Isolation of a Novel Antimicrobial Polypeptide from Aspergillus Niger Isolate LC3

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Abstract

There is a need to discover new antimicrobial agents due to increasing resistance to antibiotics. Researchers focused on AMPs (antimicrobial polypeptide) for these new agents discovered before antibiotics and did not develop resistance as much as antibiotics. In this study the extracellular proteins from isolate LC3 belonging to *Aspergillus* were purified for new AMP discovery and then tested for antimicrobial activity against *Staphylococcus aureus* (ATCC 25923) and Methicillin-resistant *Staphylococcus aureus* (MRSA). Antimicrobial activity was determined by the trypsin/proteinase K assay, which was polypeptide-based, and it was observed that this protein was a protein of about 11 kDa by gel overlay assay. Then the minimum inhibitory concentration of purified AMP molecule against *S. aureus* ATCC 25923 and MRSA was 8 µg/ml and 32 µg/ml, respectively and the AMP molecule was confirmed. ITS sequence analysis showed that isolate LC3 was identified as *Aspergillus niger*, using the Bioedit sequence assembly program, the sequence was deposited with the GenBank database with accession number MK332597. These results indicate that the purified AMP molecule has the potential to be used in infections caused by *S. aureus*.

1. Introduction

With penicillin's inhibition effect on microorganisms, antibiotics have also begun to be used in infectious diseases. However, microorganisms have gained resistance over time by various mechanisms. The main problem confronting us is that antibiotics lose their effectiveness over time. The emergence of antibiotic-resistant microorganisms and increasing concerns about the use of antibiotics have led to the development of AMPs, which have effective application prospects in medicine, food, husbandry, agriculture and aquaculture. AMPs are not affected by many antibiotic resistance mechanisms that prevent antibiotics use (Apan 2004; Huan et al. 2020). AMPs do not induce widespread resistance due to cell membrane specificity and effects on specific protein targets. Bacteria acquire existing resistance as a result of gene transfers. Due to these properties, they are attracting attention as a source of new antimicrobial agents (Zasloff 2002; Yazici et al. 2018).

AMPs are small polypeptides that are important members of the natural immune system and have 12–100 amino acids synthesized by various organisms, usually with broad-spectrum microbicidal activity (Hancock and Diamond 2000). Researches have shown that AMPs do not only have antimicrobial properties but also have immunomodulator, apoptotic, anticancer and anti-inflammatory properties (Hancock and Diamond 2000; Bachère et al. 2004). Although AMPs are generally cationic, there are also anionic ones that contain a few acidic amino acids such as aspartic acid and glutamic acid (Bachère et al. 2004; Jenssen et al. 2006). They are evolutionarily protected molecules found in many organisms, from prokaryotes to humans. The therapeutic applications of AMPs are still limited due to the high cost of production, enzymatic degradation, short half-life. Moreover, these peptides show enormous toxic side effects on mammalian cells in long-term use. Researchers focused on biological resources to overcome these problems and discover new antimicrobial resources. New biological discoveries for AMPs will also pave the way for in silico approaches in terms of providing new gene resources (Jenssen et al. 2006;

Huan et al. 2020). Lysozyme, the first reported human AMP, was described in 1922 by Alexander Fleming from the nasal mucus (Nizet et al. 2001). Since the discovery of lysozyme and other AMPs, a total of 3324 antimicrobial proteins have been identified in the Antimicrobial Polypeptide Database (ADP3). Over 2,500 polypeptides belong to mammals and plants, followed by bacteria. However, there are 22 records of the fungi. Only three of these belonged to the *Aspergillus* includes antibacterial polypeptide producer species (https://aps.unmc.edu/AP/). Filamentous fungus species found in nature produce metabolites of industrially and medically importance, such as antibiotics, AMPs. The antimicrobial agents, which are the secondary metabolites, control bacterial infections and are used in various pharmaceutical processes (Zjawiony 2004). Researchers have shown that compounds produced by fungus have approximately 126 different therapeutic functions (Wasser 2011). The majority of fungi variety have been found to have antibacterial, antiviral, antioxidant, anti-inflammatory, antitumor and immune modulation effects (Smith et al. 2003). These effects indicate that fungi are a potential source for discovering new AMPs. Only a few studies show that fungi belonging to the genus *Aspergillus* have antimicrobial properties (Gun Lee et al. 1999; Kalyani and Hemalatha 2017; Subhash et al. 2022).

The isolate LC3 of *Aspergillus*, a filamentous fungus, was chosen as the source of this study. *S. aureus* and MRSA cause clinical infections worldwide (Tong et al. 2015). There is a need for new therapeutic compounds against these microorganisms. The aim here is to report to purification of a novel AMP from the isolate LC3 to combat these microorganisms. The results exhibit a potent antimicrobial effect of a polypeptide of about 11 kDa purified from the isolate LC3.

2. Material And Methods

2.1. Microorganisms and maintenance

Antimicrobial-producer the isolate LC3 isolated from soil in the previous study was investigated for new AMP production (Yazici et al. 2021). The antimicrobial activity of the culture filtrate obtained from the isolate LC3 was confirmed by proteinase K/trypsin treatment, which was polypeptide-based. *S. aureus* ATCC 25923(clinical isolate) and Methicillin-resistant *S. aureus* (MRSA)(clinical isolate) were used as the reference pathogens. Reference pathogens were maintained in Mueller Hinton agar (MHA) at 37°C.

The isolate LC3 was incubated for 7 days at 25°C by shaking at 150 rpm in YPG medium(yeast-peptone-glucose). For the evaluation of AMP production according to days and the effect of physiological conditions on AMP production, fermenter production was carried out after flask culture. For this, inoculated 10 ml of the reference culture grown on 7 day YPG medium to the 3-liter fermenter. The isolate LC3 was grown according to the parameters pH, temperature, shake speed and dissolved oxygen level; 6.2, 25°C, 300 rpm, %10 ± 2, respectively in fermenter.

The cell-free supernatants (CFS) were prepared for use in agar diffusion, trypsin/ proteinase K treatment and protein isolation experiments. After 7 days of incubation of the isolate LC3 in YPG medium, the

mycelium was filtered with four cheesecloth layers, then centrifuged for 10 min at 9,000 rpm. The supernatant was passed through a 0.22 µm syringe filter and stored at 4°C for further analysis.

2.2. Determination of Antibacterial Effect of Isolate LC3

2.2.1. Agar Diffusion Assay

Agar diffusion assay was used to determine the antimicrobial activity of the isolate LC3. In the agar diffusion assay, a soft MHB was prepared by the pour plate method. Wells were punched out with a 6 mm cork borer and 200 µl of culture filtrates were added to these wells. The Petri plate was incubated at 30°C for 24 hours. At the end of the period, inhibition zone diameters were measured (Wiegand et al. 2008).

2.2.2. Cross Streak Assay

The isolate LC3 was first grown in YPG agar medium at 25 ° C for 7 days. Then, on the 7th-day, bacteria strains were inoculated by a single streak of inoculum to stay 1 cm to the fungus colony zone. The growth of bacteria on day 8 was evaluated (Lertcanawanichakul and Sawangnop 2011).

2.3. Protein isolation and gel overlay assay

The ultrafiltration was performed to concentrate the culture filtrates obtained from the liquid culture of the isolate LC3. 60 ml of CFS was added to 3 kDa cut-off ultrafiltration column and concentrated. For total protein isolation, concentrated CFS was mixed with an equal volume of 20% TCA (trichloroacetic acid) by pipetting and incubated at -20°C for 2 hours. At the end of the period, the mixture was precipitated by centrifugation at 13500 rpm for 10 minutes. It was washed 3 times with 70% ethanol and centrifuged at 13500 rpm for 5 minutes. Finally, after washing with acetone, the pellet was dried at room temperature and stored at -20°C (Chen et al. 2005).

The ultrafiltration process was repeated for fermenter culture for 7 days for protein isolation and antimicrobial assays. Protein samples were run at 80 V for 3 hours in SDS-PAGE. When the running was complete, the gel was removed from the cassette and stained with Coomassie R-250 for 20 minutes. It was left in the wash solution for 1 hour to display the proteins. The gel was photographed with the Bio-Rad gel imaging system (Schägger 2006). To confirm the antimicrobial activity and to determine which protein band is responsible for this activity, a gel overlay assay was used to test for antibacterial activities in CFS. For gel overlay assay, the CFS sample was run at 150 V for 1 hour in native-PAGE (non-denaturing electrophoresis). The gel was then divided into two parts after electrophoresis. The first gel was used for the image, and the second gel was used for the gel overlay assay. The gel overlay assay was applied by modifying Valore et al (1996)'s assay (Valore et al. 1996).

Briefly, a poor agarose (0.03% TSB in 0.01 M sodium phosphate, pH 7.4, with 1% agarose) with low sodium concentration was mixed with the pathogen to make up the bottom layer. A piece of the native-PAGE gel is placed on top at 37 ° for 3 hours for proteins to diffuse from the gel onto the poor agarose. The gel was then removed, and the a piece of poor agarose containing the gel was cut. Poor agarose piece placed on a rich agarose (6% TSB in 0.01 M sodium phosphate, pH 7.4, 1% agarose).

As a result of overnight incubation, the protein corresponding to the clear zone was found to be responsible for the antibacterial activity (Valore et al. 1996).

2.4. Purification of AMP

According to the gel overlay assay results, the protein responsible for antimicrobial activity was centrifuged at 13500 rpm for 10–15 minutes using the appropriate cut-off ultrafiltration column (30 kDa). Then, the protein sample was stored at -20 ° C for MIC determination.

2.5. Determination of Minimum Inhibitory Concentration (MIC)

To determine the Minimum Inhibitory Concentration (MIC) value of the polypeptide isolated from isolate LC3, the protocols received from the European Committee for Antimicrobial Susceptibility Testing (EUCAST) have been applied (http://www.eucast.org/). MIC is the smallest amount of concentration that prevents the development of microorganisms.

A broth microdilution method was performed to determine MIC value against *S. aureus* and MRSA. Briefly, 100 µl of bacterial cells at a concentration of 0.5 McFarland in 96-well plates and protein isolated at different concentrations (0.25, 0.5; 1; 2; 4; 8; 16; 32; 64; 128 µg / ml) were added. The total volume was completed to 200 µl. The plates were incubated for 24 h at 37°C. At the end of the period, MICs were determined.

2.6. Identification of Fungus

DNA isolation was performed manually from a 7-day solid culture with the phenol-chloroform assay. 1 ml of lysis buffer was added to a sample of mycelium taken into 2 ml of Eppendorf tubes. It was in the homogenizer at a maximum speed of 30 minutes. After centrifugation at 12000 rpm for 10 minutes, the supernatant was taken up in a new tube and 2 µl of RNase A was added. Incubate at 37 ° C for 15 minutes. 1 volume of phenol: chloroform: isoamyl alcohol (25: 24: 1) was added to mixture. It was vortexed and centrifuged at 12000 rpm for 10 minutes. The upper part was taken into the new tube and the DNA was precipitated with 100% ethyl alcohol at -20 ° C for 1 hour. After centrifugation at 12000 rpm for 10 minutes, the pellet was washed three times with 70% ethyl alcohol and dried in 50 µl TE buffer (Sambrook and Russell 2006).

Amplification of the ITS Region with PCR was performed to make molecular identification. ITS 1 primer (5 'TCC GTT GGT GAA CCA GCG G 3') and ITS 4 primer (5 'TCC TCC GCT TAT TGA TAT GC 3') were used to amplify the 18S-ITS1-5.8S-ITS2-28S region. The PCR conditions include; initial denaturation (95°C for 2 min); 35 cycles [95°C for 45 sec, 55°C for 45 sec, 72°C for 1 min] and a final extension at 72°C for 10 min. The amplicons were sequenced using ITS1 and ITS4 primers. Then, the Bioedit sequence assembly program was used and submitted to the BLASTN 2.13.0+ (Zhang et al. 2000).

3. Results

3.1. Determination of antimicrobial activity

3.1.1. Agar Diffusion Assay

200 µl of the culture filtrate (CFS) was added to the wells (punched out with 6 mm cork borer) containing a soft MHB medium prepared by the pour plate method. The Petri plate was allowed to incubate at 30 ° C for 24 hours. At the end of the incubation period, the inhibition zones were measured. The results show that the culture filtrate of the isolate LC3 has an antimicrobial effect against *S. aureus* (ATCC 25923) and MRSA. The agar diffusion assay results of the culture filtrates from the flask culture and fermenter culture are shown in Table 1. While CFS obtained from flask culture showed antimicrobial activity from the 4th day of growth, CFS obtained from fermenter culture started to show activity from the 6th day.

Table 1. Agar Diffusion Assay Results (flask and fermenter culture)

	Staphylococcus aureus (ATCC 25923)				MRSA			
	4.day	5.day	6.day	7.day	4.day	5.day	6.day	7.day
Flask culture	+++	+++	+++	+++	+++	+++	+++	+++
Fermenter culture	-	-	++	++	-	-	++	++

*+; Zone less than 10 mm, ++; Zone between 10–20 mm, +++; It represents zone diameters larger than 20 mm.

3.1.2. Cross Streak Assay

The cross-streak assay is performed to determine whether microorganisms have antagonistic effects against each other (Lertcanawanichakul and Sawangnop 2011). The isolate LC3, *S. aureus* and MRSA bacteria were cultured on YPG agar medium. After 1 day incubation, absence of *S. aureus* and MRSA growth confirms the antimicrobial activity of isolate LC3 against these bacteria. Figure 1 shows the Petri plate image of the cross-streak assay.

3.2. Trypsin / Proteinase K Digestion Assay

Trypsin / Proteinase K Digestion Assay was applied by modifying Yazici et al (2021)'s assay. The culture filtrate of the isolate LC3 was incubated with trypsin/proteinase K enzymes at 37 ° C for 6 hours to find out whether the zones formed in the agar diffusion test were polypeptide-based and at 100 ° C for 5 minutes to degrade enzymes. In addition to the protease treatment, the CFS was incubated at 100 ° C for 5 minutes and non-treatment CFS was used as a control. At the end of the incubation, results were determined by agar diffusion assay (Yazici et al. 2021). The antimicrobial activity in Fig. 2A and Fig. 2C indicates that CFS was not affected by temperature and temperature treatment was not affected the

protease treatment assay result. The loss of antimicrobial activity in Fig. 2B showed the antimicrobial compound to be a polypeptide.

3.3. Protein Isolation and SDS-PAGE

The CFS of isolate LC3 was concentrated with 3 kDa cut-off ultrafiltration column before protein isolation and total proteins were purified by TCA (trichloroacetic acid) method (Jiang et al. 2004). Total protein was determined by the Bradford method (Kruger 2009). According to the results given in Fig. 3., the total proteins of the fungus grown in flask culture gave the most intense band in the region of about 11 kDa.

3.4. Gel Overlay Assay

For gel overlay assay was performed SDS-PAGE and antimicrobial activity was determined using a prepared pour plate method with soft MHB, but as a result of the experiment, the antimicrobial activity could not be determined. Since the clear zone seen in the assay applied according to the gel overlay protocol was not clear, it could not be determined which protein band was responsible for the antimicrobial activity (results not shown) (Liu et al. 2012).

Proteins are separated according to their native structure in native-PAGE assay and the medium with agarose facilitated the diffusion of proteins in the gel into the medium. As a result of modifications to the assay, the native-PAGE gel was detected the protein band responsible for antimicrobial activity. This experiment adds to an effective determination of our AMP.

According to band profiles in Figure 4, the gel overlay assay showed zones of inhibition against *S. aureus* starting from about 11 kDa. According to these results, the band showing antibacterial activity is a protein of about 11 kDa.

3.5. Purification of Polypeptide

The antimicrobial polypeptide was determined with Native-PAGE and gel overlay assay. Then, it was purified by 30 kDa cut-off ultrafiltration column. Figure 5. shows the SDS-PAGE image of the purified protein. The first line in the image belongs to the marker protein and the one band in the second line is the crude protein band showing antimicrobial activity. This purified polypeptide was used for the determination of the MIC value.

3.6. Evaluation of AMP's Production According to Days

The total protein production of fungus grown in fermenter culture and antimicrobial activity of CFSs were determined according to days. SDS-PAGE of the total proteins of the filamentous fungus according to the protein band profile is shown in Figure 6A. The antimicrobial activity of the fermenter culture according to days is shown in Figure 6B. The protein band, about 11 kDa, which was responsible for antimicrobial

activity determined in Figure 6A, began to appear on the 5th day, but the antimicrobial activity in the agar diffusion assay appeared on the 6th day as shown in Figure 6B. The absence of this activity on the 5th day may be due to the fact that the about 11 kDa band responsible for the antimicrobial activity is less dense than the other days.

3.7. Determination of MIC Value

The MIC of purified AMP molecules against *S. aureus* 25923 and MRSA was 8 µg/ml and 32 µg/ml, respectively.

3.8. Identification of Antimicrobial Peptide Producer Isolate

Molecular characterization has been done for isolate LC3. rDNA sequence data was interpreted using BLASTN 2.13.0+ and Bioedit program, then isolate LC3 was identified as *Aspergillus niger* with accession number MK332597.

4. Discussion

A. niger has biotechnological importance as it produces various enzymes, organic acids and removes waste materials. Citric acid and many *A. niger* enzymes are recognized as GRAS by the United States Food and Drug Administration. *Aspergillus* species have received much attention as sources of new antimicrobial agents (Al-Fakih and Almaqtri 2019). There are only a few studies showing that *A. niger* has antimicrobial properties. Subhash et al. (2022) reported that evaluated antibacterial activity of *A. niger* culture filtrate (ACF) against enteric pathogens (*Klebsiella pneumoniae, Pseudomonas aeruginosa, Vibrio cholerae, Salmonella enterica, Shigella dysenteriae, Escherichia coli, Staphylococcus aureus, Klebsiella variicola*). They have demonstrated that ACF inhibits the growth of enteric pathogens (Subhash et al. 2022). Gun Lee et al.(1999) reported that a new antifungal peptide (named Anafp) was purified from the culture supernatant of *A. niger*. Anafp exhibited strong growth inhibitory activities against filamentous fungi as well as yeast strains in the MIC = 4 to 15 µM range. In contrast, Anafp did not show antibacterial activity against *E. coli* and *Bacillus subtilis* even at MIC = 50 µM (Gun Lee et al. 1999). Kalyani and Hemalatha (2017) reported that antibacterial and antifungal activity of crude extract of *A. niger* (MTCC-961) (Kalyani and Hemalatha 2017). There are studies in the literature on the antimicrobial effect of culture filtrates obtained by *A. niger*. However, purification of AMP has rarely been studied. According to agar diffusion results, antimicrobial efficacy difference between flask culture and fermenter culture are occurred probably due to variable parameters such as pH and dissolved oxygen level in growth conditions. Although these parameters were controlled in the fermenter culture, the results were not more effective. Fungi and other microorganisms produce metabolites that inhibit the growth of other microorganisms for nutrient competition and habitat in the natural environment (Al-Fakih and Almaqtri 2019; Cesa-Luna et al. 2020). The amount of AMP produced may vary, as the need for nutrients and oxygen is limited in the flask culture. Yazici et al. reported that (2021), CFS of the fungus identified as *A.*

tubingensis showed a zone larger than 20 mm against *S.aureus* and MRSA (Yazici et al. 2021). Kalyani and Hemalatha (2017) reported that the antimicrobial activity of *A. niger* (MTCC-961) *'s* CFS against various bacterial and fungal species was determined and the inhibition zone value for *S.aureus* (MTCC-3160) was found to be 12 mm (Kalyani and Hemalatha 2017). Al-Shaibani et al. (2013) determined the inhibitory effect of the CFS of *A. niger*, obtained from the inflamed eyes of patients against *P. aeruginosa, S. aureus, S. epidermidis, and Bacillus sp.*, which isolated from patients of microbial keratitis. The results show that *A. niger* possessed inhibitory effects against *P. aeruginosa, S. aureus, S. epidermidis*, and *Bacillus sp.*, with inhibition zones of 15, 25, 30, and 32 mm, respectively (Al-Shaibani et al. 2013). Omeike et al. reported that (2019) antibacterial activity of *Geotrichum candidum* OMON-1, *Talaromyces pinophilus* OKHAIN-12, and *Penicillium citrinum* PETER-OOA1 fungal extracts as 32 ± 0.12, 24 ± 0.2, 12 ± 0.17 mm zone of inhibition against *S. aureus*, respectively [27]. The best result from our data is the zone value of 25 mm and it is similar to the values in the literature. The results of the experiment found clear support that the fungus culture filtrates differed in antimicrobial effects against various bacterial strains. In the study of Park et al. (2008), it was determined that the production of bacterial culture at different scales and physiological conditions changed the antimicrobial activity (Park et al. 1998). In the study of Liu et al. (2012), the antimicrobial activity of Laparaxin produced from *Lactobacillus paracasei* was investigated and it was found that this polypeptide obtained from the fermenter, where pH was fixed at 6, showed less antimicrobial activity compared to the polypeptide obtained from the non-pH control fermenter (Liu et al. 2012). Based on these studies, the reason for the differences in the results of the SDS-band profile culture grown in a flask and fermenter culture can be attributed to the different physiological conditions in which they exist. A MIC is generally considered to be the most basic laboratory measurement of the activity of an antimicrobial agent against a microorganism. Drugs with a lower MIC value are more effective antimicrobial agents, as a lower MIC value indicates that less drug dose is needed to inhibit the growth of the microorganism (Kowalska-Krochmal and Dudek-Wicher 2021). The correlation was observed between molecular weights and antimicrobial activities of AMPs. However, most researchers report an increase in antimicrobial activity with decreasing molecular weight (Sultana et al. 2021). MICs of some AMPs such as LL-37(4.4 kDa), indolicidin(1.9 kDa), pexiganan(2.2 kDa) against *S. aureus* ATCC 25923 are 14 µg/ml, 16 µg/ml, 32 µg/ml, respectively (Ebbensgaard et al. 2015). Mygind et al. (2005) reported that AMP purified from *Pseudoplectania nigrella* and characterized as plectasin (4.3 kDa) gave a MIC of 4–32 µg/mL against various MRSA strains (Mygind et al. 2005). According to Mataraci and Dosler's study (2012), indolicidin and nisin (3.3 kDa) cationic polypeptides have MICs of 16 µg/ml, 16 µg/ml against MRSA, respectively (Mataraci and Dosler 2012). Yazici et al. (2020) reported that AMP(11 kDa) purified by the species identified as *A. tubingensis* has 32 µg/ml and 128 µg/ml MIC values against *S. aureus* ATCC 25923 and MRSA, respectively (Yazici et al. 2021). These studies confirm the relation between molecular weights and antimicrobial activities. However, these findings indicate that the MIC value of purified AMP in our study is consistent with other studies. Omeike et al.(2021) reported that an AMP compound characterized as Tripeptide GP-2B produced by *G. candidum* OMON-1 has 8 µg/mL, 32 µg/mL, 32 µg/mL MIC values against *S. aureus* ATCC 25923, *S. aureus* ATCC 6238, various strains of MRSA, respectively (Omeike et al. 2021). In line with previous studies MICs of purified AMP gave the same values against *S. aureus 25923* and MRSA.

5. Conclusion

To date, a total of 3324 antimicrobial proteins have been identified in the Antimicrobial Polypeptide Database (ADP3). Although fungi have the 22 molecules of the AMP identified, only three belong to the *Aspergillus* genus. According to these data, as an AMP source, there is a tendency towards *Aspergillus* isolate LC3 in this study. The antimicrobial activity was revealed by the gel overlay assay, where this antimicrobial activity originated from the about 11 kDa protein band. Then this about 11 kDa protein was purified using 3 kDa and 30 kDa cut-off ultrafiltration column and the MIC values of the crude protein were determined. As a result, the study successfully purified a novel AMP from isolate LC3. Our data indicate a new attractive therapeutic resource that provides a new light on combating infectious diseases. For future experiments, characterization of the our AMP molecule with MALDI/TOF should be carried out and determined toxicity of AMP by in vivo experiments.

Declarations

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Conflict of interest

The authors declare that they have no conflict of interest.

Author Contributions

All authors contributed to the study conception and design. Sourcing materials, performing experiments, analyzing data were performed by Serkan ÖRTÜCÜ, Ayşenur YAZICI and Ayşe ÜSTÜN. The first draft of the manuscript was written by Ayşe ÜSTÜN. All authors read and approved the final manuscript.

Data availability

All data generated or analysed during this study are included in this published article.

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Figures

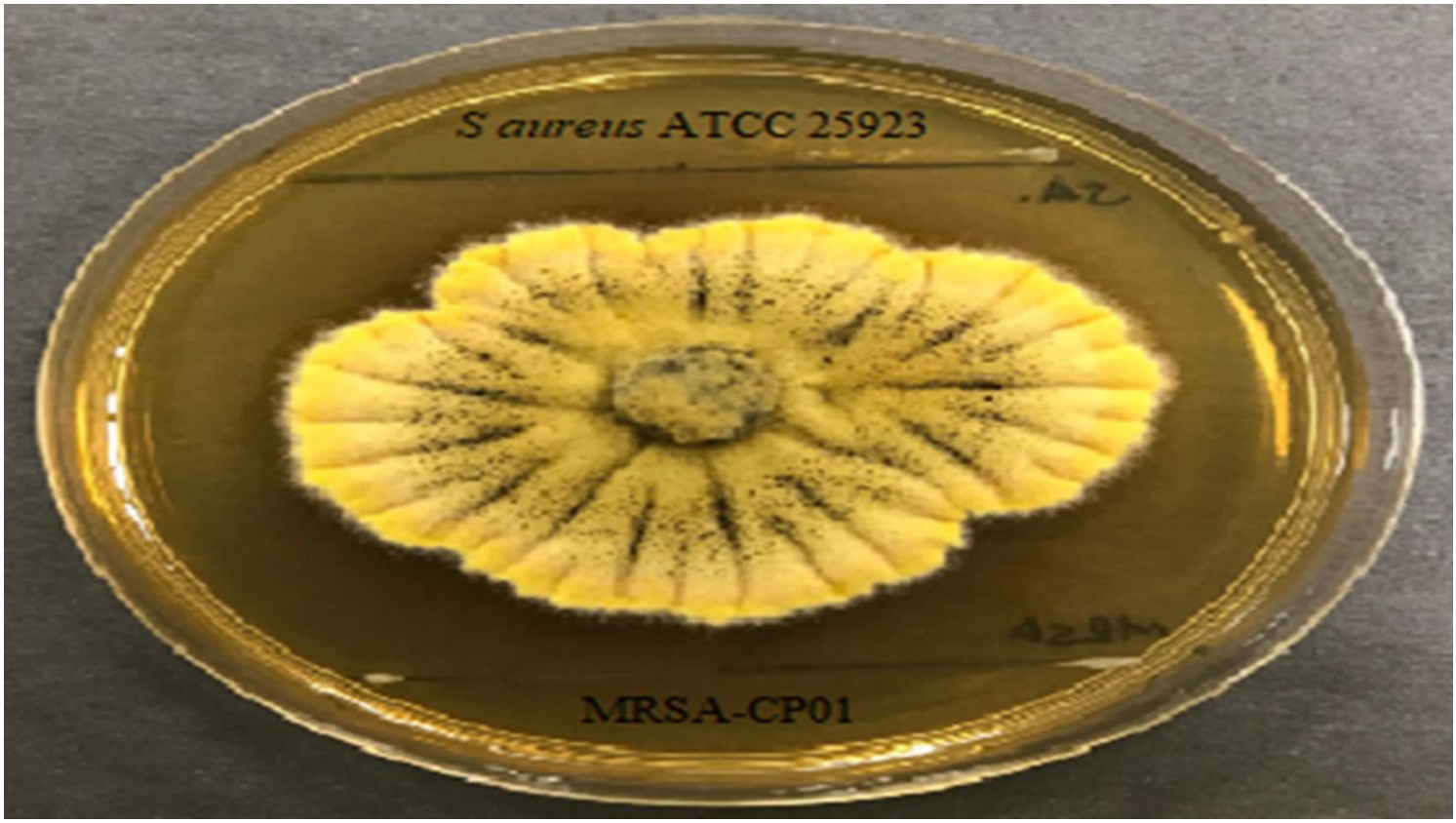

Figure 1

Cross Streak Assay Petri Image

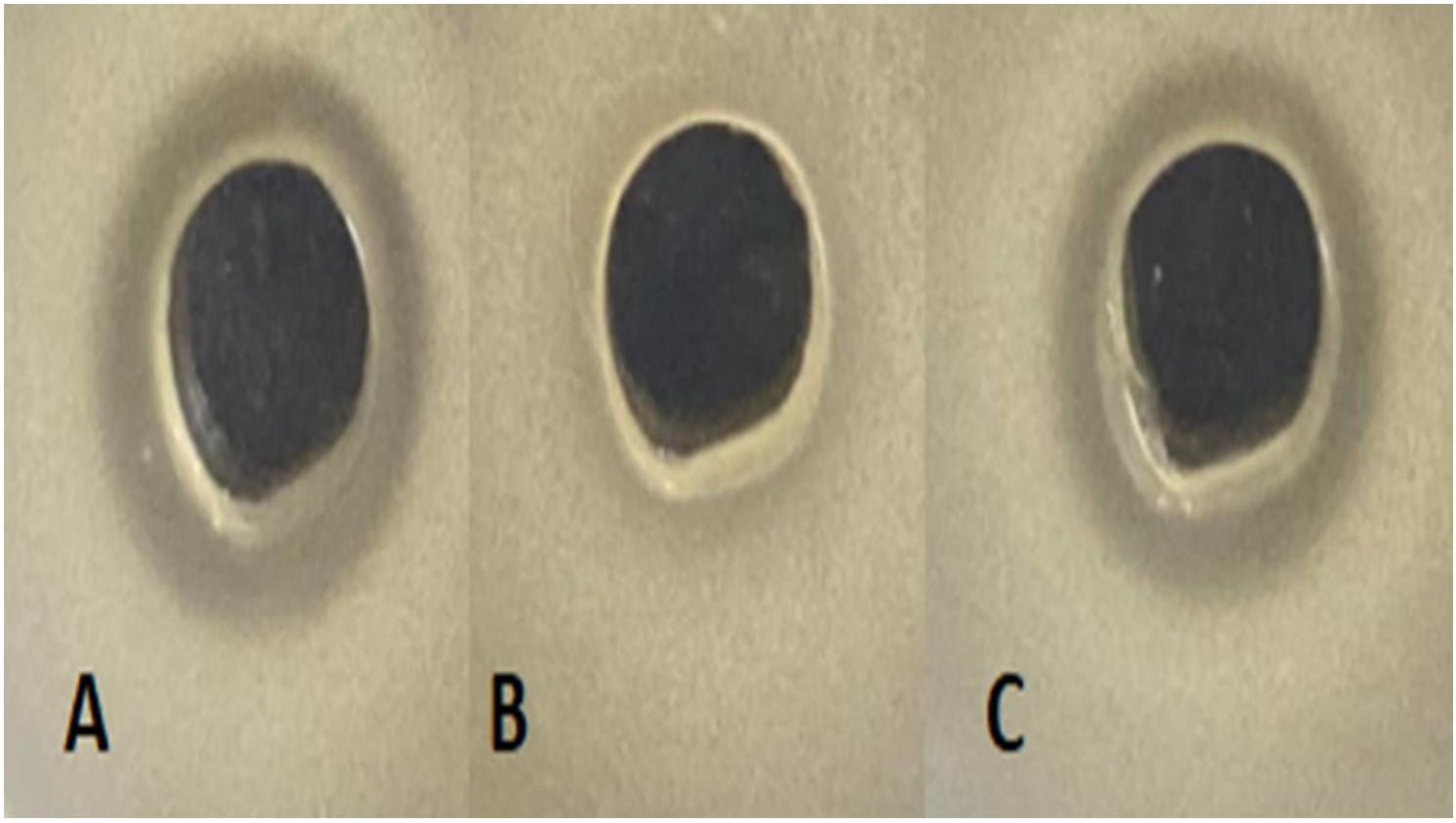

Figure 2

Agar Diffusion Assay Image of isolate LC3. **A.** Culture Filtrate **B.** Protease Treated Culture Filtrate **C.** Incubation at 100 ° C Culture Filtrate

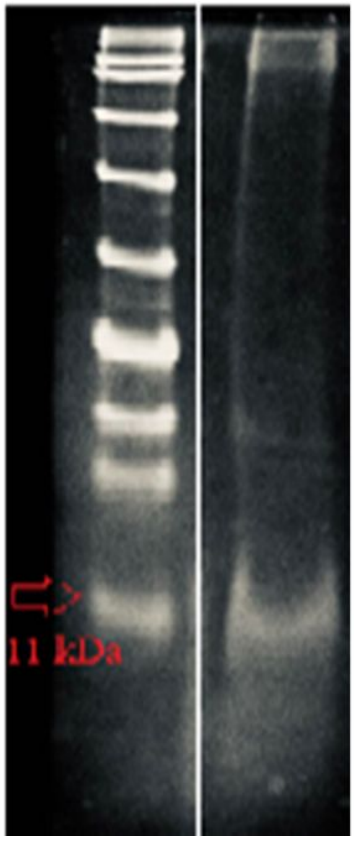

Figure 3

SDS-PAGE Profile of Extracellular Proteins of Isolate LC3

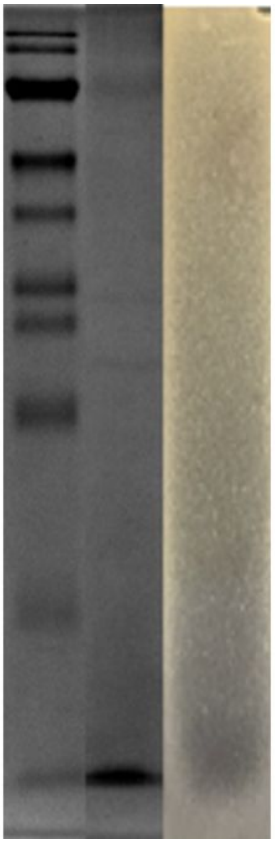

Figure 4

Result of Gel Overlay of CFS against *S. aureus*

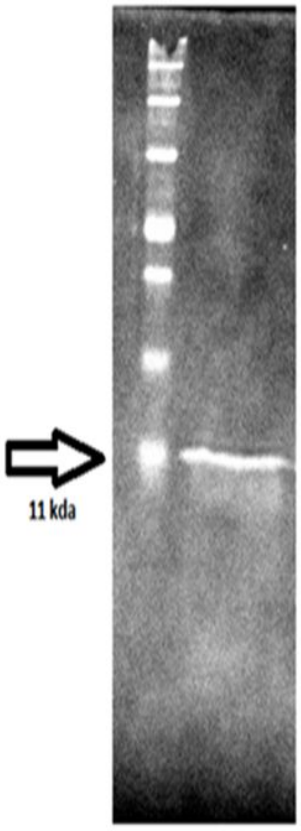

Figure 5

Crude Protein Band Showing Antimicrobial Activity

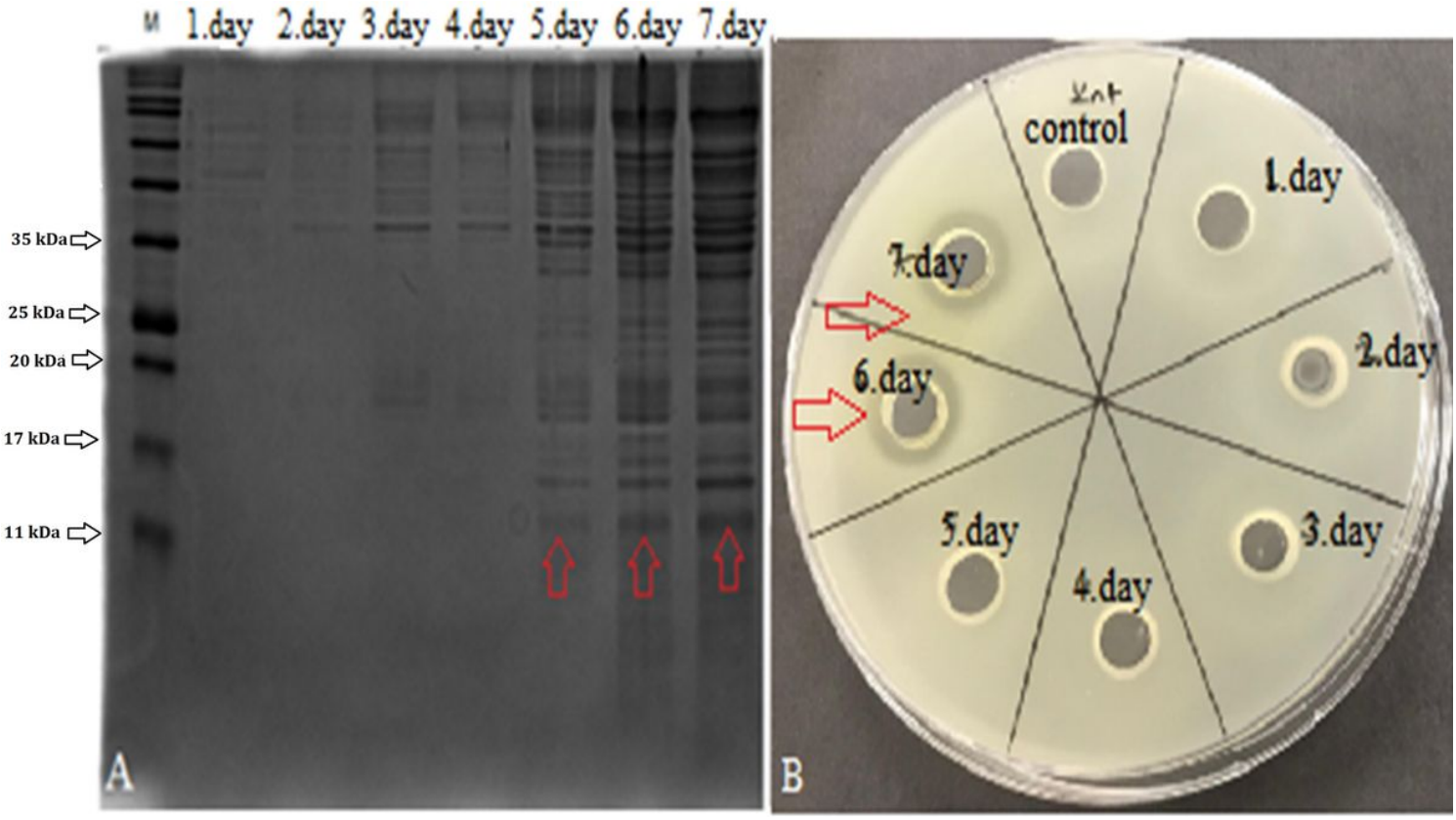

Figure 6

A. Total Proteins of the Isolate Grown in Fermenter Culture **B.** Agar Diffusion Results