Optimized antimicrobial peptide (Bacitracin) production by immobilized and free cells and of *Bacillus Spp* GU215 using Wood chips and silicon polymer beads

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Abstract: The immobilization of bacillus spp. GU215 on silicon polymer beads, wood chips was performed and antibiotic peptide (bacitracin) production, optimization of parameters were investigated. The immobilized cells presented elevated levels of activity than free cells. The silicon polymer based cells showed widest zones of inhibitions (18mm) in 72 hours and 4% concentration of glucose, PH 8 and 30°C, whereas a marginal decrease in the activity (14mm) was noticed in case of wood chips based immobilization systems and least stable immobilization in 72 hours incubation time, 4% glucose concentration, PH 8 and 30°C. This study illustrates that the silicon polymer based beads facilitate a strong interactions with bacitracin producing cells and render them suitable for excessive and long time production of antibiotic.

Keywords: Bacitracin, immobilization, hydrophobic interactions, adsorption, carbon source.

INTRODUCTION

The "antibiotics research" is a key area in both biotechnology and medical microbiology that blessed humans with variety of antimicrobial agents for ailment of multiple infections (Prakasham *et al.,* 2002). Despite the fact, since past five decades development of resistance (Palumbi, 2001) followed by a rapid fall in the effectiveness of present antimicrobial therapies urged researchers to develop and search for new antimicrobial peptides, that comprise an essential portion of the immune system of all multicellular organisms (Rivas *et al.,* 2009., Gordon *et al* 2005., Guani-Guerra *et al*., 2010). Above 700 different antimicrobial peptides have been researched from multitude of plants, animals, viruses, bacteria, fungi and so far (Reddy *et al*., 2004). Almost all antimicrobial peptides are characterized by Immense cationic behavior, small size i.e low molecular weight (between1-5 kDa) and acceptance to take amphiphatic structures that mainly facilitates association with surfaces and membranes (Perron *et al*., 2006).

To boost the productivity with optimized the economics, modification of the culture systems and whole cell immobilization technology remained the aim of researchers in the antibiotic production during fermentation (Prakasham *et al*., 2002). The cell immobilization is the physical localization or confinement of whole cells to specific region of solid support with aim to preserve desired biological activity (Karel *et al*., 1985). Immobilization is generally accomplished by using various techniques including, entrapment in the matrix, adsorption on surface of carrier, self-aggregation through flocculation and covalent linkages (Mohapatra *et al*., 2007, Pilkington *et al.,* 1998). A variety of solid supports are used for imobilization including calcium alginate, polyacrylamide, carrageenan gel and agar-agar alumina etc (Krajewska 2004, Linderholm *et al*., 2004, Phadtare *et al*., 2004).

The immobilization of whole cells provide immense industrial advantages including cost effectiveness, excessive production, time saving and inexpensive steps involved in purification and isolation of bioactive compounds including antibiotics (Ohmiya, Ohashi, Kobayashi & Shimizu, 1977). Moreover the immobilization is preferred these days due improved stability of the product (Karel *et al*., 1985, Bernal *et al*., 2007) and facility to re use the bioactive cells/molecules (Zheng *et al.,* 2004, Fernandes *et al.,* 2004, Phadtare *et al*., 2004).

In the present investigation, the peptide antibiotic bacitracin production using immobilized cells, optimization of parameters for bacitracin production and the feasibility of using simplified solid support for immobilization was attempted.

MATERIALS AND METHODS

Microorganisms and culture conditions

A bacitracin producing bacillus strain GU251 isolated in Gomal Center of Biochemistry and Biotechnology (GCBB), Gomal University D.I. Khan, KPK, Pakistan was used during investigations. This culture was preserved on agar slants at 4°C and sub cultured after specific intervals. The culture was transferred at least twice before use. The bacitracin production medium contained (g/l); $MnSO_4.H_2O$ 0.01, KH_2PO_4 0.5, $MgSO_4.7H_2O$, Glucose 10, $FeSO_4.7H_2O$ 0.01, $CuSO_4.7H_2O$ 0.01, L-glutamic acid 5.0; K_2HPO_4 0.5; NaCl 0.01; $CaCl_2.H_2O$ 0.015: PH 8) (Hafizullah *et al*., 2010).

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Preparation of cell culture

About 5 ml of sterilized distilled water was added to a well cultured (7 to 8 days) slant. The cell suspension was prepared and transferred to 45 ml of growth media in 250 ml Erlenmeyer flask. The inoculated flask was incubated at 30°C in an orbital shaker at 120 rpm. The inoculum was incubated till 72 hours and the broth culture was centrifuged. The pellets were washed using sterilized KCl 2% solution and resuspended in sterilized saline solution. This prepared cell suspension was later used in successive immobilization experiments.

Bacitracin activity

The fresh samples were taken from fermentation broth in microcentrifuge tubes and centrifuged for 15 minutes at 12,000 rpm. The supernatant was used in the agar diffusion assay for bacitracin activity against *Staphylococcus aureus* (Barefoot and Krauthammer, 1983). Briefly, about 5 µl of the two fold diluted, cell free supernatant was transferred aseptically to pre formed wells in sterilized petri plates that were seeded already with 5 µl of the *Staphylococcus aureus* (ATCC# 6538). The loaded plates were incubated for 24 hours at 30°C and observed for zone of inhibitions around each well.

Cell density measurements

The cell density measurements were performed using spectrophotometer at 600nm wavelength (OD 600nm) using a spectrophotometer (Shimadzo, Japan). For cell dry weight (CDW) determination, portions (18 ml) of cell culture was centrifuged for 15min at 12,000 rpm. The supernatant so collected was washed thrice with sterilized distilled water and dried for 24 hours in hot air oven at 70°C.

Optimization of various parameters for maximum antibiotic production

The optimized production of bacitracin was observed with respect to incubation time (0-96 hours), Glucose (2-5%) and PH o fermentation media (6-8) (Hafizullah *et al*., 2010)**.**

Immobilization of whole cells

Immobilization on Wood Chips

About 100 wood chips of 2x2 mm were autoclaved for 15min at 121ºC, and transferred aseptically into 50ml flask having 20ml of nutrient broth. To this about 5ml of cell culture was added and flask were incubated for 24 hours at 30°C in an orbital shaker incubator at 120 rpm for immobilization. Then these wood chips were thrice washed with autoclaved distilled water and inoculated into production media and incubated at 30ºC in orbital shaker at 120rpm upto 72 hours. One ml of the each sample was taken after 24 hours to determine optical density at UV spectrophotometer and antibacterial activity (zones of inhibition).

Immobilization on Silicon polymer beads

Silicon sealant (GMSA RTV Silicon sealant) was purchased commercially. The silicon sealant beads about 2mm in diameter approximately were prepared using sterilized syringe. The beads were surface sterilized with spirit and then thrice washed with sterilize distilled water. About 100 beads 2mm in diameter transferred aseptically into 50 ml flask having 20ml of nutrient broth and 5 ml microbial suspension and incubated at 30^{o}C for 24 hours orbital shaker at 120 rpm for immobilization. The beads were then thrice washed with sterilized distilled water followed by inoculation into production media and incubation at 30ºC in orbital shaker at 120 rpm upto 72 hours. One ml of the each sample was taken after every 24 hours to determine optical density at UV spectrophotometer and antibacterial activity (zones of inhibitions).

RESULTS

Bacitracin producing strain

The bacitracin producing strain GU251 (Snell *et al*., 1956; Meyers *et al.,* 1968) isolated from roots of rice previously isolated and identified using biochemical and cytomorphological techniques (MacFaddin 2000, Krier and Holt 1984) was used during investigation.

Bacitracin production by free and immobilized cells

Bacitracin production using *Bacillus spp* GU215 under free, silicon and wood chips based immobilized states was investigated in batch fermentation. Based on observation regarding zone of inhibitions at various time intervals (0-120 hours) and cell mass density, it was evident that the immobilized cells produced more antibiotic than the free cells. The free bacillus strain GU215, presented widest zones of inhibition (12mm) at optimum PH 8 after 72 hours of incubation. A steady increase in the activity was observed till 72 hours, followed by a progressive decline. At time zero no activity was evident (fig. 1). Likewise an optimum rise in the activity of the bacillus strain was reported with the increase in the glucose concentration up to 4%, whereas comparatively lesser zones of inhibitions were resulted at higher glucose concentration (fig. 2).

Never the less much encouraging results were obtained with immobilized cells. Both the silicon and wood chips immobilized cells started their activity from time zero, that was not evident in case of free cells (fig. 3). The widest zone of inhibition for bacitracin production (18mm) by test strain was reported in 96 hour by silicon immobilized cells at optimum PH 8 and 4% glucose concentration (fig. 4).

In the case of wood chips immobilized cells notably reduced activity was recorded in comparison with silicon immobilized cells. However a slight increase in bacitracin production was seen contrast to free cells (figs. 1, 5). The

wood chips immobilized cells presented peak activity (14 mm) in 72 hours with maximum zones of inhibition (14mm) at 4% glucose concentration at PH 8 (figs. 6-8).

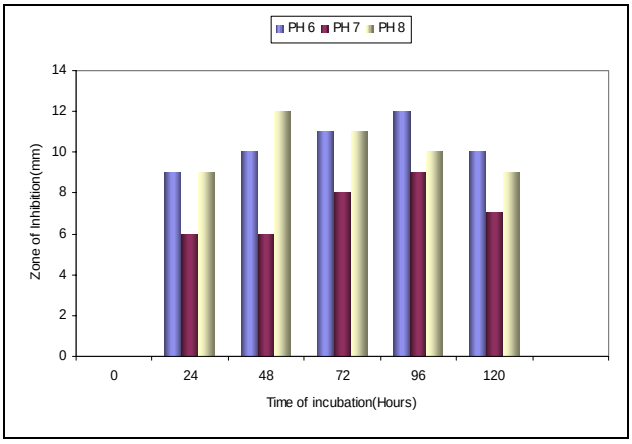

Fig. 1: Maximum antimicrobial peptide (Bacitracin) production by free cell at various PH (6-8) and time of incubation (0-120 hours).

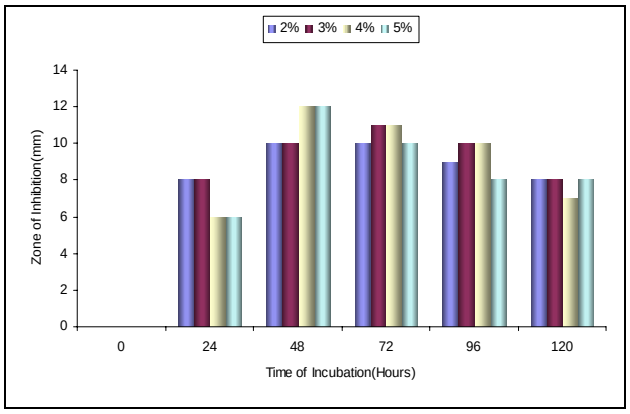

Fig. 2: Optimized antimicrobial peptide (Bacitracin) production by free cell at various glucose concentration (2-5%) and time of incubation (0-120 hours) at PH 8.

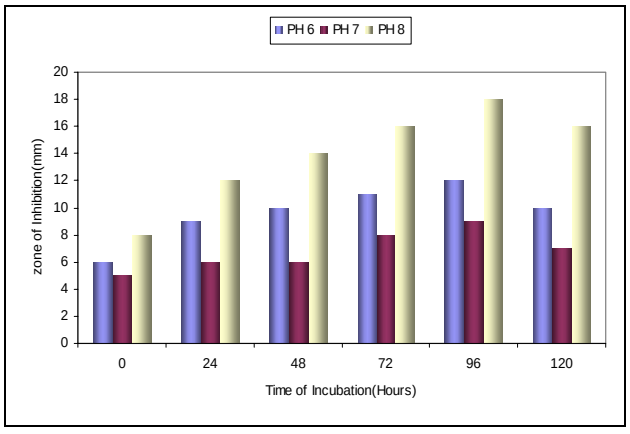

Fig. 3: Maximum antimicrobial peptide (Bacitracin) production by silicon immobilized at various PH (6-8) and time of incubation (0-120 hours).

DISCUSSION

Immobilization technology has attained a promising advancement in biotechnology and biomedical sciences. A huge set of bioactive materials like drugs, plant and animal cells, proteins, and microorganisms producing secondary metabolites like antibiotics have been immobilized on definite supports (Krajewska, 2004., Linderholm *et al.*, 2004., Phadtare *et al*., 2004). The immobilization of antibiotics producing strains is not a new dilemma and numerous successful attempts have been made in the past with the aim to produce secondary metabolites in excessive quantity (Schallmey *et al.*, 2004, Hamedi *et al.*, 2005, Mendo *et al*., 2004). In present study we investigated the effect of immobilization using silicon beads and wood chips on peptide antibiotic production by bacillus spp GU215.

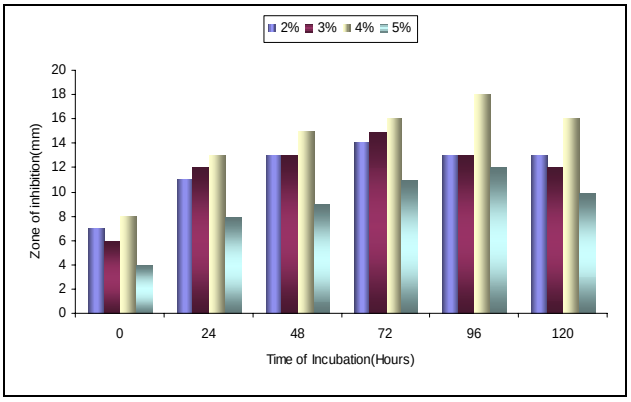

Fig. 4: Optimized antimicrobial peptide (Bacitracin) production by silicon immobilized cell at various glucose concentration (2-5%) and time of incubation (0-120 hours).

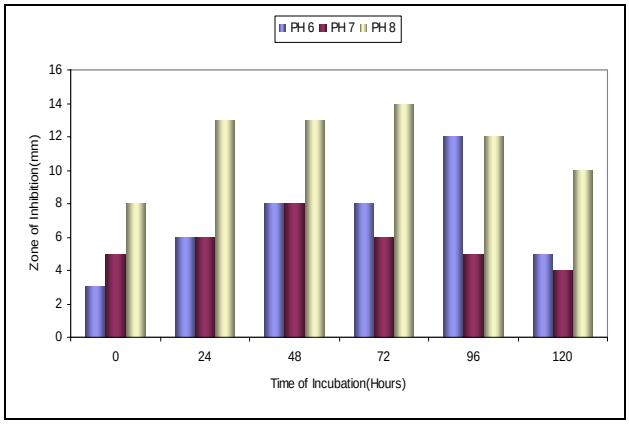

Fig. 5: Maximum antimicrobial peptide (Bacitracin) production by wood chips immobilized cells at various PH (6-8) and time of incubation (0-120 hours).

Our principle finding in this study was that, immobilized cell presented more activity compared to the free cells. The free cells presented a delayed lag phase of 24 hours,

while immobilized cells commenced the antibiotic production immediately. This is due to stress imposed during the immobilization process, not found in the free cells fermentation (Prakasham *et al.,* 2002). However it was evident that the peptide antibiotic production by bacillus spp was not equivocal in both immobilization media and the wood chips based systems presented a reduced activity. This is most likely due to weak interaction, kinetic behavior and lesser adsorption forces between the whole cell and wood chips (Razmovski and Pejin, 1996, Lampty and Moo-Young, 1987).

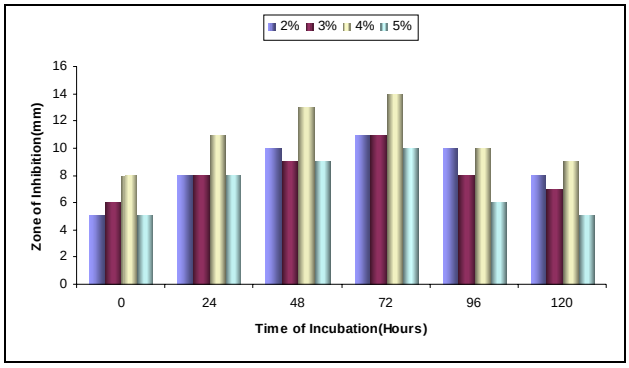

Fig. 6: Optimized antimicrobial peptide (Bacitracin) production by Wood chips Immobilized cell at various glucose concentration (2-5%) and time of incubation (0-120 hours).

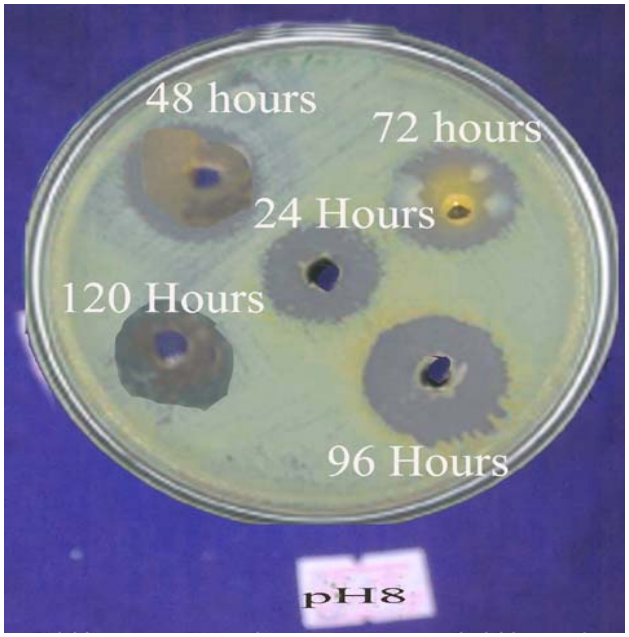

Fig. 7: Silicon Immobilized Cells with zones of inhibitions (mm)

The free cells of bacillus pp GU 215 presented maximum activity in 48 hours with immediate rise just after lag phase, followed by steady fall in activity afterwards. Most of the bacillus spp isolated from soil environment are accompanied by similar behavior. This most probably corresponds to environmental influences and habitat (Awais *et al.,* 2010, Sharga *et al.,* 2004, Costa *et al*., 2011). Likewise optimum bacitracin production was observed at alkaline PH as reported earlier (Fariha Hassan *et al*., 2009, Muhammad *et al*., 2009) however variability in activity of isolated bacillus spp is dependent on native habitat of the strain (Mendo *et al*., 2004). The effect of carbon source on production of secondary metabolites has been primary subject of many studies (Glinel *et al*., 2009, Zasloff, 2002, Bushra *et al.,* 2007, Awais *et al*., 2008, Muazz *et al.,* 2007) that propose an increase in activity with increase in carbon source (for example glucose) till optimum levels as shown during present study.

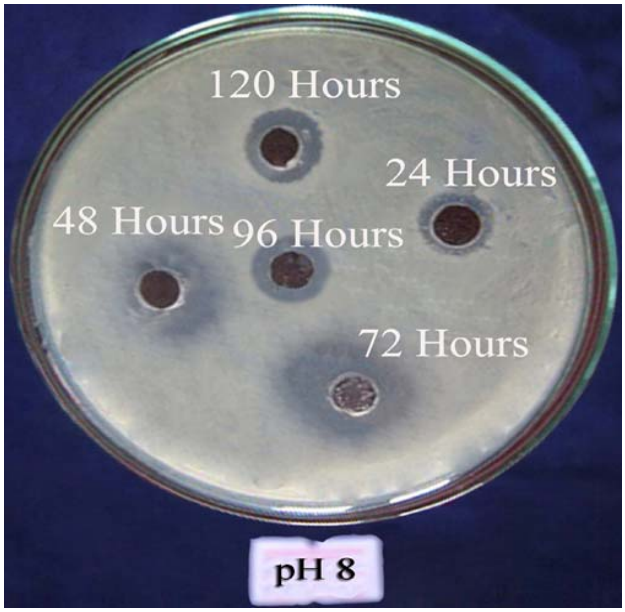

Fig. 8: Woodchips immobilized cells with Zones of Inhibition (mm).

The silicon immobilized *bacillus spp* showed maximum activity than both wood chips immobilized and free cells at initial lag time. The silicone polymer based products are hydrophobic in nature that makes it attractive in the immobilization of cell due to hydrophobic interaction (Claudino *et al*., 2008) as observed during present investigation. These results prove that silicon based immobilization system not only present immediate response to the production media but also to the increasing concentration of glucose. These findings are reported earlier (Pan *et al*., 1997, Oriel 1988, Kawakami *et al*., 1990).

Never the less, upon comparative evaluation of antimicrobial activity, the wood chips immobilized cells gave better productivity than free cells however weak adsorption capacities limited its significance (de Ory *et al*., 2004). Same as silicon beads, the log time began immediately after immobilization, and accelerated with the increase in the glucose concentration. However the

productivity did not continued for longer duration of time due to kinetic behavior and weak forces, decrease in cell viability over the time and shear stress produced by either agitation or aeration or involvement of both (Ramakrishna and Prakasham, 1999, Razmovski *et al*., 1996).

The bacillus spp GU215 showed activity nearly at all PH levels from 6 to 8, whereas at PH 8, both free and immobilized cells presented maximum activity. This corresponds to the fact that changes in PH levels, directly effect the secondary metabolite synthesis in the bacterial cells (Solé *et al.*, 1997). These finding are supported by earlier reports (Stein 2005, Srinivasulu *et al*., 2003).

The findings of present investigation proposed that the silicon immobilized cells of bacitracin reducing bacillus Spp GU215 presented encouraging antimicrobial activity at 4% glucose concentration in 72 hours at PH 8. We further conclude that silicon based hydrophobic immobilization systems can effectively indulged for immobilization of whole cells

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