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EVALUATION AND IMPROVEMENT OF AN ANTIMICROBIAL PRODUCT FROM *Bacillus cereus* MLAMC 271

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ABSTRACT

Foodborne pathogens pose risks in food products, hence the need to seek alternative antimicrobials for reducing these pathogens. The aim of this work was to define the antimicrobial features of the substances from *Bacillus cereus* MLAMC 271 against Gram negative (*Salmonella* Enteritidis MLAMC 804, *Pseudomonas aeruginosa* MLAMC 538, and *Escherichia coli* MLAMC 925) and Gram positive (*Bacillus subtilis*, MLAMC 853, *Staphylococcus aureus*, MLAMC 638 and *Listeria monocytogenes* MLAMC 163) pathogens. Bacterium *Salmonella* Enteritidis MLAMC 804 has been utilized as test organism to determine effect of several operation conditions such as time, pH (4.5 – 11.5), temperature (25–45ºC), whey powder (10–20 g/L) or glucose concentration (0–20 g/L) on the rate of the secretion of antimicrobials. The results showed antimicrobial substances of *B. cereus* MLAMC 271to be efficient against all pathogens tested except for *P. aeruginosa* MLAMC 538. Antimicrobial levels peaked at 20-27 h of bacterial growth, optimal pH was 8.5, and optimal temperature 35°C. Production of antimicrobial was highest when only nitrogen sources were present and glucose was absent. Antimicrobials were produced in the media that contained only whey powder, which suggests the potential of whey powder as a medium for satisfactory production of efficient antimicrobials using *B. cereus* MLAMC 271 against wide spectrum of the bacteria.

Keywords: *Bacillus cereus*, Antimicrobial activity, Whey powder, Pathogens, Gram negative, Gram positive

1. INTRODUCTION

Bacillus spp have been widely utilized for many decades in investigations and researches (Moszer et al., 2002; Sonenshein et al., 2002). They have ability to form various structures as antimicrobials compounds, and this potential has been recognized for several years. These bacteria are Gram-positive capable of forming endospores, and are normally found in plants and soils. The antimicrobials which formed by *Bacillus*spp usually are in the form of peptide, like subtilosin A, subtilin, sublancin and ericin, which exhibit structures described as rigid, hydrophobic, and/or cyclic (Katz & Demain, 1977). An extensive spectrum of antimicrobial activities were demonstrated by the bacteriocins produced by *Bacillus* spp. This included antiviral, antifungal, antimycoplasma and antiameobocytic agents (Stein, 2005; Awais et al., 2010; Zhao et al., 2013). In general that temperature, pH and media contents affected the bacteriocin produced by *Bacillus* spp (Motta & Brandelli, 2008; Aftab et al., 2012; Khochamit et al., 2015). Although relatively high levels of bacteriocin and abundant growth is normally supported by complex media, it is necessary to find an alternative for utilizing waste from the food industry, for which milk whey is a possible candidate (Amiali et al., 1998; Guerra & Pastrana, 2002). The content of peptides is an important feature of whey, as they can function as inducers of bacteriocin production (De Vuyst, 1995). This study was arranged, firstly to define the antimicrobial characteristics of bacteriocins produced by *Bacillus cereus* MLAMC 271 against the examined pathogens, and secondly to estimate the influence on antimicrobial production by carbon sources in the media, pH value, incubation time and temperature changes. In addition, the antimicrobial substances were determined by a method involving tricine-sodium dodecyl sulfate polyacrylamide gel electrophoresis (Tricine-SDS-PAGE).

2. MATERIAL AND METHODS

2.1. Chemicals

Whey powder was obtained from Sigma-Aldrich-USA, which comprised of 83–85% lactose, 5.5–6.5% protein, 5.5% mineral, 2% moisture, and 0% fat. Yeast extract, glucose, nutrient agar and broth, and peptone were obtained from Sigma-Aldrich-USA, and membrane filter was obtained from Millipore-Merck (Darmstadt, Germany).

2.2. Bacteria species

In the present study, a *Bacillus cereus* MLAMC 271 was kindly facilitated by the Microbiology Laboratory, King Abdul-Aziz Medical City - Makkah - Saudi Arabia. The bacterium was kept in nutrient broth with

50% glycerol in long term storage. The foodborne pathogens as Gram negative (*Salmonella* Enteritidis MLAMC 804, *Pseudomonas aeruginosa* MLAMC 538, and *Escherichia coli* MLAMC 925) and Gram positive (*Bacillus subtilis*, MLAMC 853, *Staphylococcus aureus*, MLAMC 638 and *Listeria monocytogenes* MLAMC 163), were kindly obtained from the Microbiology Laboratory, King Abdul-Aziz Medical City - Makkah - Saudi Arabia.

2.3. Media and cultivation circumstances

The seed culture was cultivated in flasks (100 mL capacity) containing nutrient broth (20 mL) placed in a shaking incubator (125 rpm) at 35°C for 24 h. Medium of bacteriocins production was made by modifying the medium given by Avci et al. (2016) containing glucose (10 g), peptone (5 g), yeast extract (5 g), K_2HPO_4 (1 g), $MgSO_4 7H_2O$ (0.1 g) in distilled water (1 L) as the basal medium. The medium's initial pH was adjusted to 7.0 with NaOH (2 mol/L) or HCL (2 mol/L) and then transferred into Erlenmeyer flasks (100 ml) as 30 ml portions for sterilization for 15 min. at a temperature of 121°C. Fresh seed culture (5%) (v/v) was used with 2.0 OD (600 nm). The effect of temperature on antimicrobial production was investigated by incubation of the strain at temperatures range from 25°C to 45°C, and pH influence by preparing the basal media at pH values ranging between 4.5 and 11.5. The influence of glucose concentration on the production was investigated by preparing the basal media with glucose concentrations of 0, 10, 15 and 20 g/L. The influence of whey powder on the production of antimicrobials was also examined in the basal media without glucose at the levels of 10, 15 and 20 g/L. Identical concentrations of whey powder solution were examined using the basal media without nitrogen sources (peptone and yeast extract) and glucose. These production experiments were conducted for 24 h at 35°C in a shaking incubator at 125 rpm, except for temperature effect investigations. Bacterium growth was measured at 600 nm spectrophotometrically (Khochamit et al., 2015). The culture broth for antimicrobial analysis was centrifuged for 15 min at 10,000 rpm to remove the cells. Subsequently a membrane filter (Millipore-Merck, Darmstadt, Germany) (0.45 mm) was used for filtering the supernatant containing antimicrobials for further experimentation.

2.4. Antimicrobial activity assay

Antimicrobial activity was tested by selecting *Salmonella* Enteritidis MLAMC 804 as the indicator organism. Tryptic Soy Broth was used to cultivate the indicator strain for 18 h at 37°C (approximately 107–108 CFU/ mL). The disc diffusion method, as detailed by Khochamit et al. (2015), was then used with a little modification for

testing antimicrobial activity against indicator organisms. The indicator culture (50 mL) was then swabbed onto the Tryptic Soy Agar and retained for absorbing the culture by the agar for 1 h. Sterile filter paper disc (6mm) (Whatman No. 1) was laid aseptically on the plate, and antimicrobial sample (5 µL) was applied onto the center of the paper. Incubation of the plates took place for 24 h at 37°C. Measurements were taken of the inhibition zone diameters. The minimum antimicrobial inhibition concentration produced by *Bacillus cereus* MLAMC 271 was defined against different pathogens, including, Gram negative (*Salmonella* Enteritidis MLAMC 804, *P. aeruginosa* MLAMC 538, and *E. coli* MLAMC 925) and Gram positive (*B. subtilis*, MLAMC 853, *S. aureus*, MLAMC 638 and *L. monocytogenes* MLAMC 163). A cell free supernatant was obtained for this purpose by cultivating the bacterium for 24 h in the basal medium at 35°C. This supernatant was serially diluted with sterile deionized water in a 2-fold manner (2^n), and each dilution was observed for antimicrobial activity against pathogen bacteria using the same disc diffusion method. Incubation of the bacteria was maintained for 24 h at 37°C, except for *L. monocytogenes* MLAMC 163 which was kept at 30°C. The following formula was applied to calculate antimicrobial activity as arbitrary units (AU per mL): 2^n x 1000 µL / sample amount in µL, where 2^n is the highest rate of dilution that gives the inhibition zone (Yamamoto et al., 2003; Gunes-Altuntas et al., 2010).

2.5. Ammonium sulfate precipitation and Tricine-SDS-PAGE analysis

Ammonium sulfate was used to precipitate the proteins in the cell supernatant. Testing was undertaken of three various concentrations (40, 60, and 80%) of ammonium sulfate for determining the optimum concentration for ideal precipitation of antimicrobial substances. These experiments were performed on a magnetic stirrer at 4°C by adding ammonium sulfate gently until it reached the specified concentrations. They were maintained overnight at a temperature of 4°C in order to the proteins be settled down, and then centrifuged for 30 min at 9000 rpm. Tris-HCl buffer (100 mM at pH 7) was used to dissolve the pellets. Subsequently, they were dialyzed against the buffer for 48 h at 4°C using 1000 MWCO of dialysis tubing (Spectrum Labs, CA, USA). This dialysis buffer was changed occasionally throughout the procedure. The disc diffusion method was used to determine the antimicrobial activity of the precipitated proteins against *Salmonella* Enteritidis MLAMC 804 and *L. monocytogenes* MLAMC 163 (Fig. 1). Estimation of the antimicrobial on the gel was made by performing Tricine-Sodium

Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (Tricine-SDS-PAGE) of the proteins, which were obtained at the optimum concentration of 60% ammonium sulfate.

2.6. Statistics

The experiments were repeated three times, and a two-way Analysis of Variance was applied via SPSS (version 13.5, SPSS Inc, USA). The means were compared by applying the Duncan Grouping test (at $P = 0.05$).

3. RESULTS AND DISCUSSION

3.1. Antimicrobial substances production by *B. cereus* MLAMC 271

3.1.1. Time course of antimicrobials production

Throughout the growth of *B. cereus* MLAMC 271 in the basal medium, samples were withdrawn periodically in order to confirm the relationship between the growth of cell and the production of antimicrobial (Fig. 2). The data shows that growth related antimicrobial production commenced after 6 h from the start of the exponential phase. Between 20 and 27 h of bacterial growth, the production of antimicrobial recorded the maximum yield, but the inhibition zone diameters began to reduce significantly ($p < 0.05$) during the later growth period. The relationship between production of antibiotic peptide by *Bacillus* spp. and the growth rate of cells has been reported previously in relation to finding the optimum growth condition (Haavik, 1975). For instance, Awais et al. (2010) reported that the antimicrobial peptide production of *B. subtilis* during the rapid growth phase only. The present work made similar results. Antimicrobial production ceased when the incubation times was prolonged, which was indicated by smaller inhibition zones.

3.1.2. Effects of temperature and pH on antimicrobial production

For determining optimal temperature for production of antimicrobial, *B. cereus* MLAMC 271 was cultivated at temperatures between 25 and 45°C (Fig. 3). The inhibition zone measured against *Salmonella* Enteritidis MLAMC 804 was supposed to be correlated with antimicrobial amount in the culture. Antimicrobial metabolite production was therefore recorded only in the temperature range of 30 to 40°C. The ideal temperature for antimicrobial production was found to be 35°C, and maximum cell growth was achieved at 37°C. When the cultivation temperature exceeded 39°C, antimicrobial production ceased despite evidence of some growth continuing. This finding is similar to that obtained by Khochamit et al. (2015) who detected antimicrobial substances produced by *Bacillus* spp. when the cultures were grown at similar temperatures of 30, 37 and 42°C, but not at 45°C. Likewise, minimal bacterial growth and

antimicrobial activity were evident at 42°C relative to lower temperatures. The degradation of antimicrobial substances by extracellular proteases could explain the elimination of antimicrobial activity at this temperature of 42°C (Bizani & Brandelli, 2002). Otherwise, it may be due to an unsuitable growth circumstances leading to cessation of antimicrobial synthesis. At a higher temperature of 50°C, slight growth of the culture was evident, but it was insufficient to produce antimicrobial substances, which suggests observation of a relationship between antimicrobial production and regular physiological function unless the bacteria experience heat stress.

Fig. 4 shows the initial pH effect on antimicrobial production of *B. cereus* MLAMC 271. The highest inhibition zone of 11 mm was recorded when the medium's initial pH value was 8.5. As well as, the species grew at pH 11.5 and produced antimicrobials, while the inhibition zone was 7 mm. Maximum bacterium growth occurred at a pH value of 7.5 when the zone of inhibition was 9 mm despite lower antimicrobial production. Antimicrobial production and growth decreased significantly ($p < 0.05$) as the pH level decreased. At a pH value of 5.5, bacterial growth was exhibited, but antimicrobial production was not detected. Neither growth nor antimicrobial production was evident at a pH value of 4.5. The importance of pH value and temperature variation on antimicrobial substance production by *Bacillus* sp. is established in other studies (Cladera-Olivera et al., 2004; Motta & Brandelli, 2008). As in the present work, maximum antimicrobial activity has been recorded previously at initial pH ranging between 6.0 and 8.0 (Cladera-Olivera et al., 2004; Motta & Brandelli, 2008). In addition, any relation between optimal bacterial growth and antimicrobial production could not be demonstrated.

3.1.3. The influence of substrate concentration on antimicrobial production

The influence of various glucose concentrations between 0 and 20 g/L on antimicrobial production was examined (Fig. 5 A). In the absence of glucose, antimicrobial production in the medium was greatest at the inhibition zone of 10 mm. The results were similar for media which contained glucose for all different concentrations examined. There was no evidence of a correlation between glucose amount in the medium and antimicrobial production. However, a slight gain in cell growth was observed when glucose concentration was increased ($p > 0.05$). Biosynthesis of antibiotic usually is organized by other activities that induce starvation, such as genetic competence development, sporulation, and extracellular

derivative enzyme production (Katz & Demain, 1977; Losick et al., 1986). In the present work, glucose absence necessarily induces greater antibiotic production relative to glucose presence, which may be due to nutritional stress. It was reported how starvation induced production of antimicrobial parallel to the formation of endospores (Marahiel, 1992). In the case of limited energy source for the microorganisms (normally carbon), catabolism is combined closely and there is completion of high yields of biomass on the carbon source (antimicrobial or extracellular production of polymers) (Saier et al., 1993). Carbon limitation generally shows less carbon consumption rates and large biomass yields relative to excess cultures of carbon, and therefore show high efficiency in terms of energetic growth (Pennock& Tempest, 1988; Teixeira de Mattos&Neijssel, 1997). A significant reduction in the energetic performance of *B. subtilis*growth was also shown by Dauner et al. (2001) in their study on growth of cultures in a condition of excess glucose, as compared to growth with limited glucose. In two various media influence of whey powder on antimicrobial production was investigated. The first set of experiments for testing antimicrobial production involved preparing the basal media by substituting glucose with whey powder (10, 15 and 20 g/L). Production of the same antimicrobial amounts was significant ($p > 0.05$), as it is for glucose media (Fig. 5B). Bacterium growth in the glucose medium is also significant to the same degree. Antibacterial production and growth is unaffected by increasing whey powder concentration. The second set of experiments involved removal of peptone, yeast extract and glucose from the basal medium, and the addition of whey powder (10, 15 and 20 g/L). Bacterial growth was relatively lower than in the first set of experiments, and the inhibition zones were unchanged (Fig. 5C) ($p > 0.05$). The main carbon source in whey (lactose) can only be hydrolyzed by certain microorganisms that synthesize lactase or β-galactosidase, an enzyme which hydrolyzes lactose. *Bacillus*sp, which contains β-galactosidase is capable of utilizing lactose as a source of carbon. A previous report exhibited 60 out of 130 strains of *Bacillus* spp Produced gluconate from a laboratory medium which contained 2% lactose (Stauffer & Leeder, 1978). Another study confirmed lactose hydrolyzation resulting from increased populations of vegetative cells and reduced pH values in whey powder solution (Cagri-Mehmetoglu et al., 2012). Similarly, the pH value of the fermentation medium was reduced from 7.0 to within the range 5.7–5.5 during growth of *B. cereus* MLAMC 271 on whey powder over a period of 24 h. The media prepared in the current work

with whey powder (in proportions of 10, 15 and 20%) contained whey protein as a source of nitrogen (around 0.55, 0.82 and 1.1%, respectively) and lactose as a source of carbon (8, 12.5 and 17.5%, respectively). Growth of *B. cereus* MLAMC 271 occurred more slowly in the nutrient deficient media, as was expected and relative to growth in the medium with a nitrogen source (10%) and glucose (10%). No influence of nutrient availability was evident on antimicrobial production on both media types. Antimicrobial production might be induced by low nutrient related stress as opposed to growth for regulating pressure (Marahiel, 1992). The present work showed limited whey protein amount without peptone supplementation and any yeast extract is able to stimulate antimicrobial production in *B. cereus* MLAMC 271. A similar finding of no increased antimicrobial production as a result of adding yeast extract to cheese whey was obtained by Cladera-Olivera et al. (2004), they claimed to have observed maximum bacteriocin activity of *B. licheniformis* when cultivating in cheese whey and other media, such as grape peel, feather meal and industrial fibrous soybean residue. On the contrary, antimicrobial production by *Bacillus*sp has also been observed not to occur in whey although it did in peptone, soybean protein, infusion broth and trypticase soy broth (Motta & Brandelli, 2008).

3.2 Antimicrobials influence by *B. cereus* MLAMC 271 on Selected Foodborne Pathogens

Inhibitory influences of antimicrobial products by *B. cereus* MLAMC 271 were investigated against both Gram positive and Gram negative bacteria (Fig. 6). The isolated antimicrobial substances had an inhibitory influence on Gram positive and Gram negative bacteria that were tested. Inhibition was greatest in the case of *L. monocytogenes* MLAMC 163, *E. coli* MLAMC 925 and *B. subtilis*, MLAMC 853 followed by *Salmonella* Enteritidis MLAMC 804 and *S. aureus* MLAMC 638. The antimicrobial substances were unable to inhibit the growth of *P. aeruginosa* MLAMC 538. Previous studies are in conformity with the current work as regards observing a similar inhibitory effect of antimicrobial substances formed by *Bacillus*sp against a wide spectrum of Gram positive and less Gram negative bacteria, where antimicrobial activity was observed in a novel strain of *Bacillus* BP6 in response to different foodborne pathogens, such as *Micrococcus luteus*, *Vibrio parahaemolyticus*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Escherichia coli* O157 (Lim & Kim, 2009). It is reported that antimicrobial (bacteriocin) from *B. subtilis* KIBGE IB-17

possess an inhibitory influence against *Micrococcus* sp., *S. aureus*, *B. stearothermophilus*, *Enterococcus faecalis*, *L. monocytogenes*, *E. coli* and *Salmonella* typhi A (Ansari et al., 2012).

3.3. Gel electrophoresis

An investigation was made of ammonium precipitation and dialysis using the Trisin SDS-PAGE method with respect to the proteins obtained by measuring their molecular weight. On the first lane of the polyacrylamide gel shown (Fig. 7), the protein bands show signs of standard proteins, on the second and third lanes, these bands show evidence of proteins in the media, without (second) and with 80% ammonium precipitation (third). However, there is insufficient evidence confirming the bands with molecular weight almost 23, 29 and 30 kDa belong to bacteriocins that were produced by *B. cereus* MLAMC 271. More investigations are planned for specifying antimicrobial substances.

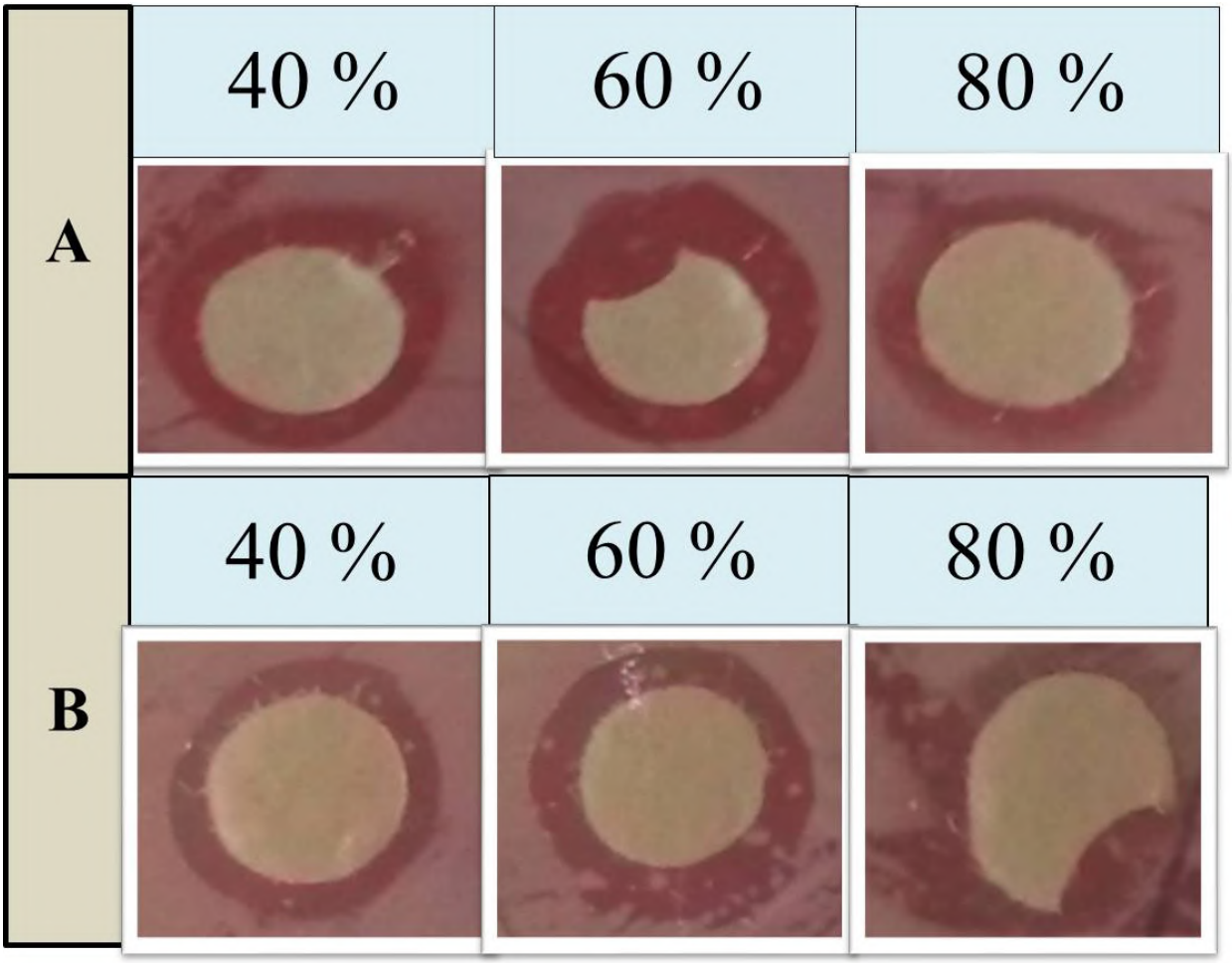


Fig. 1: Antimicrobial activities of the proteins from the supernatant of *B. cereus* MLAMC 271 obtained after the precipitation with varying concentrations of ammonium sulfate (40, 60, 80%), against *L. monocytogenes* MLAMC 163 (A) and *Salmonella*Enteritidis MLAMC 804 (B)

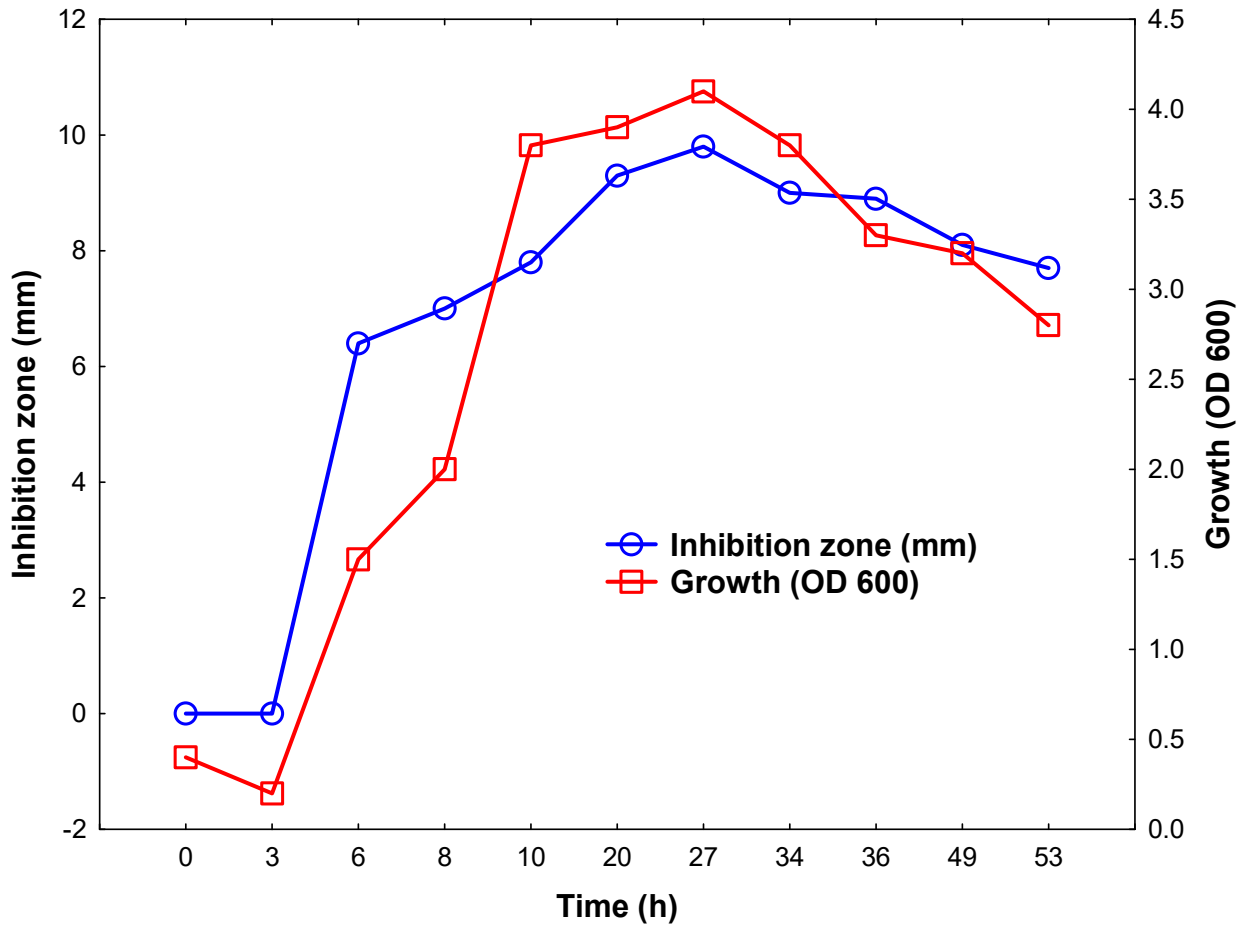


Fig. 2: Time course of the production of antimicrobial substances by *B. cereus* MLAMC 271. Antimicrobial activity was tested on *Salmonella*Enteritidis MLAMC 804

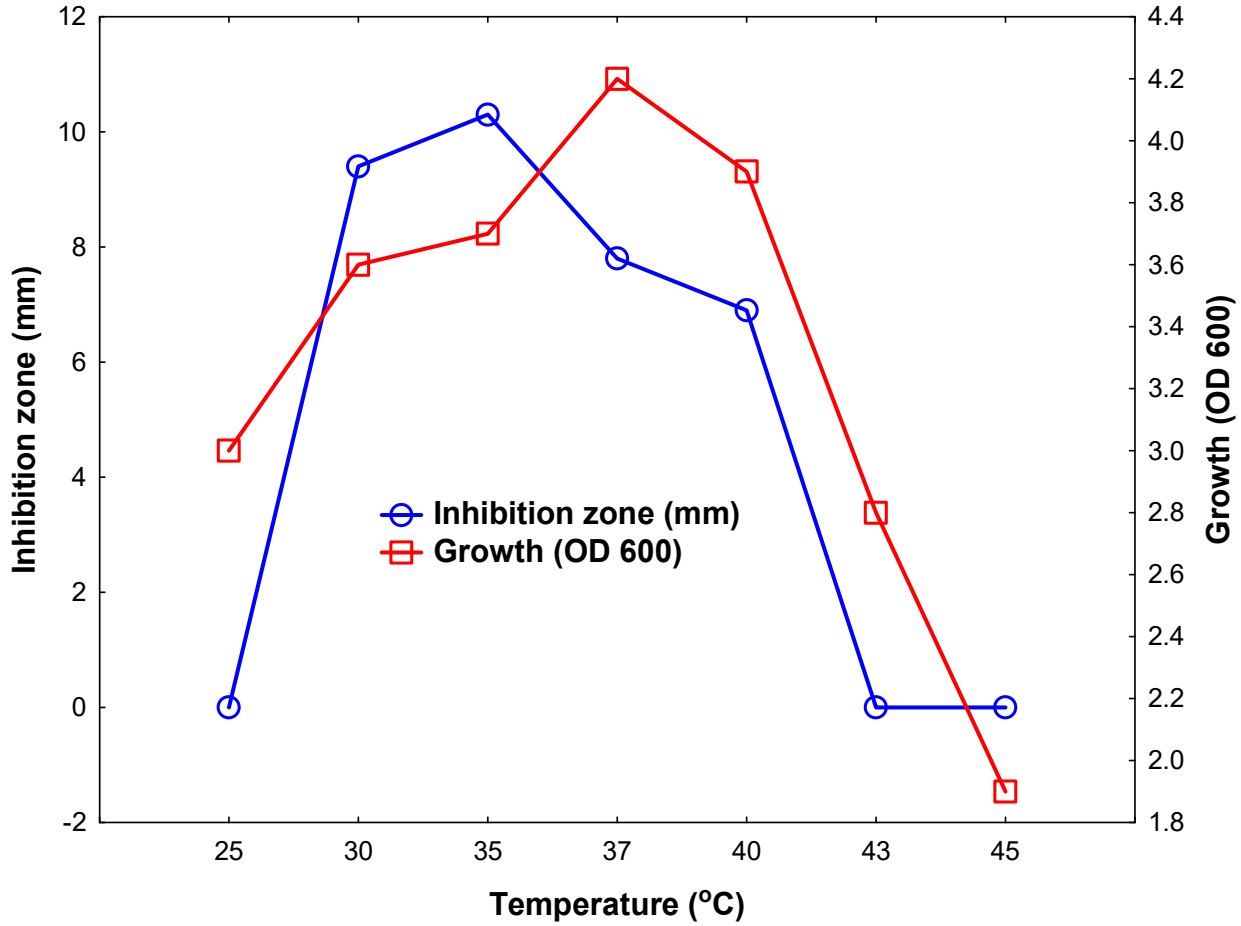


Fig. 3: The effect of temperature on the antimicrobial substance production by *B. cereus* MLAMC 271 grown on the basal medium for 24 h. Antimicrobial activity was tested on *Salmonella* Enteritidis MLAMC 804

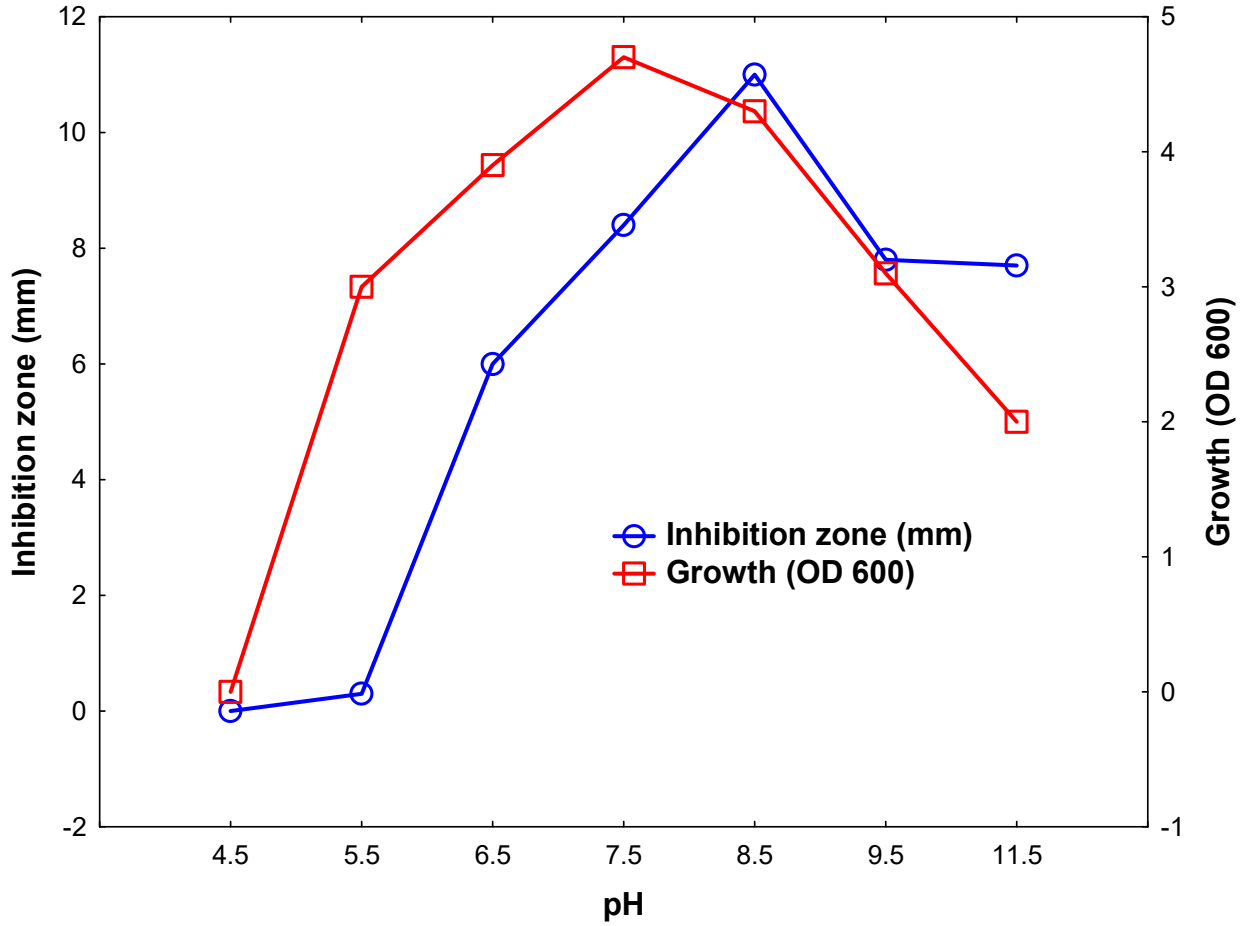
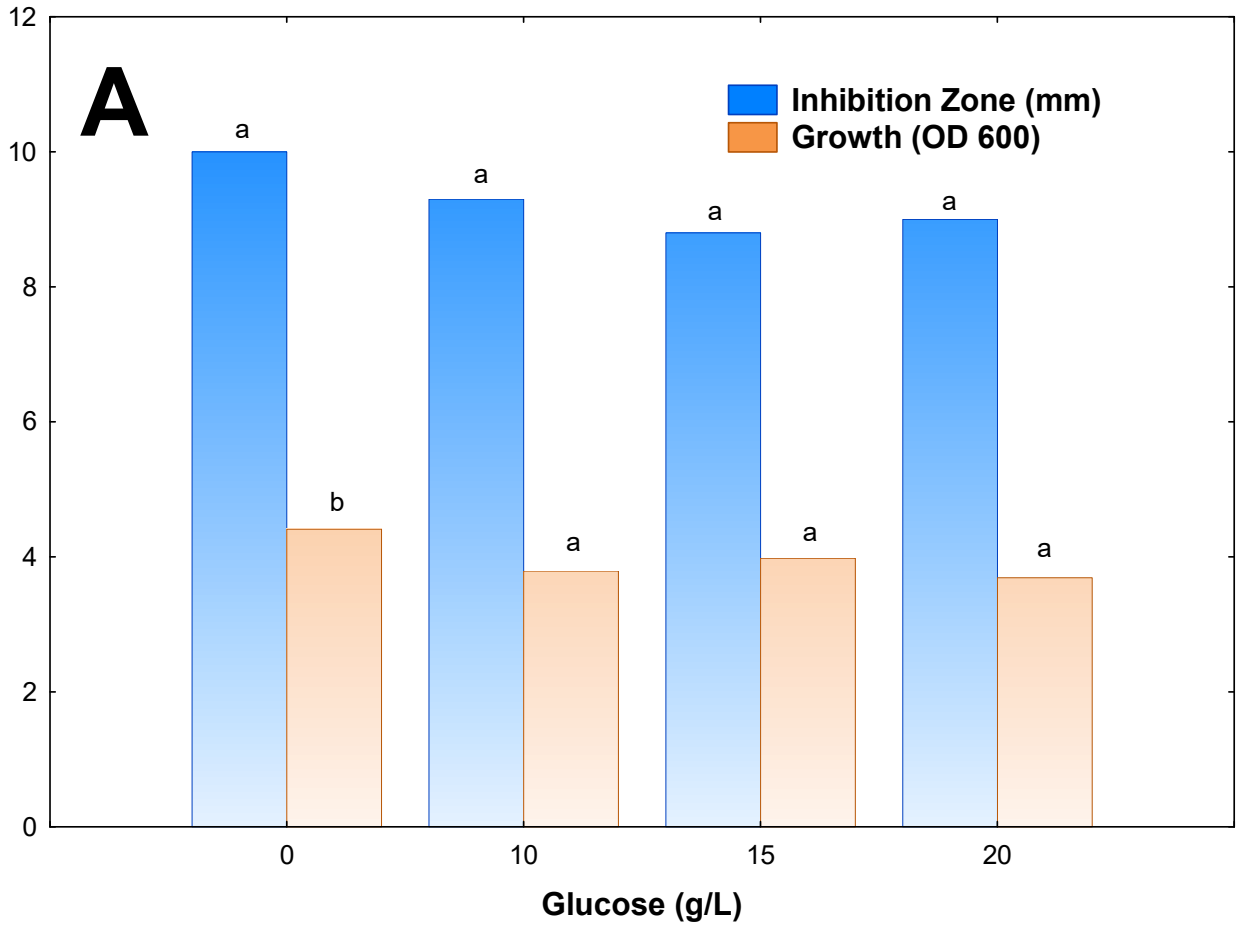


Fig. 4: The effect of initial medium pH on the antimicrobial substance production by *B. cereus* MLAMC 271 grown on the basal medium at 35 ºC for 24 h. Antimicrobial activity was tested on *Salmonella* Enteritidis MLAMC 804


A
Inhibition Zone (mm)
Growth (OD 600)
12
10
8
6
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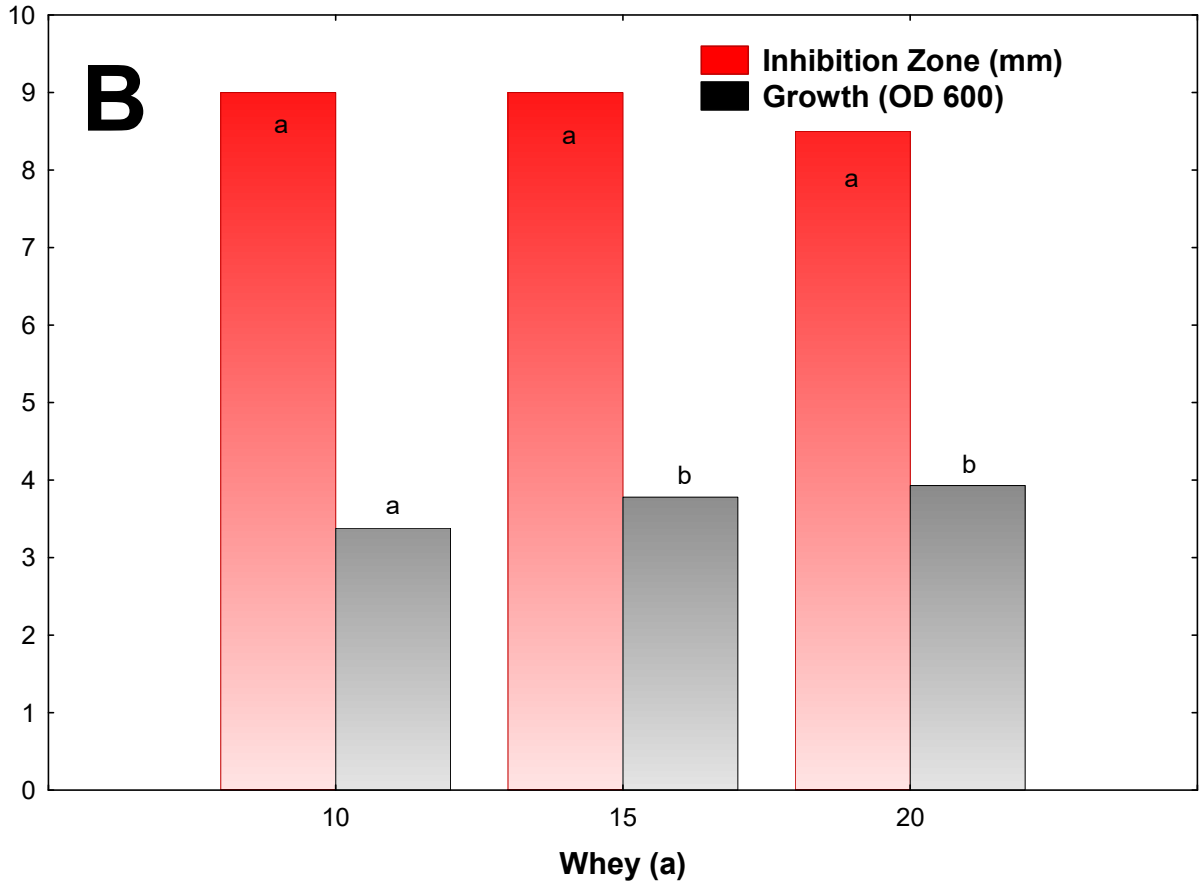
a
a
a
a
0
10
15
20
Glucose (g/L)


B
Inhibition Zone (mm)
Growth (OD 600)
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Whey (a)

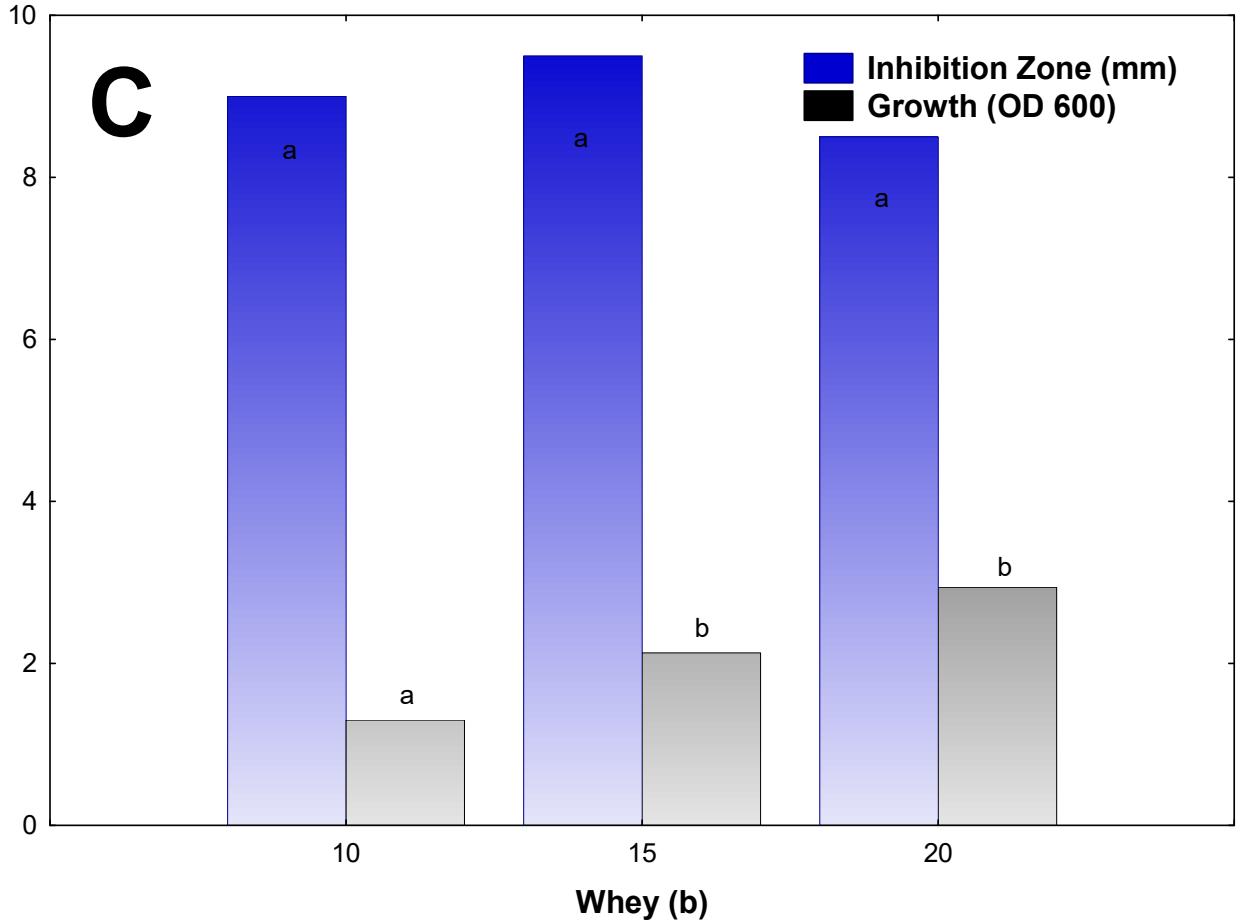

Fig. 5: The effects of glucose (A) and whey (B & C) concentrations on the antimicrobial substance production by *B. cereus* MLAMC 271 grown on basal medium at 35 ºC for 24 h. Antimicrobial activity was tested against *Salmonella* Enteritidis MLAMC 804. (Mean ± standard deviation n = 3. Means in same Bar with different superscript are significantly different ($p < 0.05$). (a): Glucose in the basal medium was replaced with whey powder. (b): Glucose and nitrogen sources were removed from the basal medium).

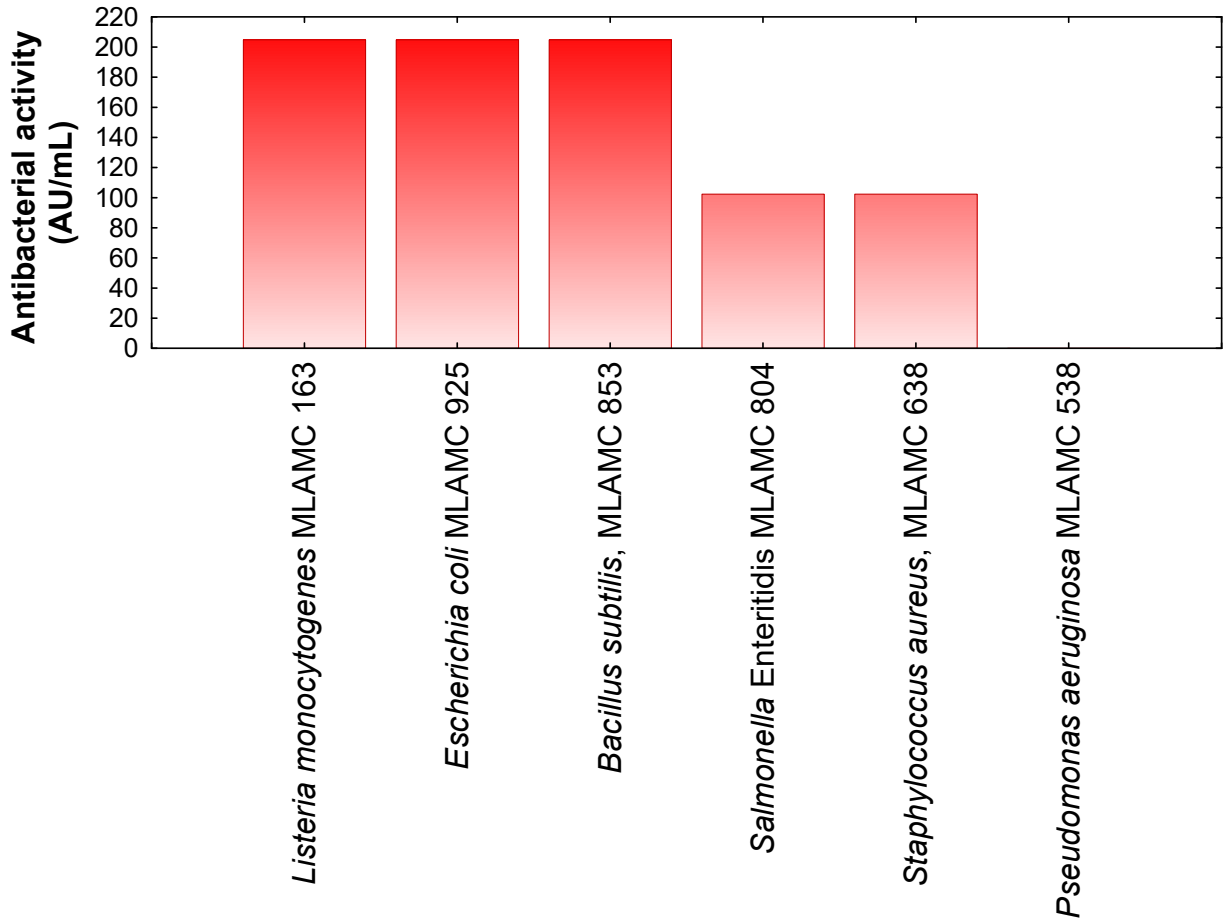

Fig. 6: Antibacterial activity of *B. cereus* MLAMC 271 on some pathogen bacteria

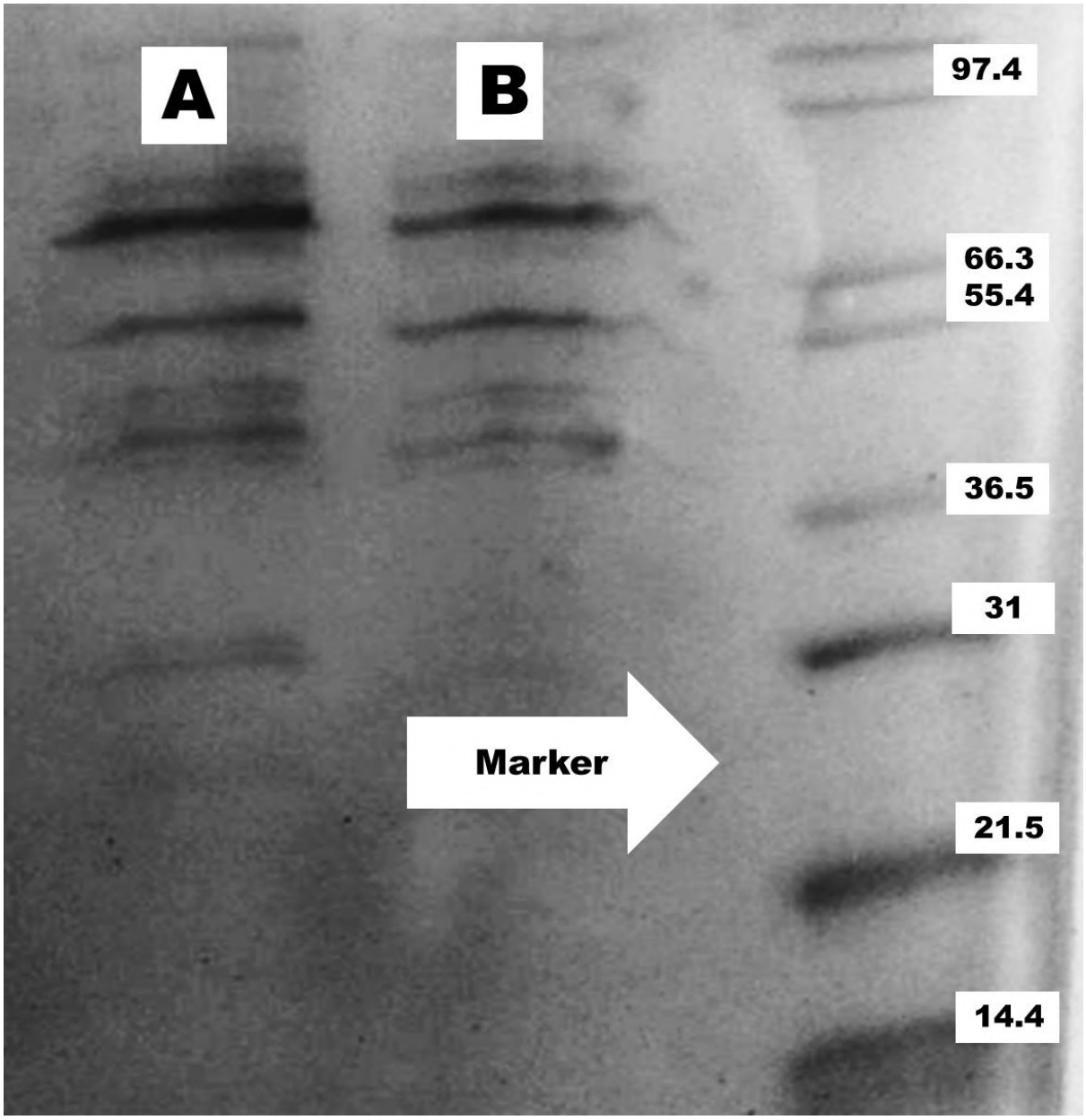

Fig. 7: Tricine-SDS-PAGE gel of the supernatant (A) and proteins precipitated by ammonium sulfate (B)

4. CONCLUSIONS

An investigation was conducted of antimicrobial production by *B. cereus* MLAMC 271. The antimicrobials exhibited an inhibitory influence on *Salmonella* Enteritidis MLAMC 804, which was maximized at pH 8.5 and a temperature of 35°C. Antimicrobial production is increased by limitation of the nutrient in the medium. A similar influence on antimicrobial substance production by *B. cereus* MLAMC 271 was shown when whey powder was utilized as the medium compared to production in the basal medium. For economic antimicrobial production, this waste material can be utilized as an affordable alternative medium. Apart from *Salmonella* Enteritidis MLAMC 804, the antimicrobials produced by *B. cereus* MLAMC 271 inhibited growth

of different Gram positive and Gram negative foodborne pathogens. There is potential for industrial applications of wide antimicrobial spectrum of by *B. cereus* MLAMC 271.

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