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EFFECT OF INCUBATION PERIOD ON ANTIBACTERIAL ACTIVITY OF CRUDE FERMENTATION BROTH OF A GUAVA PLANT ASSOCIATED *BACILLUS* SP. MTZ-1 AGAINST CLINICAL PATHOGENS

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ABSTRACT

The cell free culture supernatant (crude extract) of a *Bacillus* sp. MTZ-1 isolated from rhizosphere soils of *Psidium guajava* (guava) grown under submerged fermentation showed antibacterial effect against a number of clinical pathogens. The maximum activity was obtained against *Pseudomonas sp*. showing zone of inhibition of 27 mm followed by that of 24 mm against *E. coli* and 22 mm *against Salmonella* sp. Moreover, Vancomycin Resistant *E. coli* (VRE), *Bacillus subtilis, Staphylococcus epidermidis* showed zone of 20 mm in each case. The zone of inhibition against *Acinetobacter*, Enteropathogenic *E. coli* (EPEC), Methicillin Resistant *Staphylococcus aureus (MRSA), Klebsiella* sp., *Shigella* sp. and *Staphylococcus aureus* was found to be 15mm, 8mm, 13mm, 17mm, 12mm and 18mm, respectively.

KEYWORDS: *Bacillus* sp. MTZ-1, Antibacterial effect, Clinical pathogens.

INTRODUCTION

A number of pathogenic bacterial and fungal strains causes many infectious diseases and the treatment of which has been a major problem in the medical field (Mehrgan *et al*., 2008; Bazzaz *et al*., 2005; Calvin and Kunin, 1993). This leads to the increased necessity and synthesis of more effective inhibitory compounds (like azoles and quinolone derivatives), but the adverse effect of synthetic compounds has enforced the study of natural metabolites from microorganisms (Emami *et al*., 2008; Shafiee *et al*., 2008; Mehrgan *et al*., 2008; Bazzaz *et al*., 2005). The use of synthetic compounds has also led to the increased emergence of microbial resistance which in turn increase the demand for new antimicrobial agents for which there is a need to explore new promising microorganisms capable of producing new inhibitory metabolites (Jean *et al*., 2011; Thakur *et al*., 2007). For this purpose, first step is the screening of fungal and bacterial strains which are capable of producing inhibitory compounds (Imada *et al*., 2007). Bacillus is an important genus to search for the production of inhibitory compounds as it is able to produce several antimicrobial peptides (Bizani *et al*., 2005). *Bacillus* strains have been reported to produce secondary metabolites which have shown antibacterial and antifungal activity against phytopathogens and food spoiling microorganisms (Shirokov *et al*., 2002; Touré *et al*., 2004; Czaczyk *et al*., 2000). The parameters used for cultivation have been known to greatly influence the production of secondary metabolites by microorganisms, even minute changes in the medium can not only influence the amount of the specific compound but also effect the general metabolism of the organism (Scherlach and Hertweck, 2009). Therefore, the nutrients and their concentration present in the production medium are very important, among which the carbon source has been the focus of study by industries and research groups (Sanchez *et al*., 2010). The current study was aimed to explore the antibacterial potential of *Bacillus* sp., MTZ-1 by using its cell free culture supernatant.

MATERIALS AND METHODS

The inhibitory effect of crude fermentation extract of *Bacillus* sp. MTZ-1, previously isolated from rhizosphere of *Psidium guajava* (guava) plant by Microbiology and Biotechnology Research Laboratory, Department of Microbiology, Federal Urdu University of Arts, Science and Technology, Karachi, Pakistan, was tested against a number of Gram negative and Gram positive bacteria by Agar Well Diffusion Method (Zaidi *et al*., 2013: Naz and Rasool, 2013). *Bacillus* strain was first grown into the nutrient broth followed by inoculation of the 24 h culture in to the fermentation medium containing g/L of Glucose (0.1), Peptone (10), Starch (5),Yeast extract (5), KH_2PO_4 (0.05), $MgSO_4$ (0.01), $CaCl_2$ (0.01), $FeSO_4$ (3) and Skim milk (10), for the production of metabolites. The inoculum in production medium was kept under static fermentation conditions with a vigorous shaking after every 24 h manually. The samples were taken aseptically from the production medium at 24 h intervals and centrifuged at 3000 rpm for 30 minutes to obtain the cell free crude fermentation extract and effect of incubation period was demonstrated.

The bacterial cultures to be tested were first refreshed in nutrient broth for 2h, the turbidity was compared with 0.5% Mac Farland's solution and then seeded on the nutrient agar plates with the help of a sterile cotton swab, respectively. Wells of 6mm diameter were made on the plates with the help of a borer. A total of 100µl of the crude fermentation extract, taken at different time intervals was poured in to the wells marked respectively. The plates were incubated at 37°C for 24h and antibacterial activity was determined by measuring the zone of inhibition around each well.

RESULTS AND DISCUSSION

Effect of Antibacterial activity of crude extracts of *Bacillus* sp. MTZ-1 taken at different time intervals (24-96 h) was observed against *Vancomycin resistant Enterobacter* (VRE), *Bacillus subtilis, Pseudomonas* sp., *Acinetobacter, Enteropathogenic E. coli (EPEC), Methicillin resistant Staphylococcus aureus* (MRSA), *Klebsiella* sp., *Salmonella* sp., *Shigella* sp., *Staphylococcus epidermidis, E. coli* and *Staphylococcus aureus* with maximum activity against *Pseudomonas* sp., showing zone of inhibition of 27 mm followed by of 24mm against *E. coli* and 22 mm *against Salmonella* sp. Moreover, VRE, *Bacillus subtilis, S. epidermidis* showed maximum zone of 20 mm in each case. The zone of inhibition against *Acinetobacter,* EPEC, MRSA, *Klebsiella* sp., *Shigella* sp. and *S. aureus* was found to be 15mm, 8mm, 13mm, 17mm, 12mm and 18mm, respectively (Fig. 1). *Bacillus* species have been known for the potential production of significant antibiotics and zone of inhibition against *S. aureus* and *K. pneumoniae* have been observed to be 5 and 6 mm, respectively, in previous studies (Kuta *et al*., 2008). Another study showed no antimicrobial activity of *Bacillus cereus* against *Pseudomonas aeruginosa* and *S. aureus*, which is contrary to the present study. It also reported 12 mm zone of inhibition against *Klebsiella pneumoniae* (Quoba *et al*., 2007). *Bacillus* species are spore formers thus the maximum production of metabolites take place during 0-48h after that they start producing endospores, therefore zone of inhibition is small in 72h, later when the spores started to re-germinate (at 96h) they regain their metabolic activity, start producing metabolites again and zone of inhibition again attains its peak, (Egorov *et al*., 1986) this is probably the reason for highest activity at 96h in our study. Other reports also showed the maximum metabolite production at post-stationary phase (Lee *et al*., 2008; Hassanien *et al*., 2009). However, some other studies revealed that the maximum activity was found only during rapid growth phase (24-48h) by *Bacillus licheniformis*ATCC-14580 (Yousaf, 1997; Haavik and Froyshov, 1975). Egorov *et al*. (1986), reported the maximum activity of bacitracin at the end of log phase and start of spore formation, which is in contrast to our findings in the present study.

The effect of incubation period on antibacterial activity was found in most cases that inhibitory effect of cell free culture supernatant was directly propotional to the incubation period. In case of VRE, the zone of inhibition was 16 mm after 24 h which followed a decline to 8 mm and 14 mm at 48 and 72 h, respectively, and attained maximum antibacterial activity with 20 mm zone on further incubation for 96 h. The same pattern was shown by *Bacillus subtilis* (Fig. 2) and *Pseudomonas* sp., where maximum activity was observed at 96 h of growth of the producer strain with zones of 20mm and 27mm, respectively. In case of *Acinetobacter* sp., EPEC, MRSA, *Klebsiella* sp., *Shigella* sp., *S. epidermitidis, E. coli* and *S. aureus* the crude cell free filtrate failed to show any inhibitory effect during initial hours (24-48h) while late incubation resulted in increased production of inhibitory substances in the fermentation broth. However, the pattern was reverse in *Salmonella typhi* which was found to be sensitive by 24 h crude filtrate but later developed resistance and no sensitivity was observed against 48-96 h samples. These findings showed that the concentration of inhibitory metabolites of *Bacillus* sp., MTZ-1 in fermentation broth varied at different incubation periods with increased production at late hours resulting in increased sensitivity of test organisms.

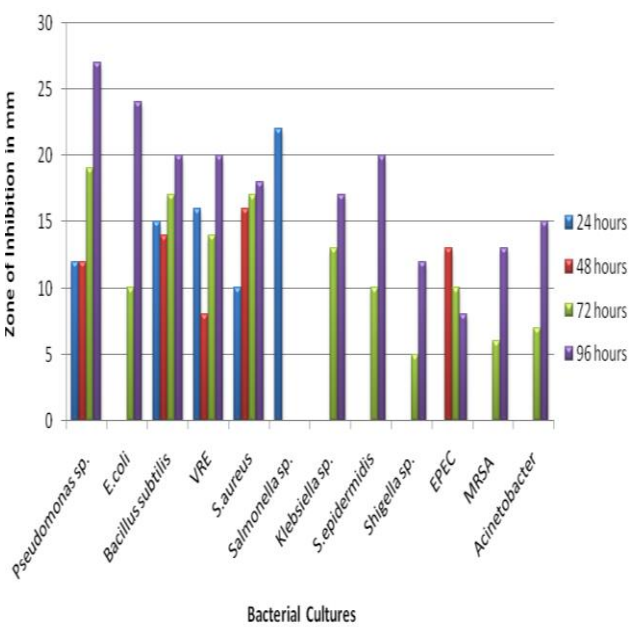

Fig. 1. Antibacterial activity of *Bacillus* sp. MTZ-1 against clinical pathogens at different time intervals.

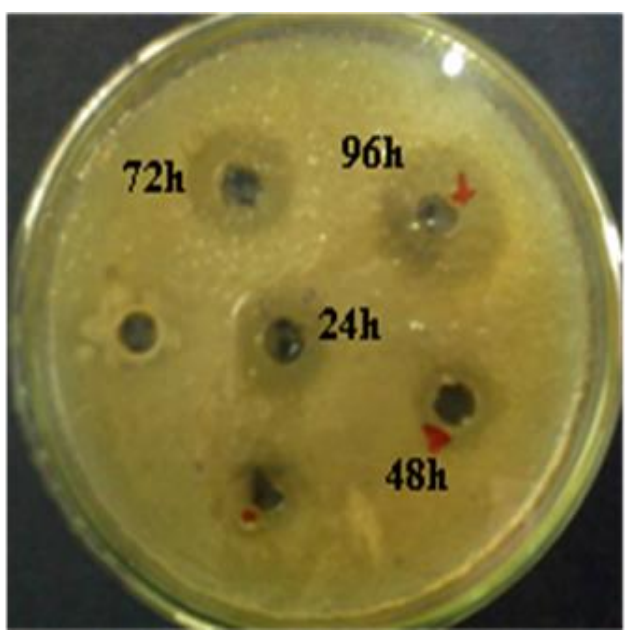

Fig. 2. Antibacterial activity of crude extract of differentially aged *Bacillus* sp. MTZ-1 against *B. subtilis*.

CONCLUSION

The current study represents *Bacillus* sp. MTZ-1 as a potent candidate for the production of antibacterial substances and its potential can be further utilized for the enhanced production, recovery and identification of antibacterial compounds.

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