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Estimation of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Antimicrobial peptides of *Saccharomyces boulardii* against Selected Pathogenic Strains

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Estimation of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Antimicrobial peptides of *Saccharomyces boulardii* against Selected Pathogenic Strains

Abstract

Saccharomyces boulardii is used for antimicrobial peptide production and so can be termed as bacteriocin molecules with probiotic potential. Antimicrobial peptides are extracted from the culture broth and followed the purification using Sephadex G-50 column. Further, the inhibition activity was determined against selected microorganisms and inhibition zone against *streptococcus pneumoniae* was found to be highest among selected microbes. The fourth fraction of the peptide after purification was shown a 26 mm bacterial inhibition zone determined by agar well diffusion method. SDS-PAGE analysis revealed the protein band corresponding to 18 kDa was observed. The MBC/MIC study proved that the peptide extract of *S. boulardii* has bactericidal activity.

Keywords

S.boulardii, antimicrobial activity, anti-oxidant activity, S. aureus, Antimicrobial peptides

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Authors Contribution All authors have contributed equally in carrying out research work

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1. Introduction

Antimicrobial peptides (AMPs) are oligopeptides with a varying number of amino acids. AMPs are very active against multidrug resistant gram positive bacteria and kill them by disrupting membrane integrity via interaction with negatively charged cell membrane [1,2]. Probiotics are microorganisms that are beneficial to humans and animals. Probiotics are used in multifarious subjects and their branches ranging from depression to autoimmune diseases and are being used as *bio-therapeutic* agent [3]. Probiotics reduce gastrointestinal disorders, allergies, diarrhea, bacterial vaginal infections, cholesterol levels, necrotizing enterocolitis, auto-immune diseases and skin ailments. *Saccharomyces boulardii* is a probiotic microbe obtained from lychee fruit and mangosteen fruit and it can be treated as natural yeast [4]. It is found in yoghurt which is traditionally taken after meals to relieve constipation, diarrhea and other abnormalities in bowel moments. It is also found in other foods like kefir, kamahi, kombucha, miso etc. It can be used against antibiotic-induced diarrhea and *Clostridium difficile* infections [5]. The supplement of probiotic *S. boulardii* along with antibiotics is used to reduce diarrhea and stomach pain in patients suffering from amoeba-related infections. It can also help in treating lactose intolerance, vaginal yeast infections, canker sores irritable bowel syndrome (IBS), crohn's disease etc. [6]. *S. boulardii* synthesizes the inhibition factors such as, polyamines, alkaline phosphomonoesterases, protease serine enzyme and short-chain fatty acids. The probiotics has numerous attributes namely: production of short-chain fatty acids, immunomodulation, bile-salt metabolism and epithelial barrier maintenance which makes them viable for the extraction of antimicrobial peptides. Antimicrobial peptides (AMPs) are increasingly coming into the focus as a new treatment strategies for bacterial infections [7]. Only a few reports are available on the antimicrobial peptides of probiotics and hence the present study is aimed to extract the peptides from *S. boulardii* and then its potency is screened for antimicrobial activity against selected pathogenic microorganisms.

2. Materials and methods

2.1. Antimicrobial peptides from *S. boulardii*

S. boulardii was isolated from probiotic product G-norm using MRS broth medium by enrichment method

and pure culture was developed on MRS agar plates. *S. boulardii* was inoculated in yeast extract peptone dextrose medium and incubated at 37 °C for 48 h. The cells were removed by centrifugation at 13,000 RPM for 20 Min., and supernatant was collected separately and then filtered using 0.45 µm membrane, followed by dialysis (10–12 kDa). The dialyzed crude extract was lyophilized. For purification of the antimicrobial peptides, lyophilized crude sample was applied on sephadex G-50 column. The fractions were collected at regular intervals using 0.1 M phosphate buffer and absorption values were recorded at 280 nm. The antimicrobial peptides present in lyophilized and purified fractions were separated by SDS-PAGE (12%) analysis [8].

2.2. Proteolytic activity

The proteolytic activity of antimicrobial peptides was confirmed by treating with protease. The cell free supernatant is mixed with enzyme at concentration 1.0 mg/mL and then incubated at room temperature for 1 h. After incubation, the antimicrobial activity was evaluated by well diffusion assay using selected indicator organisms. The cell free supernatant without the enzyme was kept as a control [9].

2.3. Antimicrobial activity

The antimicrobial activity of extracted peptides was determined by agar well diffusion assay. Wells were created aseptically on sabouraud dextrose agar (SDA) for determining antifungal activity and *Mueller-Hinton agar* (MHA) for antibacterial activity respectively, crude and partially purified antimicrobial extracts were added to the wells from 1 to 10 µg/ml (w/v) concentrations. The fungal and bacterial cultures were inoculated and then the plates were incubated at room temperature for 48 and 24 h, respectively. Chloramphenicol (10 µg/mL) and sterile distilled water were used as positive and negative controls [10]. Antimicrobial compound activity was measured in terms of both MIC (Minimum inhibitory concentration) and MBC (Minimum bactericidal concentration).

2.4. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Agar well diffusion method was carried out for MIC determination. Wells were created aseptically on

Sabouraud Dextrose Agar (SDA) for determining antifungal activity and Muller-Hinton agar (NA) for antibacterial activity was used in the study. The crude and partially purified antimicrobial protein extracts were added to the wells in the concentration ranges from 10 to 100 $\mu\text{g/mL}$ (w/v). Fungal and bacterial cultures were inoculated and incubated at 30 °C in different time intervals i.e., 48 h for fungal and 24 h for bacterial strains respectively. Chloramphenicol (10 $\mu\text{g/mL}$) and distilled water were used as positive and negative controls. The clear zone around well is indicated as zone of inhibition and the diameter was measured.

For MBC, the broth micro dilution assay was performed by serially diluting the 96-well micro titer plate with extracted peptide fraction and then the plate is inoculated with test strains followed by incubation at 30 °C for 48 h and 24 h respectively. Bacterial growth was estimated by measuring the absorbance at 660 nm using microtiter plate reader. The wells showing >90% inhibition were taken into consideration by transferring 5 μl of the contents onto a nutrient agar plates followed by the incubation at similar conditions. The least concentration with no revival was considered as MBC. Known antibiotics were taken as control. Further, MBC/MIC is also calculated to determine the bacteriocidal effect of peptide extract.

Statistical analysis ANOVA analysis and Turkey's post hoc methods were used to analyze the data normalization. P-values less than 0.05 are considered significant. The Standard errors of the mean values were conveniently represented as \pm symbol and the obtained results with standard error of the mean were tabulated.

3. Results and discussion

3.1. Partial purification of antimicrobial peptides from *S.boulardii*

Antimicrobial peptides extracted from *S. boulardii* were purified and each of the active fractions was screened for the antimicrobial activity against selected microbes. Probiotic yeast has attracted the interest of scientists to work on in recent years and the probiotic microbes are used to explore antimicrobial properties. The cell free supernatant of probiotic yeast contains the antimicrobial peptides is purified by dialysis and gel permeation chromatography. The fractions are collected from the chromatography column and the total concentration of protein in each fraction was estimated. The SDS-PAGE analysis revealed that the fraction-4 containing antimicrobial peptide obtained after purification process from the extract of *S. boulardii* has shown good inhibition activity against selected bacteria and fungi. The protein concentration in each the fraction has shown in Fig. 1 and highest concentration was found to be 79 $\mu\text{g/mL}$ in fraction-4. The molecular weight of the purified peptide fraction was found to be 18 kDa. Crude extract was shown various protein bands and disappeared after purification (Fig. 2). The concentration of total proteins in crude and purified samples was shown in Table 1.

3.2. MIC and MBC

The antimicrobial activity of peptides extracted from *S. boulardii* was tested against different bacterial

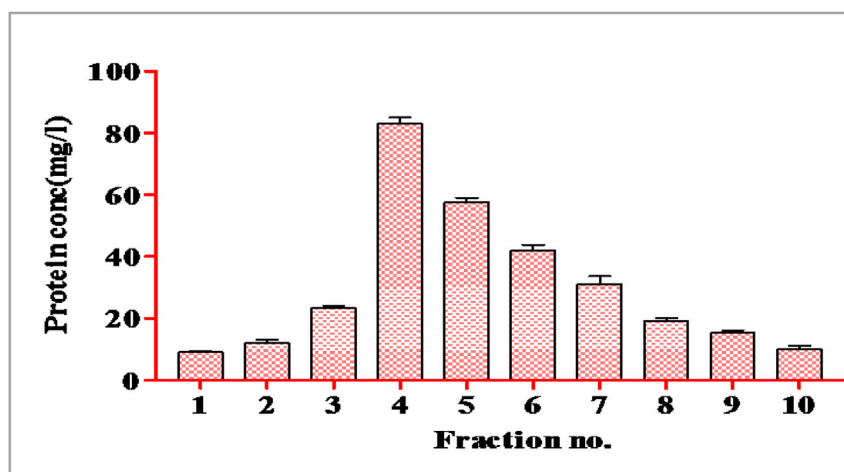


Fig. 1. Elution profile of antimicrobial proteins on Sephadex G-50 column chromatography.

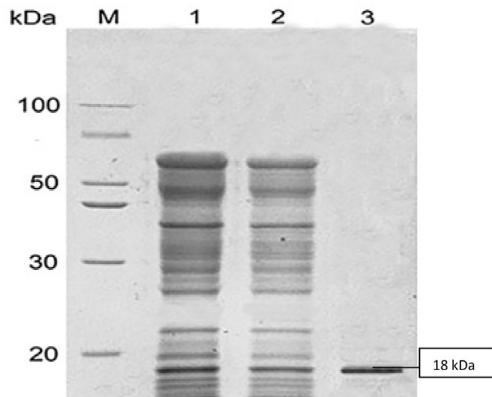


Fig. 2. The molecular weight of antimicrobial peptides determined by SDS-PAGE method compared with standard proteins (Lane M-marker; Lane 1&2- crude cell free extract; Lane 3- purified band at 18 kDa (fraction-4).

Table 1
Total protein concentration of peptide fractions from *S.boulardii*.

Volume (ml)	Sample	Total Protein (mg/L)
20	Dialyzed Crude	167 ± 3.1
20	Sephadex G-50	79 ± 2.9

and fungal strains in terms of MIC and MBC respectively. The cell free supernatant when treated with protease enzyme found no zone of inhibition against selected indicator organisms which indicates that the peptide is proteinaceous in nature. Results have shown that the highest activity was recorded against *staphylococcus aureus* (26 mm zone). The previous studies

reported that the peptides from *Paenibacillus alvei* AN5 strain showed zone of inhibition ≤ 21 mm with MIC 64 $\mu\text{g/ml}$ against *Staphylococcus aureus* [11]. The peptide extracted from *S. boulardii* showed highest antimicrobial activity (26 ± 0.71 mm) with 40 $\mu\text{g/ml}$ compared to findings of Alkotaini et al. (2013). Peptide fraction showed significant antimicrobial activity against *K. pneumoniae*, *P. Vulgaris*, *P. aeruginosa*, *H. pylori*, *B. cereus*, *S. aureus* and inhibitory activity was not found against *Asperigillus niger* and minimal effect was found against *Candida albicans*. The MICs of extracted peptide ranging from 10 to 250 $\mu\text{g/mL}$ obtained after susceptibility tests represented good antimicrobial activity against the tested pathogens. MBCs of peptide clearly indicated bactericidal effect against pathogens especially on *S. aureus*. The effect of peptide extract on selected indicator organisms has showed the bactericidal activity. The zone of inhibition values was found to be low for *A. tumefaciens* and *C. albicans* at 500 $\mu\text{g/ml}$ and 100 $\mu\text{g/ml}$ respectively. The antibiotic chloramphenicol is used standard and its effect for MIC and MBC against the selected pathogens was also studied and the findings of the study were included in Table 2. In previous studies, AL-Saadi et al., 2016 reported that the cell-free extracts of *Bifidobacterium* species are effective against the methicillin-resistant *Staphylococcus aureus* [12]. The $\text{MBC/MIC} \leq 2$ and $\text{MBC/MIC} \geq 4$ implies the bactericidal and bacteriostatic effect respectively [13]. Further, ratio of MBC/MIC is calculated and the concentration of MBC/MIC is ≤ 2 for peptide extract of *S. boulardii* against the indicator organisms which indicates that the extract has bactericidal activity.

Table 2
MIC and MBC analysis for the antimicrobial peptide fraction of *S. boulardii* and chloramphenicol.

Name indicator organisms	Antimicrobial activity (MIC-Zone of inhibition (100 $\mu\text{g/ml}$))	Concentration of peptide in $\mu\text{g/mL}$ for MIC	CFS treated with Protease enzyme	Concentration of peptide in $\mu\text{g/mL}$ for MBC	Chloramphenicol (10 $\mu\text{g/ml}$)	MIC with Chloramphenicol ($\mu\text{g/mL}$)	MBC with Chloramphenicol ($\mu\text{g/mL}$)
<i>K. pneumoniae</i>	20 ± 1.21	10	0.0	20	24 ± 0.25	3	5
<i>P. vulgaris</i>	22 ± 1.16	25	0.0	50	22 ± 1.12	2	4
<i>P. aeruginosa</i>	18 ± 0.85	20	0.0	40	24 ± 0.78	2	4
<i>H. pylori</i>	16 ± 0.21	30	0.0	60	26 ± 0.56	3	5
<i>A. tumefaciens</i>	04 ± 0.08	250	0.0	500	20 ± 1.12	20	40
<i>B. cereus</i>	20 ± 0.65	10	0.0	15	18 ± 0.45	8	16
<i>S. aureus</i>	26 ± 0.71	40	0.0	70	26 ± 1.16	1	2
<i>A. niger</i>	ND	ND	0.0	ND	12 ± 0.44	80	100
<i>C. albicans</i>	08 ± 0.21	70	0.0	100	16 ± 0.32	50	100

4. Conclusion

The extracted peptide from *S.boulardii* confirmed the antimicrobial activities against pathogenic strains. The peptide has shown high bactericidal effect against *S. aureus*. Therefore, the extracts from *S. boulardii* could be used source for developing antimicrobial agents.

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