ISSN (Online) 2456 -1304

International Journal of Science, Engineering and Management (IJSEM)
Vol 2, Issue 12, December 2017

Fractional Characterization of Bacteriocin-Like Material of Pseudomonas aeruginosa AND Determination of Its Antibacterial Activity Against b. Cereus and s. typhimurium

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Abstract: **This study presents the fractional characterization of bacteriocin-like material (Inhibiting molecule showing properties of bacteriocin) produced by Bacteriocin-like material was found to be active against Bacillus cereus and Salmonella typhimurium.. The maximum antibacterial activity of Pseudomonas aeruginosa was recorded at 16th hour incubation in nutrient broth at 370C. The antibacterial activity was found to be extracellular since, cell-free supernatant showed inhibition against test organism. The inhibitor was found to have proteinaceous nature and same has been confirmed by inactivation of inhibitor after addition of proteolytic enzymes (trypsin and proteinase K) and 0.2N NaOH for alkaline hydrolysis. After prolonged refrigeration and incubation at high temperature (600C, 1200C and under autoclave) for 15 minutes, inhibitor was found to be active against Bacillus cereus and Salmonella typhimurium. Therefore, inhibitor produced by the Pseudomonas aeruginosa can be bacteriocin-like material and may find application in medicine and in minimally processed food stuffs.**

Keywords:-- Pseudomonas aeruginosa, Bacillus cereus, Salmonella typhimurium, antibacterial activity, bacteriocin-like material.

I. INTRODUCTION

Worldwide number of bacterial strains rapidly developed resistance against available antibiotics (Reinert et al., 2005; Furuya et al., 2006) and as a result, huge numbers of people are at high risk of developing severe sepsis and septic shock (D.N. Cook et al., 2004; R.J. Ulevitch et al., 2004). Since the broad-spectrum antimicrobials inhibit almost all bacteria in body including the body's normal flora [jay]. Therefore, there is a pressing necessity to develop narrow-spectrum antibiotics that will be prevalent when a rapid technique for clinically diagnosing microbial infection is widely available in the near future (C. Walsh, 2003). This implies that more attention should be focused on narrow-spectrum bacteriocins. Bacteriocins and bacteriocin-like material are small, extracellularly released antimicrobial peptides or peptide complexes (usually 30–60 amino acids) produced by a number of different bacteria that are usually effective against closely related species (Baugher and Klaenhammer, 2011). Bacteriocins have potential for being used as the next generation of veterinary and human antimicrobials (P.D. Cotter et al., 2005; O. Gillor et al., 2005). However, instead of using native bacteriocins for the development of antimicrobials, fusion of narrow-spectrum bacteriocins with specific recognition domains of target microorganisms (Y. Michel-Briand, 2002; J.M. Rodriguez,, et al., 2003) would be effective alternative. In last decade, some bacteriocins that are generally recognized as safe (GRAS) have received great attention due to their potential applications in food preservation, probiotics in the human health and as therapeutic agents against pathogenic microorganisms (xx Riley and Wertz, 2002). (Sullivan et al., 2002; Cotter et al., 2005). The bacterium possesses a wide range of secretion machineries and extracellularly exports numerous proteins considered to be important in the pathogenesis of clinical strains like Pseudomonas aeruginosa (Hardie et al., 2009). The Pseudomonas species having biocontrol properties against plant pathogens (Chin-A-Woeng TF et al., 2000) and some strain can produce antagonistic compound against gram-positive microorganisms. (Esipov et al., 1975). In this paper, we report on the screening, optimum conditions for production and partial characterization of bacteriocin-like material produced by Pseudomonas aeruginosa. The antibacterial activities were tested against.

II. MATERIALS AND METHODS

2.1 Isolation of Microorganisms

The milk sample was collected from local milk collection center and mixed with sterile DI water (1:1 v/v)

ISSN (Online) 2456 -1304

International Journal of Science, Engineering and Management (IJSEM)
Vol 2, Issue 12, December 2017

then 0.1 mL of suspension was spread inoculated on to nutrient agar plate. After incubation at 37oC for 24 hour single colonies were isolated and screened for antimicrobial activity. The isolated bacterial strain was identified by 16S rRNA partial sequencing.

2.2 Strains and culture conditions

The isolated and identified strain of Pseudomonas aeruginosa was cultured on nutrient agar slants at 37oC for 24 hour and stored in refrigerator at 4oC. Standard bacterial species used as indicator microorganisms; Bacillus cereus and Salmonella typhimurium was cultured on nutrient agar slants at 37oC for 24 hour and stored in refrigerator at 4oC.

2.3 Screening for antibacterial activity

The antibacterial activity of Pseudomonas aeruginosa was tested qualitatively by agar well diffusion assay (AWDA) [jay] on Bacillus cereus and Salmonella typhimurium. The Muller-Hilton (MH) agar plates were spread inoculated with overnight grown cultures of B. cereus and S. typhimurium. On the surface of agar plates, wells of 5 mm in diameter and of 18 µL in capacity were formed by using sterile gel borer. The 15 µl of an overnight grown culture of the Pseudomonas aeruginosa were placed in each well and plates were incubated at 370 C for 24 hours. The antibacterial activity was related to the area (mm2) of the inhibition zone measured around well (Salim Ammor et al., 2006).

2.4 Determination of inhibitor site of synthesis

Overnight grown culture (1×105 CFU/ mL) of Pseudomonas aeruginosa underwent centrifugation (10,000 rpm for 10 min at 4oC) to obtained cell-free supernatant and culture pellets in sterile DI water (T. Ghrairi et al., 2008). The antibacterial activities of the two samples of Pseudomonas aeruginosa were determined against test organisms to find out whether the inhibitor is intracellularly or extracellularly synthesized.

2.5 Determination of optimum pH value for growth of Pseudomonas aeruginosa

To determine the optimum pH at which Pseudomonas aeruginosa exhibited the maximum growth, overnight grown culture (1×105 CFU/ mL) of Pseudomonas aeruginosa were inoculated in different flask of nutrient broth with different pH (6,7,8,9) at 37oC. At intervals of 0, 4, 8, 12, 16, 20, 24, and 28 hour absorbance of samples at 600 nm were measured.

2.6 Estimation of rate of growth, protein and bacteriocin production

Bacterial culture (1×105 CFU/ mL) of Pseudomonas aeruginosa were inoculated in nutrient broth for estimating growth rate, protein and bacteriocin production. At intervals of four hours bacterial culture were tested for biomass production by taking absorbance at 650 nm (T. Ghrairi et al., 2008). The proteins produced by bacteria were quantitatively determined by Lowery's method and checked each sample for its antimicrobial activity by AWDA method.

2.7 Fractional characterization of the inhibitor

Sensitivity of inhibitor to heat was examined by treating the culture supernatant of Pseudomonas aeruginosa in water bath at 600C, 1200C and under autoclave for 15 minutes. The effect of extended storage at low refrigerated temperature on supernatants stability was evaluated by placing supernatants at 40C up to 15 days. (T. Ghrairi et al., 2008; A.G. Ponce et al., 2008). To evaluate the nature of inhibitor the supernatant was treated with trypsin, proteinase K, and 0.2N NaOH (jay). The absence of inhibition zone in presence of the proteolytic compounds confirmed polypeptide nature of inhibitor (Lewus, C et al., 1991).

III. RESULTS

3.1 Screening for antibacterial activity

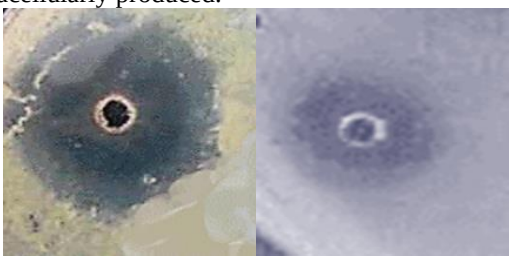
Pseudomonas aeruginosa were shown to produce inhibition zone against Bacillus cereus and Salmonella typhimurium. (Figure 1)

(a) *(b)*

Figure 1: Antimicrobial activity of Pseudomonas aeruginosa against (a) B. cereus (b) S.typhimurium

3.2 Detection of inhibitor site of synthesis

The cell-free supernatant and culture pellets dissolved in sterile DI water both were shown antibacterial activity against Bacillus cereus and Salmonella typhimurium. Since, cell-free supernatant of Pseudomonas aeruginosa showed inhibition (Figure 2) it proves that the inhibitor was extracellularly produced.



(a) (b)

Figure 2: Cell-free supernatant of Pseudomonas aeruginosa showed inhibition against (a) B. cereus (b) S.typhimurium

3.3 Determination of optimum pH value for growth of Pseudomonas aeruginosa

ISSN (Online) 2456 -1304

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The growth rate of Pseudomonas aeruginosa in medium having different pH values with respect to time shown in figure 3. The Pseudomonas aeruginosa has shown maximum growth at 20th hours in medium having pH 7.0. Therefore, the optimum pH for growth of Pseudomonas aeruginosa isolate was found to be 7.0.

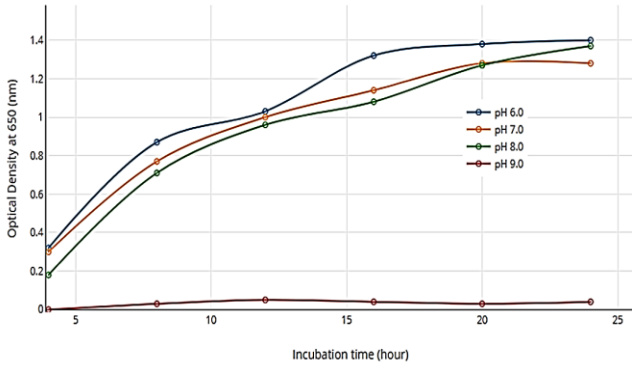

Figure 3: The growth rate of Pseudomonas aeruginosa at different pH

3.4 Estimation of rate of growth, protein and bacteriocin production with respect to time

The protein concentration and bacteriocin production with respect to antibacterial activity of Pseudomonas aeruginosa was estimated over a growth and time (Figure 4). The Pseudomonas aeruginosa has shown maximum growth at 20th hours and maximum activity of inhibitor and protein concentration at 16th hour. Therefore, the optimum incubation time for maximum inhibitor and protein production of Pseudomonas aeruginosa was at16th hour.

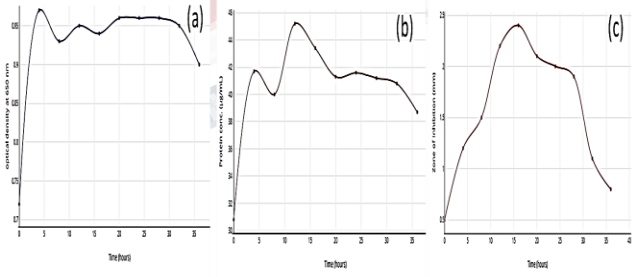

Figure 4: Rate of production of (a) biomass, (b) protein and (c) Inhibitor

3.5 Fractional characterization of the inhibitor

Cell-free supernatant of Pseudomonas aeruginosa showed greater stability to high temperature and retained antibacterial activity after 15 min incubation at 600C, 1200C and under autoclave (Table 1). Since, inhibitor was found to be heat stable and also bacteriocin show same property, inhibitor can be considered as bacteriocin-like material.

Table 1: Effect of temperature on activity of inhibitor against B. cereus and S.typhimurium

Temperature	*B. cereus*	*S.typhimurium*
60^{0}C	+	+
120^{0}C	+	+
Autoclave	+	-

Note: + inhibition zone present, - inhibition zone absent

Application of Trypsin and Proteinase K at final concentration of 1 mg/mL and 0.2N NaOH led to inactivation of the antibacterial activity of inhibitor (Table 2). Therefore, it was proved that the inhibitor produced by Pseudomonas aeruginosa was proteinaceous in nature.

Table 2: Effect of proteolytic compounds on activity of inhibitor against B. cereus and S.typhimurium

Proteolytic compound	*B. cereus*	*S.typhimurium*
Trypsin	+	+
Proteinase K	+	+
0.2N NaOH	+	+

Note: + inhibition zone present, - inhibition zone absent

IV. DISCUSSION

The area where prevalence of B. cereus and S.typhimurium is more, we restricted our investigation in the following sections to the study antibacterial activity of bacteriocin-like material against B. cereus and S.typhimurium. Therefore, characterizing the inhibitor responsible for the inhibition of B. cereus and S.typhimurium was of interest. Pseudomonas aeruginosa is able to produce proteinaceous inhibitor that inhibits the growth of B. cereus and S.typhimurium, it may also be due to the production of bacteriocin or bacteriocin-like compounds (A.G. Ponce et al., 2008; Gonza´ lez, L et al., 2007). Since, bacterial strains need to withstand the competition of other microorganisms to survive in their hostile natural environment, so they often produce antimicrobials (Ayad, E et al., 2002). The antagonistic activity Pseudomonas aeruginosa was sensitive to proteolytic enzymes, indicating that it was due to proteinaceous nature of inhibitor. The bacteriocin produced by producer strains was thermostable, and thus would be a very useful characteristic if it was to be used as food preservative and other biotechnological processes. It is also stable over a wide range of pH and may be useful in acid as well as nonacid environment. Like most bacteriocins (Campos, A et al., 2006; Parente, E et al., 1999), Pseudomonas aeruginosa bacteriocin was

produced during the exponential growth phase, with the greatest production occurring during the beginning of the stationary phase. However, additional experiments need to be designed to test the allergenicity and pathogenicity of bacteriocin- like material produced by Pseudomonas aeruginosa for further use in food or feed or anywhere in medications. Therefore, its immediate use in food preservation and medicine is not recommended. But bacteriocin- like material of Pseudomonas aeruginosa can be used if it does not show any allergenic and pathogenic effects on human and other animals. However, antibacterial activity displayed by Pseudomonas aeruginosa against B. cereus and S. typhimurium encouraged us to consider it for further investigations.

V. CONCLUSION

The Pseudomonas aeruginosa inhibits the growth of B. cereus and S. typhimurium by producing bacteriocin- like material. The maximum antimicrobial activity of pseudomonas aeruginosa was recorded at 16th hrs incubation in nutrient broth at 370C. The antimicrobial activity was found to be extracellular since, cell-free supernatant showed inhibition. The antimicrobial activity of pseudomonas aeruginosa was inactivated by the addition of proteolytic compounds, thus confirmed the proteinaceous nature of the inhibitor. The bacteriocin activity was stable after extended refrigerated storage and at high temperature (600C, 1200C and under autoclave for 15 minutes). Therefore, the bacteriocin like- material of Pseudomonas aeruginosa can be used as a barrier against the undesirable microorganisms in complex ecosystem.

Conflict of Interest: The authors declare that they have no conflict of interest.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent: Not applicable

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