiology of denture stomatitis is multifactorial. Mucosal trauma from ill-fitting denture, elderly denture wearers, the age of dentures, denture nocturnal use,

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bacterial and fungal infection and poor oral hygiene of denture wearers are associated with denture stomatitis [4]. There was the study identified that *F. nucleatum* and several species of *Streptococcus* were powerfully associated with denture stomatitis [24].

Management strategies for denture stomatitis [16, 25, 26]

Denture care by daily cleaning with running water and brushing then daily soak in the following solutions; 0.12% chlorhexidine gluconate for 20 minutes, commercial denture cleanser such as Polident® or Efferdent® as manufacturing recommendation, or 0.5% sodium hypochlorite overnight

Oral mucosa treatment by topical antifungal as a following;

Nystatin oral suspensions 4-6 ml rinse 2 minutes then spit 4 times per day for 2 weeks duration of use.

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Clotrimazole troches 10 mg suck until dissolved 4 times per day for 2 weeks duration of use.

For all above antifungal, remove denture before use medication and clean denture before reinsertion.

Due to the etiology of denture stomatitis is multifactorial, so the management is included plaque and denture hygiene control and the use of

antiseptic and antifungal products such as denture relining which contained antifungal, antiseptic mouth rinse and soaking denture in antimicrobial solutions.[27]

Denture cleaning methods

As previously mentioned, a critical risk for denture stomatitis is poor denture care and hygiene. Poor oral hygiene of denture wearers promotes anaerobic and low pH conditions that lead to opportunistic growth to pathogenic microbes such as *C. albicans* [4]. Therefore, the proper maintenance of denture hygiene is significant for reducing the risk of microbial infection in patients who wear dentures.

Denture cleaning methods can be divided into mechanical and chemical methods. The most commonly recommended methods to patients are water and brushing; mechanical cleaning. However, it is an ineffective method against microbial biofilms on denture and it removes only the large debris [28]. In contrast, chemical disinfectant solutions are more effective in reducing the microbes by soaking the denture in the solutions, especially for elderly patients [28, 29].

Chemical denture cleansers are commercially available and divided into five categories; alkaline peroxides, alkaline hypochlorite, enzyme mixtures which dissolve organic components of denture debris, disinfectants such as chlorhexidine solution and abrasive paste [5, 29, 30]. Alkaline peroxide is the most commonly used denture cleansers such Polident®, Efferdent®. The debris is loosened, and light stains

removed by an oxygen-liberating mechanism of alkaline peroxides. Alkaline hypochlorite such as sodium hypochlorite has bleaching ability to remove debris and light stain. These solutions classed into immersion type cleansers. They do not contain abrasive particles [30]. On the contrary, the abrasive paste contain abrasive particles, such as calcium carbonate and dicalcium phosphate, can increase surface roughness of acrylic denture base and promote accumulation and retention of plaque and microbes [5].

Ultrasonic cleaning is classed into mechanical cleaning methods. It is effective to clean the denture by detaching, dispersing and emulsifying the debris through sonication energy. It does not change the denture base properties. The recommendation for ultrasonic cleansing is sonicated the denture for 5-10 minutes [19].

There are several studies about antifungal activity of chitosan. Chitosan is a natural nontoxic polymer derived from chitin. Chitosan and chitosan oligomer proved as a natural antimicrobial compound. It can be obtained from shells of crustaceans such as crab, shrimp and crayfishes and it can be a product of some fungi such as *Aspergillus niger* and *Penicillium notatum* [31, 32]. It is used for commercial applications in biomedical, food and chemical industries. The study of Tayel and colleagues reported the antifungal action of chitosan against *C. albicans* from the high susceptibility interaction with chitin of its cell wall [33]. The positively charged chitosan

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interacts with the negative cell membrane of *C. albicans* and alters the permeability then causes the intracellular material leakage. The study concluded that chitosan, had effectiveness for safely and ecofriendly control of *C. albicans* invasion and it can alternative to chemical antifungal agents [33]. Therefore, this study will apply chitosan as antifungal solutions for chemical denture cleaning.

CHAPTER III
RESEARCH AND METHODOLOGY

POPULATION AND SAMPLE

1. **Population**

   Polymethyl methacrylate

2. **Study population**

   Polymethyl methacrylate which used for fabricating acrylic-based removable prosthesis.

3. **Study sample**

   Polymethyl methacrylate which used for fabricating acrylic-based removable prosthesis and was fabricated by the dental laboratory in Faculty of Dentistry, Chulalongkorn University.

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SAMPLE SIZE CALCULATION

G* Power 3.0 was used for the sample size calculation. The number of specimens in each group which obtained from the calculation was 6 specimens.

DATA COLLECTION

The viable cells of *Candida albicans* after cleaning.

STUDY PROTOCOL

SPECIMEN PREPARATION

Cylindrical clear acrylic resin (Rodex, SPD, Italy) with diameter 15 mm and 20 mm in length were fabricated by the loss-wax technique with the cylindrical silicone in the plaster stone mold and metal flask. Heat-activated acrylic resin was mixed according to manufacturing's recommendation and pack into a flask with 1200 Psi of hydraulic press. Acrylic resins were polymerized with conventional heat method. Specimens were deflasked after cooled overnight at room temperature, then finishing and polishing the specimens with standard procedures. Cylindrical acrylic resins were cut into 2 mm of thickness by low speed cutting machine (IsoMet™ Low Speed, Beuhler,USA) using 200 rpm of speed and 50 g load then polished with sand paper number 500, 800 and 1000, respectively. (Figure 1) Before culturing with *C. albicans*, all specimens were disinfected in 70% alcohol for 10 minutes, washing with distilled water and then sterilized with ethylene gas.

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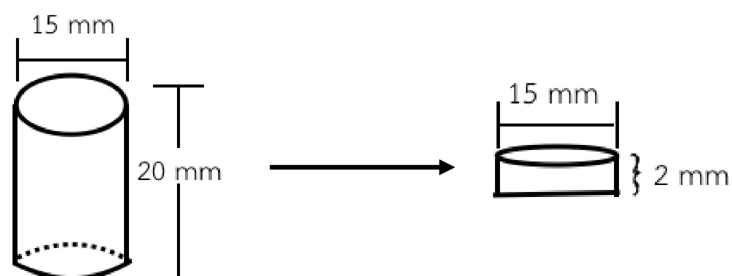

Figure 1 The specimen before and after cutting.

Candida albicans **CULTURED AND BIOFILM FORMATION ON SPECIMEN**

C. albicans (SC5314) from the frozen stock was cultured on yeast peptone dextrose (YPD) agar and incubated at 30°c for 48 hours in the incubator (MyTemp™, Benckmark Scientific, USA). A single colony of this culture was inoculated into 10 ml of liquid YPD and incubated at 30°c overnight in the orbital shaker (WiseCube®, CS witeg, Germany). After that, the cultures were adjusted to optical density (OD) 0.1. The cultures were incubated for 4-6 hours until log phase at OD 0.4-0.6 at 600 nm as measured by spectrophotometer (GENESYS™ 20, Thermo Fisher Scientific, USA). The cell suspension was adjusted for the following experiments.

Each acrylic resin specimen was placed in 24-well tissue culture plates with 1 ml of *C. albicans* suspension and incubated at 37°c for 24 hours. All procedures were carried out in a biological safety cabinet.

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The contaminated specimens were then randomly placed in a new 24-well tissue culture plate and randomly assigned to one of the following cleaning methods (n=6 per group) by immersing the specimen in denture cleaning solutions as listed. There are total 20 experimental groups including negative control as the following and summarized in the Table 1.

Group 1: distilled water for 1 hour (negative control for 1 hour, 5 minutes and 15 minutes experimental groups)

Group 2: distilled water for 12 hours (negative control for 12 hours experimental groups)

Group 2: 0.1% acetic acid (EMD Millipore Corporation, Germany) for 1 hour

Group 4: 0.1% acetic acid (EMD Millipore Corporation, Germany) for 12 hours

Group 5: 0.2% acetic acid (EMD Millipore Corporation, Germany) for 1 hour

Group 6: 0.2% acetic acid (EMD Millipore Corporation, Germany) for 12 hours

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Group 7: 3 mg/ml oligomer chitosan 7-9 kDa (Taming Enterprise, Thailand) for 1 hour

Group 8: 3 mg/ml oligomer chitosan 7-9 kDa (Taming Enterprise, Thailand) for 12 hours

Group 9: 6 mg/ml oligomer chitosan 7-9 kDa (Taming Enterprise, Thailand) for 1 hour

Group 10: 6 mg/ml oligomer chitosan 7-9 kDa (Taming Enterprise, Thailand) for 12 hours

Group 11: 3 mg/ml 30 kDa chitosan (Marine Bio Resources, Thailand) for 1 hour

Group 12I: 3 mg/ml 30 kDa chitosan (Marine Bio Resources, Thailand) for 12 hours

Group 13: 6 mg/ml 30 kDa chitosan (Marine Bio Resources, Thailand) for 1 hour

Group 14: 6 mg/ml 30 kDa chitosan (Marine Bio Resources, Thailand) for 12 hours

Group 15: distilled water with denture cleansing tablet (Polident®, GlaxoSmithKline, Thailand) for 1 hour

Group 16: distilled water with denture cleansing tablet (Polident®, GlaxoSmithKline, Thailand) for 12 hours

Group 17: distilled water with denture cleansing tablet (Polident®, GlaxoSmithKline, Thailand) for 5 minutes, following the manufacturing's instructions

Group 18: 0.2% chlorhexidine (Faculty of Dentistry, Chulalongkorn University, Thailand) for 1 hour

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Group 19: 0.2% chlorhexidine (Faculty of Dentistry, Chulalongkorn University, Thailand) for 12 hours

Group 20: 0.2% chlorhexidine (Faculty of Dentistry, Chulalongkorn University, Thailand) for 15 minutes

Table 1 Experimental groups

Group	Duration			
	1 h	**12 h**	**5 mins**	**15 mins**
Distilled water	✓	✓	-	-
0.1% acetic acid	✓	✓	-	-
0.2% acetic acid	✓	✓	-	-
3 mg/ml oligomer chitosan	✓	✓	-	-
6 mg/ml oligomer chitosan	✓	✓	-	-
3 mg/ml 30 kDa chitosan	✓	✓	-	-
6 mg/ml 30 kDa chitosan	✓	✓	-	-
Polident®	✓	✓	✓	-
0.2% Chlorhexidine	✓	✓	-	✓

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MTT COLORIMETIC ASSAY

MTT colorimetric assay is the cleavage of MTT (3-(4, 5-dimthylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) into the formazan crystals which has a purple color by mitochondrial activity of living cells enzyme. The amount of formazan is corresponded with the number of viable cells.

To examine the viable cells of *C. albicans* in acrylic resin after cleaning, MTT solutions were prepared and warmed at 37°c before use. Each specimen was incubated with MTT at 37°c for 3 hours. The formazan crystals were formed in the viable cells and were dissolved by submerged in dimethyl sulfoxides (DMSO). The optical density (OD) of the solutions were determined at 540 nm using microplate spectrophotometer (Epoch 2, BioTek®, USA). Dimethyl sulfoxides without specimen was used for the blank control. The OD of viable cells after cleaning were calculated into the percentage relative to the negative control group as equation:

$$\text{\% of viable cells} = \frac{\text{OD of each specimen}}{\text{mean of control group}} \times 100$$

The calculation was normalized the data and it can be assumed that the initial amount of cells before cleaning were equal in all experimental groups.

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CELLS DETECTION BY FLUORESCENCE STAINING

The specimens with 12 hour-immersion time of all cleaning solutions were stained by fluorescence staining for detected the live and dead cells after cleaning. Calcofluor white was used for all cells staining for 1 minute and propidium iodide was used for dead cells staining for 1 minute. After staining, the specimens were washed with distilled water to remove the excess staining. Then the cells were

detected under fluorescence microscope with 40x magnification of objective lens. The blue of calcofluor white was stained all cells and the red of propidium iodide was stained the dead cells.

STATISTICAL ANALYSIS

All statistical computations were performed by SPSS software (IBM SPSS statistics version 22.0). The percentage of viable cells after cleaning compared with the negative control groups were presented as means and standard deviations. Normality of the data was determined by Shapiro-Wilk then the data was analyzed by One-Way of ANOVA followed by Tukey post-hoc test. A *p*-value of 0.05 was considered statistically significant.

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CHAPTER IV

RESULTS

The OD of viable cells after cleaning was calculated in to the percentage relative to the negative control group. The means and standard deviations of the percentage of viable cells after cleaning were presented in Table 2. The results from this study showed that all of experimental groups were significant difference compared with both distilled water with 1-hour and 12-hour immersion time ($p < 0.05$). There was no significant difference among all groups of Polident® ($p > 0.05$). The results of 0.2% chlorhexidine were no significant difference 15-minute and 1-hour immersion time ($p > 0.05$), but these groups had significant difference with 12-hour immersion time ($p < 0.05$). All groups of 0.1% acetic acid had no significant difference with all groups of chitosan ($p < 0.05$). The group of 0.2% acetic acid with 1-hour immersion time had no significant difference with the groups of chitosan with 1-hour immersion time ($p > 0.05$), whereas the group of 0.2% acetic acid with 12-hour immersion time had significant difference with the groups of chitosan with 12-hour immersion time ($p < 0.05$). There was no significant difference among all groups of chitosan with 1-hour immersion time and it also no significant difference with 0.2% chlorhexidine with 12-hour immersion time too ($p > 0.05$). There was no significant difference among all groups of chitosan with 12-hour immersion time ($p > 0.05$). The

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results showed that there was significant difference when compared the groups of chitosan with 1-hour and 12-hour immersion time ($p < 0.05$).

The results from Figure 2 and Table 2 showed that all of chitosan experimental groups had lower percentage of viable cells when compared with others cleaning solutions, especially the groups which had 12-hour immersion time. The group which had the lowest percentage of viable cells after cleaning is 3 mg/ml oligomer chitosan with 12-hour immersion time, the mean percentage of this group is 6.22±4.30% and it had significant difference when compared with distilled water with 12-hour immersion time (negative control) ($p < 0.05$).

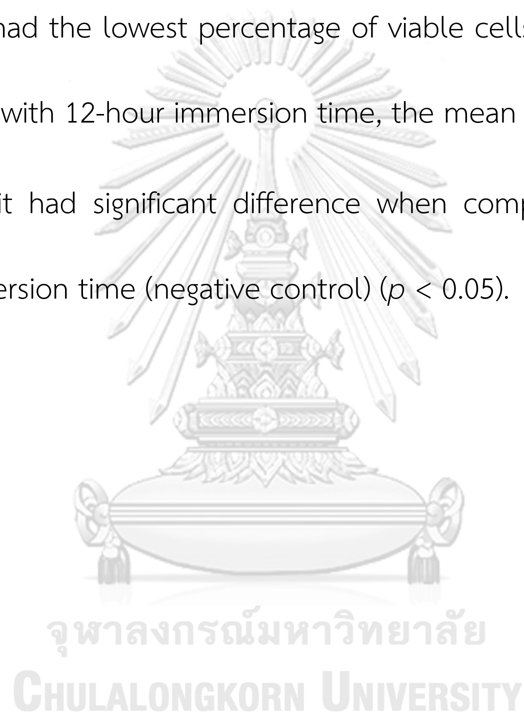
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Figure 2 The percentage of viable cells after cleaning

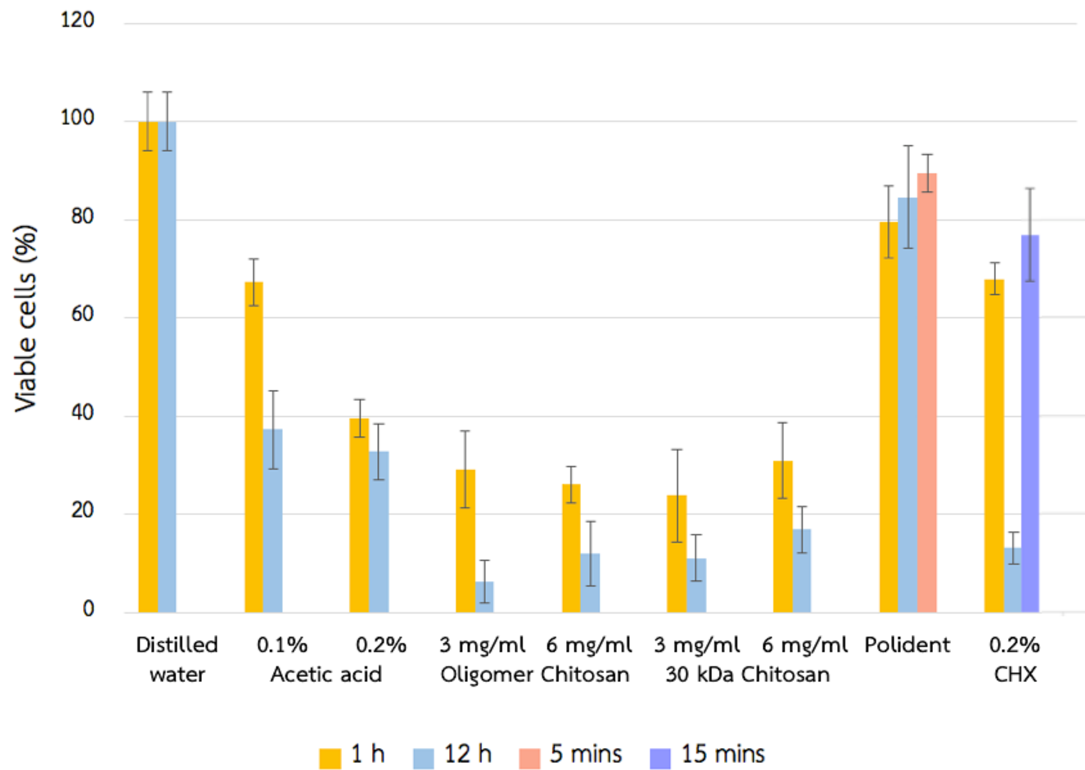

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Table 2 The percentage of viable cells after cleaning

Group/Time	Mean±SD (%)			
	1 h	12 h	5 mins	15 mins
Distilled water	100.00±5.88	100.00±5.88	-	-
0.1% acetic acid	67.28±4.71	37.20±7.89	-	-
0.2% acetic acid	39.62±3.88	32.73±5.70	-	-
3 mg/ml oligomer chitosan	29.08±7.77	6.22±4.30	-	-
6 mg/ml oligomer chitosan	26.08±3.70	12.07±6.58	-	-
3 mg/ml 30 kDa chitosan	23.86±9.45	11.09±4.68	-	-
6 mg/ml 30 kDa chitosan	30.89±7.69	16.85±4.67	-	-
Polident®	79.50±7.40	84.60±10.37	89.43±3.83	-
0.2% Chlorhexidine	67.96±3.30	13.10±3.15	-	76.90±9.43

CHAPTER V
DISCUSSION AND CONCLUSION

DISCUSSION

One of the three most common denture-related problems in Thai geriatric patients is denture stomatitis [34]. Improper denture care and oral hygiene is one of multifactorial etiologies of denture stomatitis and it has the critical risk which promoting of anaerobic and low pH condition and it is lead to the growth of opportunistic pathogens such as *C. albicans* [4]. Therefore, the proper maintenance of denture hygiene is significant for reducing the risk of microbial infection in denture wearers.

This study used distilled water with denture cleansing tablet (Polident®) which is the most common available in market, 0.2% chlorhexidine, 3 mg/ml oligomer chitosan, 6 mg/ml oligomer chitosan, 3 mg/ml 30 kDa chitosan and 6 mg/ml 30 kDa oligomer chitosan as the denture cleansers. Polident® was classed in alkaline peroxides denture cleanser, the oxygen-liberating mechanism of alkaline peroxides was loosened the debris and biofilm and it also removed the light stain. The effervescent effect of alkaline peroxides produced hydrogen peroxides which contained of active oxygen when contacted with water, this effect had important role to removing debris and antimicrobial from oxygen [30, 35]. The effect of hydrogen peroxides to *C. albicans* is to induce the hyphal differentiation and the increased

amount of hydrogen peroxides is the contrast of the biofilm growth situations which occurred in anaerobic conditions [35, 36]. This study supported the several previous studies that Polident® can reduced *C. albicans* compared with distilled water, the distilled water referred as patients had no any cleaning their dentures and it would lead to the poor denture hygiene [28, 35]. The manufacturer's instructions of Polident® used in this study was 5 minutes. There have been several reports about the immersion time of alkaline peroxides denture cleansers. The study of Shay [37] showed that 15, 30 and 60 minutes of alkaline peroxides immersion time were insufficient, and the study stated that the overnight used of denture cleansers would had more effective. The used of alkaline peroxides denture cleanser did not alter the properties of acrylic resins [38]. There was the report supported Shay's study, it found that the used of alkaline peroxides denture cleanser for 60 minutes immersion time can be reduced the amount *C. albicans*, but it was not completely removed [29]. On the other hand, the results of overnight used of alkaline peroxides denture cleanser (Polident®) from the present study did not supported the previous studies, there had no different efficacy among all of immersion time in Polident® groups due to the limited time of effervescent effect.

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0.2% chlorhexidine is classed in disinfectant cleansers. Disinfectants used for treatment and prevent fungal infection beneath the removeable prosthesis, it not commercially found as alkaline peroxides denture cleanser [35]. Chlorhexidine is widely

used for against the wide range of organisms included *C. albicans*. The antimicrobial effect of chlorhexidine is from its positive charged bind to the negative charged of cell wall, then the leakage of cell substances was initiated [39-42]. When chlorhexidine was exposed to *C.* albicans, the loosened fragment of the cell wall would occur [39]. There were the reported mentioned that chlorhexidine can be used as immersion solution to reduced microbial growth on dental prosthesis and it is also used as a denture cleansing to reduced biofilm [41, 43]. McCourtie, et al [40] demonstrated that the pretreatment of chlorhexidine to acrylic was reduced the adherence of *C. albicans*. The study of Pusateri, et al [42] showed that the biofilm of *C. albicans* on acrylic denture was sensitive to be killed and inhibited growth by chlorhexidine, the study stated that chlorhexidine was the therapeutic application. In the other hand, the frequently use of chlorhexidine has staining ability to acrylic resins [35]. The results of our study showed that 12-hour of immersion time had the highest efficacy among all of 0.2% chlorhexidine groups.

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In this study, 0.1% acetic acid and 0.2% acetic acid was used as a solvent for chitosan which had 3 mg/ml and 6 mg/ml of concentration, respectively. The used of acetic acid had the reason of chitosan is insoluble in almost of solvents, whereas it is soluble in diluted organic acid such as acetic acid, formic acid, succinic acid and lactic acid [44]. The results from this study showed that acetic acid decreased the vitality of *C. albicans*. It was supported the study of Pinto, et al which demonstrated

that the amount of *C. albicans* was decreased after soaking the denture in 10% vinegar solution for overnight [45]. There are the reports mentioned that acetic acid is a main composition of vinegar, it has been used as antifungal and antimicrobial agents since Greece era. The strongly low pH of acetic acid acts as therapeutic effect for antimicrobial by entering into the cell membranes and lead to the fatality of cells [46, 47]. Our study proved that acetic acid decreased the vitality of *C. albicans*.

The antifungal activity of chitosan is electrostatic interaction between positively charged of chitosan and negatively charged of cell membrane phospholipids, then hydrolytic enzymes causing the penetration of chitosan to the nuclei and inhibit DNA/RNA and protein synthesis. It is also the cause of intracellular leakage [44, 48]. Chitosan which used as denture cleansing solutions in this study are chitosan oligomer and 30 kDa chitosan. Chitosan oligomer has the degree of polymeriztion less than 50-55 and average molecular weight (MW) less than 10 kDa [49].

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From the groups of chitosan in this study, it had the different of type or MW of chitosan, concentration and immersion time. Molecular weight of chitosan oligomer in this study is 7-9 kDa. Chitosan oligomer has lower viscosity that make it is favorable for use as therapeutic agent [49]. The small units of chitosan oligomer has a better antifungal effect more than the larger units because of the smaller chains are easily to travelling and binding than the bigger chains to cause the ionic interaction

on the cell membrane and its microbial activity is more sensitive to fungi more than bacteria [44, 50]. The antifungal activity of LMW is likely to be more effective when compared with high molecular weight (HMW) because of LMW easily penetrate through fungal cell wall and it big enough for electrostatic interaction that cause the intracellular leakage [48]. This study supported the study of Tikhonov et al which demonstrated that LMW (4.6 kDa) had better activity to against yeast and fungi [51]. Moreover, the study of Hongpattarakere T. showed that the test of LMW chitosan against *C. aibicans* had increased antifungal activity [52].

This study showed that there was no difference of antifungal activity between oligomer chitosan and 30 kDa chitosan.

The lower concentration of chitosan had sufficient positively charged which bind to microbial surfaces [48]. Minimum fungicidal concentration of chitosan is 3 mg/ml.

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From the results of this study, there was no difference of antifungal activity between 3 mg/ml and 6 mg/ml of chitosan concentrations.

The prolong of chitosan exposure time with the yeast cell wall proved that the swelling and asymmetric shape of cells was occurred, so chitosan recommended for *C. albicans* control as strongly chemical fungicides [33].

The results of this study showed that the groups of chitosan with 12-hour immersion time had more antifungal effect than the groups of chitosan with 1-hour immersion time.

This study demonstrated only the effective of denture cleaning solutions to against *C. albicans*, while in a real situation chemical cleaning may have the effect to denture materials. So, the properties alteration of acrylic resins after soaking in denture cleaning solutions should have the further research, especially chitosan which is possible to use as newly denture cleanser.

CONCLUSION

As reported in this study, different chemical cleaning solutions had difference efficacy to removed *C. albicans* from polymethyl methacrylate. Polident® with 5 minutes of immersion time had the lowest efficacy. 0.2% chlorhexidine with 12-hour immersion time and chitosan with 1-hour immersion time had the same efficacy. Therefore, it can conclude that chitosan showed more efficacy than 0.2% chlorhexidine with lesser immersion time. All types of chitosan with 12-hour immersion time had the highest efficacy. Accordingly, chitosan is possible to use as a denture cleanser to reduce amount of *C. albicans*. The longer immersion time of all cleaning solutions showed the better antifungal effect.

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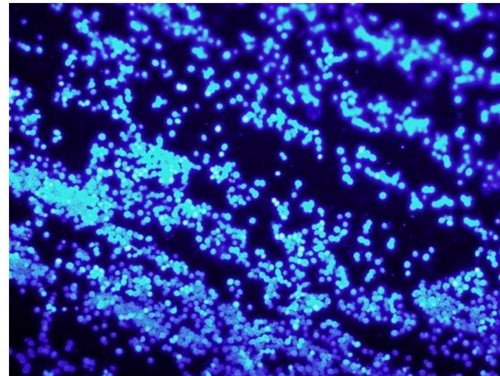
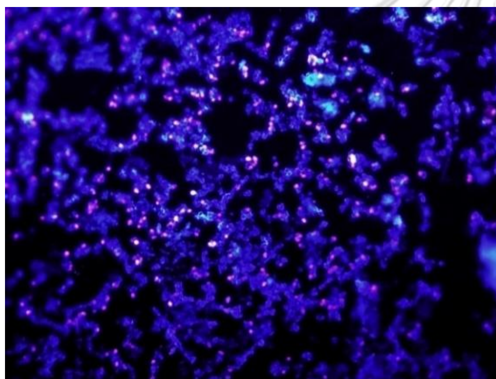
APPENDIX

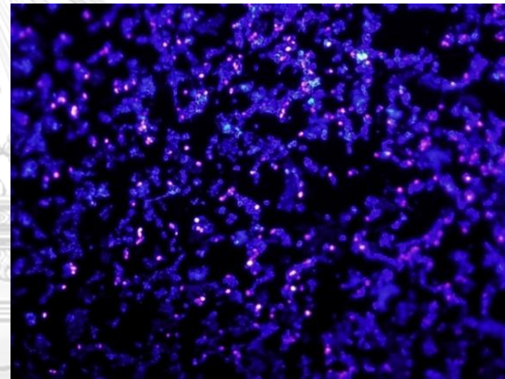
Appendix A. Photograph of *Candida albicans* after cleaning from fluorescence microscope



Distilled water



0.1% acetic acid



0.2% acetic acid

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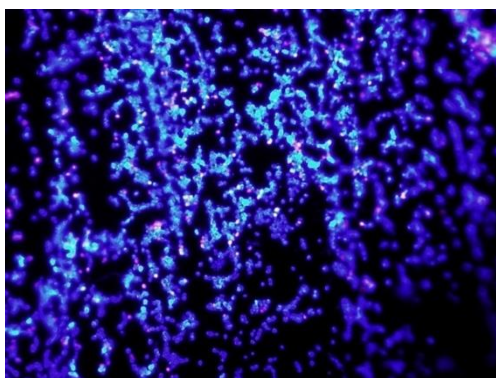
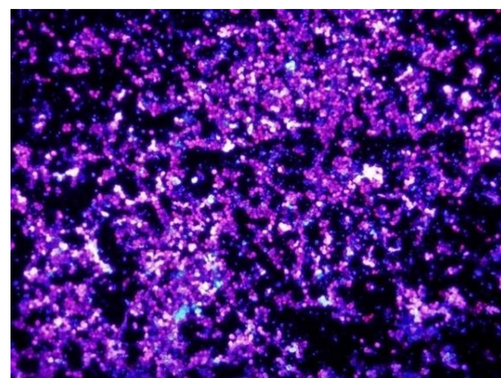
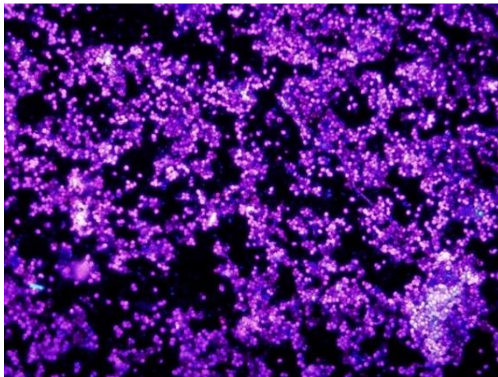


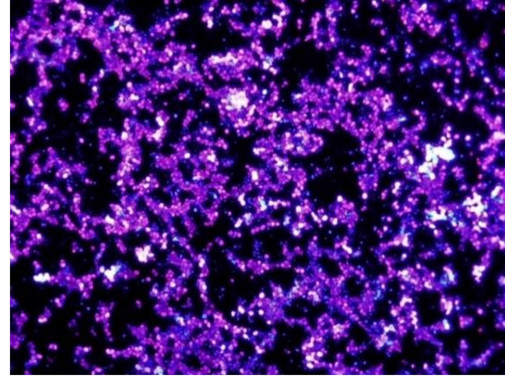
Polident®



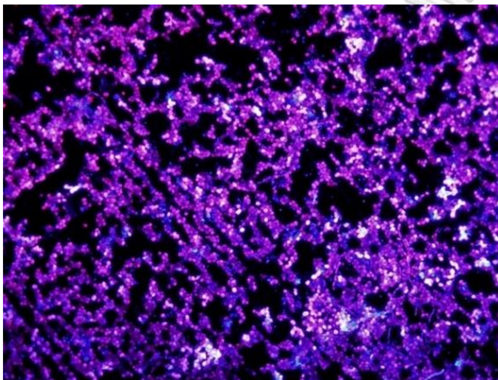
0.2% chlorhexidine



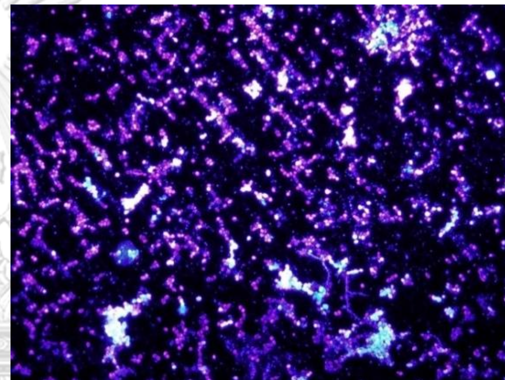
3 mg/ml oligomer



6 mg/ml oligomer



3 mg/ml 30 kDa chitosan



6 mg/ml 30 kDa chitosan

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The red of propidium iodide dye indicated the dead cells

The blue of calcofluor white dye indicated the live cells

Appendix B. The OD and percentage of *Candida albicans* viable cells after cleaning

Group/Time	1h			12h			5 mins			15 mins		
	OD	mean	%	OD	mean	%	OD	mean	%	OD	mean	%
Distilled water	0.336	0.367	91.55	0.336	0.367	91.55	-	-	-	-	-	-
	0.398		108.45	0.398		108.45	-	-	-	-	-	-
	0.388	0.402	96.52	0.388	0.402	96.52	-	-	-	-	-	-
	0.416		103.48	0.416		103.48	-	-	-	-	-	-
	0.389	0.383	101.70	0.389	0.383	101.70	-	-	-	-	-	-
	0.376		98.30	0.376		98.30	-	-	-	-	-	-
0.1% acetic acid	0.243	0.258	66.21	0.127	0.142	34.60	-	-	-	-	-	-
	0.247		67.30	0.153		41.69	-	-	-	-	-	-
	0.282		70.15	0.117		29.10	-	-	-	-	-	-
	0.248		61.69	0.112		27.86	-	-	-	-	-	-
	0.286		74.77	0.163		42.61	-	-	-	-	-	-
	0.243		63.53	0.181		47.32	-	-	-	-	-	-
0.2% acetic acid	0.153	0.152	41.69	0.111	0.126	30.25	-	-	-	-	-	-
	0.138		37.60	0.117		31.88	-	-	-	-	-	-
	0.182		45.27	0.111		27.61	-	-	-	-	-	-
	0.148		36.82	0.125		31.09	-	-	-	-	-	-
	0.159		41.57	0.168		43.92	-	-	-	-	-	-
	0.133		34.77	0.121		31.63	-	-	-	-	-	-
3 mg/ml oligomer chitosan	0.156	0.111	42.51	0.015	0.024	4.09	-	-	-	-	-	-
	0.118		32.15	0.032		8.72	-	-	-	-	-	-
	0.083		20.65	0.039		9.70	-	-	-	-	-	-
	0.106		26.37	0.005		1.24	-	-	-	-	-	-
	0.113		29.54	0.008		2.09	-	-	-	-	-	-
	0.089		23.27	0.044		11.50	-	-	-	-	-	-
6 mg/ml oligomer chitosan	0.111	0.100	30.25	0.067	0.046	18.26	-	-	-	-	-	-
	0.072		19.62	0.048		13.08	-	-	-	-	-	-
	0.098		24.38	0.004		1.00	-	-	-	-	-	-
	0.111		27.61	0.056		13.93	-	-	-	-	-	-
	0.102		26.67	0.069		18.04	-	-	-	-	-	-
	0.107		27.97	0.031		8.10	-	-	-	-	-	-

Group/Time	1h			12h			5 mins			15 mins		
	OD	mean	%	OD	mean	%	OD	mean	%	OD	mean	%
3 mg/ml 30 kDa chitosan	0.129	0.091	35.15	0.046	0.043	12.53	-	-	-	-	-	-
	0.067		18.26	0.036		9.81	-	-	-	-	-	-
	0.107		26.62	0.078		19.40	-	-	-	-	-	-
	0.032		7.96	0.022		5.47	-	-	-	-	-	-
	0.105		27.45	0.04		10.46	-	-	-	-	-	-
	0.106		27.71	0.034		8.89	-	-	-	-	-	-
6 mg/ml 30 kDa chitosan	0.162	0.118	44.14	0.038	0.065	10.35	-	-	-	-	-	-
	0.126		34.33	0.064		17.44	-	-	-	-	-	-
	0.107		26.62	0.056		13.93	-	-	-	-	-	-
	0.103		25.62	0.061		15.17	-	-	-	-	-	-
	0.088		23.01	0.085		22.22	-	-	-	-	-	-
	0.121		31.63	0.084		21.96	-	-	-	-	-	-
Polident	0.298	0.304	81.20	0.334	0.324	91.01	0.324	0.343	88.28	-	-	-
	0.322		87.74	0.348		94.82	0.335		91.28	-	-	-
	0.295		73.38	0.264		65.67	0.346		86.07	-	-	-
	0.273		67.91	0.361		89.80	0.362		90.05	-	-	-
	0.316		82.61	0.314		82.09	0.366		95.69	-	-	-
	0.322		84.18	0.322		84.18	0.326		85.23	-	-	-
0.2% chlorhexdine	0.241	0.261	65.67	0.041	0.050	11.17	-	-	-	0.329	0.295	89.65
	0.26		70.84	0.049		13.35	-	-	-	0.228		62.13
	0.29		72.14	0.063		15.67	-	-	-	0.326		81.09
	0.254		63.18	0.045		11.19	-	-	-	0.305		75.87
	0.262		68.50	0.068		17.78	-	-	-	0.31		81.05
	0.258		67.45	0.036		9.41	-	-	-	0.274		71.63

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Appendix C. The test of normality

Tests of Normality

Group		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
percent	distilled water 1h	.114	6	.200*	.996	6	.999
	0.1% acetic acid 1h	.165	6	.200*	.970	6	.890
	0.2% acetic acid 1h	.198	6	.200*	.950	6	.744
	[3] oligomer chitosan 1h	.180	6	.200*	.937	6	.636
	[6] oligomer chitosan 1h	.230	6	.200*	.920	6	.506
	[3] 30 kDa chitosan 1h	.282	6	.148	.914	6	.461
	[6] 30 kDa chitosan 1h	.211	6	.200*	.917	6	.481
	0.2% CHX 1h	.142	6	.200*	.981	6	.955
	Polident 1h	.257	6	.200*	.923	6	.527
	0.2% CHX 15min	.170	6	.200*	.974	6	.920
	Polident 5min	.148	6	.200*	.951	6	.745
	distilled water 12h	.114	6	.200*	.996	6	.999
	0.1% acetic acid 12 h	.215	6	.200*	.920	6	.508
	0.2% acetic acid 12h	.393	6	.004	.745	6	.018
	3] oligomer chitosan 12h	.219	6	.200*	.902	6	.387
	[6] oligomer chitosan 12h	.228	6	.200*	.900	6	.371
	[3] 30 kDa 12 h	.220	6	.200*	.919	6	.498
	[6] 30k Da 12 h	.197	6	.200*	.934	6	.613
	0.2% CHX 12h	.227	6	.200*	.943	6	.682
	Polident 12h	.238	6	.200*	.880	6	.268

*. This is a lower bound of the true significance.

Appendix D. Test of Homogeneity of Variances

Test of Homogeneity of Variances

percent

Levene Statistic	df1	df2	Sig.
1.263	19	100	.225

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Appendix E. The one-way ANOVA tests

ANOVA

percent

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	118626.608	19	6243.506	153.031	.000
Within Groups	4079.899	100	40.799		
Total	122706.507	119			

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