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Antifungal activity of microbial chondroitin sulfate against *Candida albicans*

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Abstract

A vaginal yeast infection called vaginal candidiasis is the most commonly seen type of *Candida* infections with a rate of 37%. Vaginal candidiasis affects three fourth (75%) of women in their lifetimes, and this is the most common cause of women seeking gynecological care. Vaginal candidiasis can result in serious symptoms such as discharge, itching, burning sensation, pain, rash and irritation in the vagina and vulva. *Candida albicans*, in particular, is the most common cause of vaginal candidiasis. Therefore, the treatment and control process of *Candida* species is very important in terms of health. In this study, as a first step, Microbial Chondroitin Sulfate was produced from Escherichia coli C2987 strain by using a specific microbial system and reliable biotechnological methods. After that, the efficacy of Microbial CS as an antifungal agent on *C. albicans* strains was tested by using the agar dilution method. As a result, it was determined that the Microbial CS was particularly effective against the yeasts *C. albicans*, and the minimum inhibitory concentration of Microbial CS was 0.32 g/mL. Our findings showed that Microbial CS can be used in the content of various biomedical products that can be used as an antifungal agent for *Candida* sp. Microbial CS can also be used to treat the disease alone or to support the treatment that increases the efficacy of antibiotics.

Keywords: Microbial chondroitin sulfate, *Candida* infection, *Candida albicans*, biotechnological drugs

Introduction

The severity of fungal diseases varies greatly depending on the type of yeast and the region of the body that is infected. In a study conducted in 2017, it was reported that more than $ 7.2 billion was spent on the treatment of fungal diseases in USA between 2005 and 2014 [1]. At the same time, it was also documented that *Candida* infections constitute the most substantial part of fungal diseases. Besides that, vaginal *Candida* is the most commonly seen type of *Candida* infections with a rate of 37% (Total cost $ 368 million) [1]. Moreover, published researches between 1985 and 2016 reported that vaginal candidiasis affects around 138 million women per year all around the world with a global annual prevalence of 3871 per 100.000 women [2].

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At least 75% of women suffer from vaginal candidiasis once in their lives. The most common yeast that causes vaginal candidiasis is *Candida albicans* following by non-*albicans Candida* species [3]. More than 85-90 % of cases of vaginal candidiasis originate primarily from *C. albicans*, followed by *C. glabrata* at 4-5%, and less from *C. tropicalis* and *C. parapsilosis* [4,5].

Vaginal candidiasis commonly seen in women of reproductive age and causes symptoms such as discharge, itching, irritation, burning sensation, vaginal pain, and rash [5,6]. Several factors predispose women to vaginal candidiasis, including uncontrolled diabetes mellitus, the use of high-estrogen contraceptives, steroids, and broad-spectrum antibiotics [5]. Imidazole antifungals (butoconazole, clotrimazole, miconazole), triazole antifungals (fluconazole, terconazole) and polyene antifungals (nystatin) are used intravaginally or orally for treatment of vaginal candidiasis [6-8].

Chondroitin Sulfate (CS) is a sulfated glycosaminoglycan (GAG) that is linear polysaccharides comprising repeating disaccharide units [9-11]. CS is used as an anti-inflammatory, antiviral, antioxidant, anti-infective agent in the field of medicine,

veterinary medicine, pharmacy, and cosmetics. It plays a role in tissue regeneration and blood lipids regulation. It is also foreseen using as a biomarker in cancerous cells and tissues [11-13].

Commercial CS is mostly produced from animal sources (pigs, sheep chicken keel, shark cartilage and other fish). Because of various risks such as bovine spongiform encephalopathy (BSE), H7N9 avian influenza and other food chain crises, researchers are dedicated to the synthetic production of CS [12].

The capsular polysaccharide of *E. coli* shows a very similar structure to chondroitin, and therefore chondroitin can be obtained from this structure [9,11]. Because of that, *E. coli* C2987 strain was used to produced Microbial CS in this study.

In addition to Microbial CS production, this study focused on antifungal activity of Microbial CS that was produced by a reliable biotechnological process.

Materials and Methods

Media

Luria-Bertani (LB) medium with ampicillin (80 mg/mL) was used for growth of recombinant *E. coli* which is source of Microbial CS. Luria-Bertani (LB) medium, consisting of 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl. Muller Hinton Agar (Merck K GaA- Germany) was used for agar dilution method to observe inhibitory effect of Microbial CS against *C. albicans*.

Strains

E. coli C2987 was obtained from NEB (New England Biolabs) and used for production of Microbial CS after transformation with pUC8:15. The plasmid pETM6_PACF transformed into *E. coli* C2987 strain responsible for capsular chondroitin synthesis (kfA, kfoC and kfoF genes) was obtained from Mattheos Koffas, Rensselaer Polytechnic Institute, Troy, NY. *C. albicans* ATCC 14053 purchased from American Type Culture Collection.

Production of Microbial Chondroitin

Plasmid pUC8: 15 and plasmid pETM6-PACF were transformed into a non-pathogenic *E. coli* strain. Transformed cells are inoculated into LB-amp agar plate and incubated at 37 ˚C for an overnight. Two colonies from each plate were picked and transferred to a 2-liter Erlenmeyer flask and incubated with shaking at 140 rpm at 37 ˚C overnight.

For purification of capsular chondroitin, cell pellet was re-suspended in water and autoclaved in the liquid cycle for 15 min. The supernatant was collected and centrifuged to remove insoluble material. Both autoclaved supernatant from the cell pellet and cell culture supernatant were precipitated with 80 vol % cold ethanol and stored in an explosion-proof refrigerator at 20 ˚C overnight allowing the recovery of both intracellular and extracellular chondroitin. After precipitation, pellet was collected and re-suspended in digestion buffer (100 mM Tris, pH 7.5, 50 mM MgCl2, 10 mM CaCl2). DNAse (1 mg/l, Sigma) was added and the sample incubated at 37 ˚C for 1 h. Protease K (2.5 mg/mL, Sigma) was then added and the sample was incubated at 56 ˚C for 2 h. A second precipitation from 80% cold ethanol was then carried, out and the dry pellet was collected, re-dissolved in water and filtered through a 10 KDa spin column to remove residual small peptides and salt.

Chondroitin sulfation method

Firstly, 4-6 g of the synthesized microbial chondroitin are dissolved in 80-120 mL of aqueous DMF (Dimethyl Formamide) and the solution is cooled to 0-5 ºC. Secondly, 12-15 grams of pyridine sulfotrioxide was added to the solution. The solution in room temperature was precipitated by the addition of 400-800 mL of NaCl saturated acetic acid. The mixture was filtered. This solid was dissolved in 200-300 mL of deionized water and the produced solution was neutralized with 1 N NaOH. After neutralization, the solution is heated to 30-50 °C and 0.2-0.3 N 60 mL NaOH is added. After waiting for 1-3 hours at this temperature, the solution was neutralized with 1 N HCl acid. This solution was then filtered through a membrane with a conductivity of less than 10 µS. Eventually, the solution is lyophilized and dried under vacuum. As a result, the produced CS was analyzed and confirmed by one-dimensional 1H nuclear magnetic resonance (NMR). NMR experiments were performed on a Bruker Advance III HD 600 MHz spectrometer.

Antifungal activity assay

Antifungal activity of Microbial CS was determined by using the agar dilution procedure. Agar dilution method was carried out by using different concentrations of the Microbial CS into a Muller Hinton agar medium. We add 3.6, 3.0, 2.4, 1.8, 1.2, 0.6 and 0.3 g. Microbial CS to 7.5 mL Muller Hinton agar to obtain seven different levels of 48 %, 40 %, 32 %, 24 %, 16 %, 8 % and 4 % (w/v) respectively.

C. albicans was subcultured on Muller Hinton Agar. Direct colony suspension is made in distilled water and turbidity adjusted to 0.5 McFarland standard (1x106 cfu/mL). A loopful (10 µl) of the standardized inoculum of the yeast cells inoculated to the surface of agar plates. Growth is assessed after incubation at 37 ˚C for two days (48 h).

Results

The results of antifungal activity assay are shown in Figure 1. After two days of incubation at 37 ˚C, the petri dishes of the control group (0 g/mL), 0.04, 0.08, 0.16 and 0.24 g/mL Microbial CS groups were entirely covered with white colonies of *C. albicans*. Furthermore, the colonies observed in the 0.24 g/mL Microbial CS petri dishes were wholly covered with white colonies in spite of having a one-day delay in their growth compared to the control. No colonies were detected on the 0.32, 0.4 and 0.48 g/mL and beyond Microbial CS-treated petri dishes during the two-day incubation (Figure 1). The lowest concentration of Microbial CS that prevented visible growth was the minimal inhibitory concentration (MIC). Therefore, the MIC of Microbial CS against *C. albicans* was determined to be 0.32 g/mL.

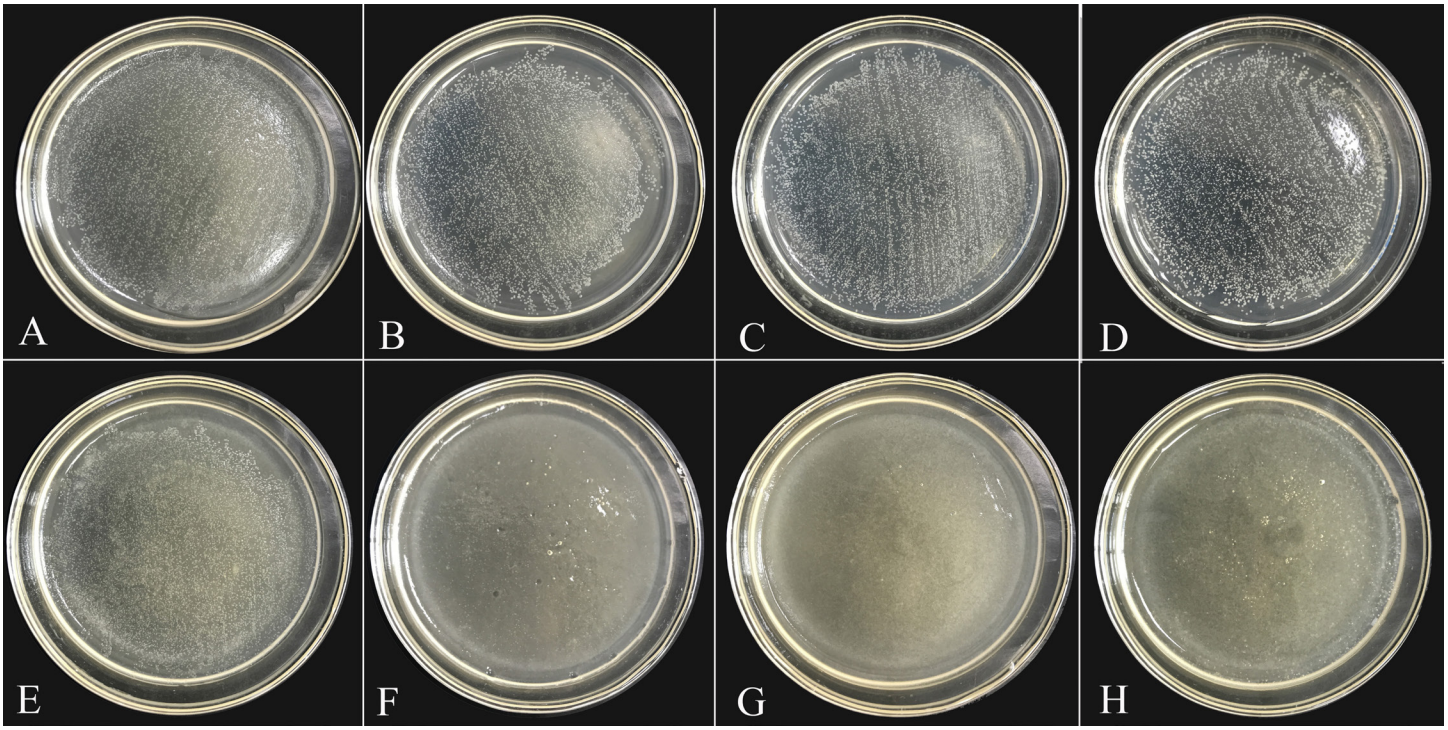

Figure 1. Petri dish pictures of Microbial CS antifungal activity assay with different concentrations (g/mL) against *C. albicans* in overnight incubation using agar dilution method. (A) Control, (B) 0.04, (C) 0.08, (D) 0.16, (E) 0.24, (F) 0.32, (G) 0.4, (H) 0.48 g/mL.

Discussion

Microbial CS that is observed as an effective antifungal agent in this study, can be used as an alternative therapy in patients with vaginal candidiasis.

Antifungal drug resistance is an important problem in vaginal candidiasis and many other fungal diseases. Moreover, its incidence rate has increased in several medical centers during the last few decades [6,14]. At the same time, the long duration of treatment of fungal diseases and the challenges in treating patients directed the researchers to alternative channels. For these reasons, it is important to use alternative antifungal agents instead of antifungal drugs.

The frequency of vaginal candidiasis is more frequent in pregnant patients, especially after treatment with antibiotics [2]. In addition to that, higher levels of estrogen and glycogen in vaginal secretions in the case of pregnancy increase the risk of developing vaginal candidiasis [15]. Microbial CS is natural, antiallergic and biocompatible compound. So, these features make it more preferable to antifungals. Therefore, it can be used as effective support for the treatment of vaginal candidiasis especially in pregnant patients after examination of possible fetal effects on experimental animal models.

Vaginal candidiasis is usually treated with oral antifungal therapy. However, a chronic subtype described in some studies might be more difficult to treat and may need to be supported by intravaginal drugs [16,17]. In this case, Microbial CS, which adheres well to tissue surface, might be a good alternative agent for intravaginal therapy because of its structural features. Microbial CS can be used to treat the disease alone or to support the treatment that increases the efficacy of antibiotics with its adhesion and diffusion facilitating properties. It is also estimated that these structural properties of Microbial CS may be an important cause of antimicrobial properties.

In a cohort study, it was found out that non-*albicans Candida* infections had not affected by recent antibiotics use, diabetes, and probiotic use [18]. In addition to that, vaginal candidiasis caused by non-*albicans Candida* can be more difficult for treatment, and usually require more aggressive remedy [6,19]. Microbial CS which is observed as an effective antifungal compound in this study can also be used against non-*albicans Candida* which are difficult to treat. If the activity of Microbial CS on non-*albicans Candida* is also proven, we can reduce both the long- term treatment process and the patient's suffering from this issue and decrease the economic impact of the treatment.

Millions of dollars are spent worldwide for the treatment of vaginal candidiasis. Hospitalizing patients, using more antifungal medicines cause lost time from work and unnecessary effort. The use of Microbial CS for treatment may reduce the economic damage and protects the patient from the side effects of antifungal drugs.

Conclusion

Results of antifungal activity assay showed that Microbial CS was particularly effective against the yeasts *C. albicans*.

As a future study, MIC value of Microbial CS against *C. albicans* at concentrations between 0.24 and 0.32 g/mL can be detailed. And, MIC of Microbial CS can investigate against non-*albicans Candida* strains.

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Conflict of interest
We declare that we have no conflict of interest.

Financial Disclosure
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Ethical approval
Ethics committee approval was obtained

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