

PRODUCTION OF PEPTIDE ANTIBIOTICS BY *BACILLUS SP. GU 057* INDIGENOUSLY ISOLATED FROM SALINE SOIL

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ABSTRACT

A total of 112 soil samples were taken from different areas of district D.I.Khan and Kohat (KPK) Pakistan and screened for production of antibiotics against the *Micrococcus luteus* and *Staphylococcus aureus*. Widest zone of inhibition (18mm) was produced by microorganism isolated from saline soil. The strain was later identified as *Bacillus* GU057 by standard biochemical assays. Maximum activity (18mm inhibition zone) was observed against *Staphylococcus aureus* after 48 hours of incubation at pH 8 and 4% concentration of glucose. The antibiotic was identified by autobiography as bacitracin. The *Bacillus* strain GU057 was confirmed as good peptide antibiotic producer and can effectively be indulged as biocontrol agent.

Key words: Peptide antibiotics, *Bacillus* species, screening, optimization of parameters.

INTRODUCTION

Antibiotics are the secondary metabolites produced by the microorganisms via enzymatic catalysis involving a series of biosynthetic pathways (9). Naturally the antibiotics produced by microorganisms are responsible for the regulation and control of microbial population in soil, compost and water. Majority of such antibiotics are toxic in nature and only few find a use in medicine for treatment of multiple ailments due to high margin of safety (7, 27). Such antibiotics are mainly produced by *Penicillium*, *Streptomyces*, *Cephalosporium*,

Micomonospora and *Bacillus* accounting for more than 5,000 compounds (4, 26). These antibiotics are low molecular weight polypeptides produced by ribosomal or non ribosomal mechanisms (17).

The *Bacillus* species are known for the synthesis of secondary metabolites with remarkable diversity both in structure and function (22). For instance cerecin 7, Tochicin, Thuricin 7, thuricin 439 and entomocidus 9 (7,8) and few may be ribosomal in origin including sublancin (18), subtilosin A (3), subtilin (27) and TasA (24) are highly active against variety of microorganisms (10).

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Bacitracin is most important cyclic polypeptide antibiotic produced *Bacillus* species and is primarily active against the Gram-positive bacteria. The bacitracin is used as anti-infective agent in a variety of pharmaceutical preparations including aerosols, topical lotions, skin ointments and creams (2, 25). The bactericidal activity of bacitracin is the result of cell wall inhibition.

The purpose of this study was to isolate antibiotic producing *Bacillus* strain from extreme conditions (saline soil) and suggest the optimum conditions for maximum activity.

MATERIALS AND METHODS

Screening of antibiotic-producing *Bacillus* species from soil

About 112 soil samples were taken from different locations of district D.I.Khan and Kohat (KPK) Pakistan and assessed for antimicrobial potential. The lawn of test strains *Staphylococcus aureus* and *Micrococcus luteus* was applied gently on surface of freshly prepared Muller Hinton agar (oxid) plates and was sprinkled with 10-25 particles of each soil sample separately. These agar plates were incubated at 30°C for 24 to 48 hours. After incubation, the plates were observed for antimicrobial activity by observing the zones of inhibition around microbial colonies (2). These microbial colonies were picked up by a sterilized wire loop and subcultured many times to obtain isolated and purified bacterial colonies.

Antibiotic production

The bacterial isolates with widest zone of inhibition was inoculated in the Muller Hinton (Oxoid) broth at pH 7.3 in a flask and incubated for 48-72 hours at 150 rpm at 30°C. Sterilized production medium was prepared according to instructions (2) (Table 1). The inoculum was shifted to the production medium and incubated for 192 hours at 150rpm in an orbital shaker. After every 24 hours, samples were drawn up to 192 hours. Each sample was centrifuged separately to obtain

cell-free culture supernatant followed by sterilization through 0.2 µm. filter paper. The supernatant so obtained was subjected to agar well diffusion assay for antibiotic production. Similar procedure was repeated for the optimization of the identified *Bacillus* specie at various pH, temperature and glucose concentrations (optimization parameters) and results were recorded.

Table 1. Composition of production medium (g/L).

| Contents | Quantity |
|--------------------------------------|----------|
| L-Glutamic acid | 5.0 |
| MgSO ₄ .7H ₂ O | 0.2 |
| MnSO ₄ .H ₂ O | 0.01 |
| NaCl | 0.01 |
| FeSO ₄ .7H ₂ O | 0.01 |
| CuSO ₄ .2H ₂ O | 0.01 |
| CaCl ₂ .2H ₂ O | 0.015 |
| KH ₂ PO ₄ | 0.5 |
| K ₂ HPO ₄ | 0.5 |
| pH | 7 |

Agar well diffusion assay

The agar well diffusion method was employed to determine the antibiotic production by the strain (20). Briefly the turbidity of 24 hours old cultures of test strains (*S. aureus* and *M. luteus*) was adjusted to 0.5 McFarland turbidity standard. The test strains was then applied on the surface of Muller Hinton agar plates (30ml) using sterilized cotton swab and allowed to dry for 15 minutes. Wells were made by using sterilized cork borer (8mm). About 100µl of the cell-free culture supernatants was transferred into each well. The agar plates were then incubated at required temperatures and observed for the development of zones of inhibitions around each well (Fig 1).

Identification of *Bacillus* specie

The strain producing maximum zones of inhibition was identified both morphologically and biochemically (5) as *Bacillus specie* (Table 2). The results were later confirmed by API 20E.

Table 2. Biochemical Tests for Identification of *Bacillus* spp.

| Test | Result |
|----------------------|------------|
| Catalase | -ve |
| TSIA | -ve |
| SCA | +ve |
| Motility | +ve |
| Indole production | -ve |
| Oxidase | +ve |
| Starch hydrolysis | +ve |
| Gas production | +ve |
| MacConkey agar | Light pink |
| Eosin methylene blue | Colorless |
| Gram staining | +ve |
| Shape | Rod |
| Spore formation | +ve |

-ve negative
+ve positive
TSIA Tripple sugar iron Agar, SCA simmon's citrate agar.

Identification of peptide antibiotics by paper chromatography

The antibiotic produced by *Bacillus* strain was identified by bioautography (16) using Whatman No. 1 filter paper. Briefly the supernatant of each sample was applied separately 2 cm above the base of the paper and dried thoroughly. Afterwards the filter paper was dipped in the solvent system (acetone: acetic acid: water; 50:3:47). After the solvent migration till the end of the paper (16-24 cm), the chromatogram was air-dried by exposure to hot air with air drier. The developed papers were placed for 30 minutes on Muller Hinton agar inoculated with *S. aureus* and *M. luteus*, and the plates were then incubated at desired temperature and time. Finally the antibiotic was identified by measuring the Rf value (Retardation factor i.e the ratio of the distance traveled by the center of a spot to the distance traveled by the solvent front) and comparing it with Rf values of different antibiotics (23). Autobiography procedure was repeated for standard bacitracin purchased commercially (Oxoid).

Optimization of various parameters for maximum antibiotic production

For the optimization of microbial growth various parameters like time of incubation (up to 192 hours), the pH (4, 5, 6, 7 and 8) and the glucose concentration (2, 3, 4, 5 and 6%)

were determined (in triplicate) and results were recorded.

RESULTS

The bacterial strain and peptide antibiotic identification

About 112 different soil samples were screened for presence of peptide antibiotics producing bacteria. Of them, the strain GU 057 was selected for further screening seeing that this strain presented widest zones of inhibitions (18mm) against test strains (Fig 1). The strain GU057 was isolated from saline soil in the roots of Terminalia Arjuna roots in District D.I.Khan at pH 8.2. The strain was later identified as *Bacillus* sp. GU 057. The strain was subjected to batch fermentation and the samples were taken to identify the antibiotic by bioautography. The Rf values of the antibiotic produced by *Bacillus* spp was compared with standard antibiotic (bacitracin) Rf values and confirmed as bacitracin.



Figure 1. Widest zone of inhibitions (18mm) of *Bacillus* GU215 against *Staphylococcus aureus*

Optimization of various parameters for antibiotic production

Maximum activity of the bacitracin was observed after 48 hours of the incubation against *Staphylococcus aureus*. Afterwards a steady decline in the activity was noted till 192 hours, while *Micrococcus luteus* presented comparatively lesser activity (Fig 2).

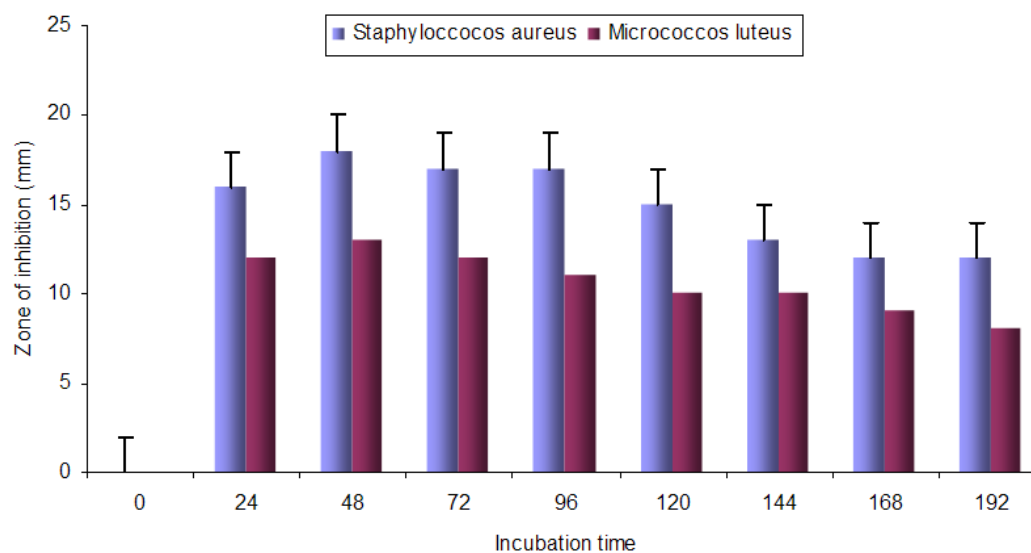


Figure 2. Development of inhibition zones produced by *Bacillus spp.* GU057 antibiotic against *Staphylococcus aureus* and *Micrococcus luteus* at different incubation time.

A gradual increase in the activity of the bacitracin was observed by increasing the glucose concentration and maximum inhibitory zone was recorded at 4% concentration of the glucose but after wards a decline in the activity was noted (Fig 3). Like wise maximum activity was observed at pH 8

after 48 hours of incubation. Comparatively lesser activities were bacitracine was observed at pH 4 to pH 7 (Fig 4). Similarly the effect of different temperatures (30, 35, 40, 45, 50, 55°C) on the production of antimicrobial in fermentation broth highlighted a maximum activity at 40°C (Fig 5).

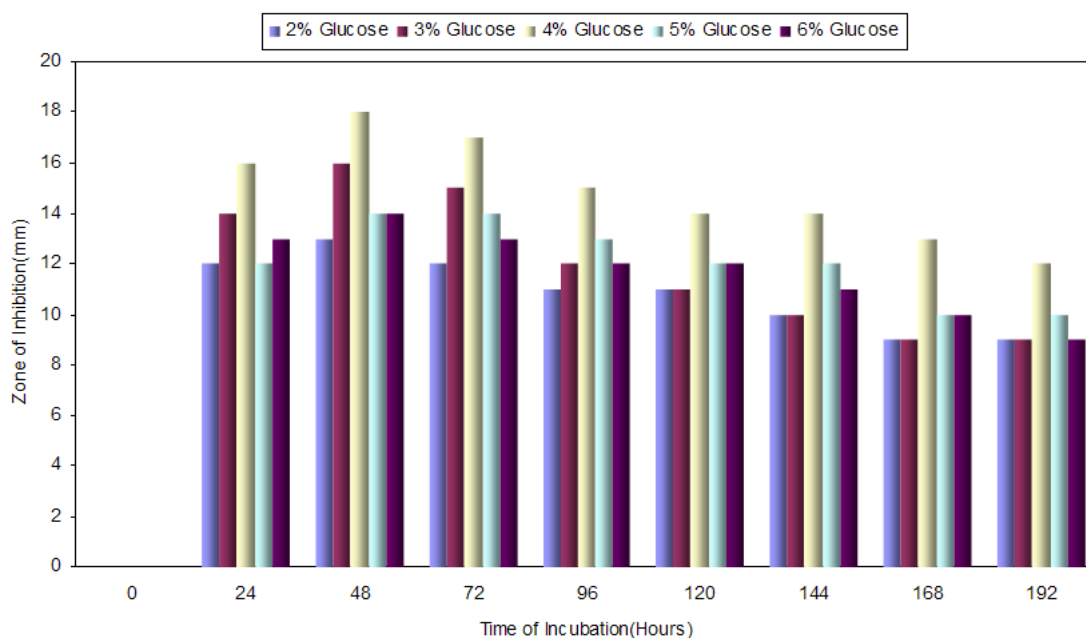


Figure 3. The effect of increasing glucose concentration on the zone of inhibition produced by *Bacillus spp.* GU057 against *Staphylococcus aureus*.

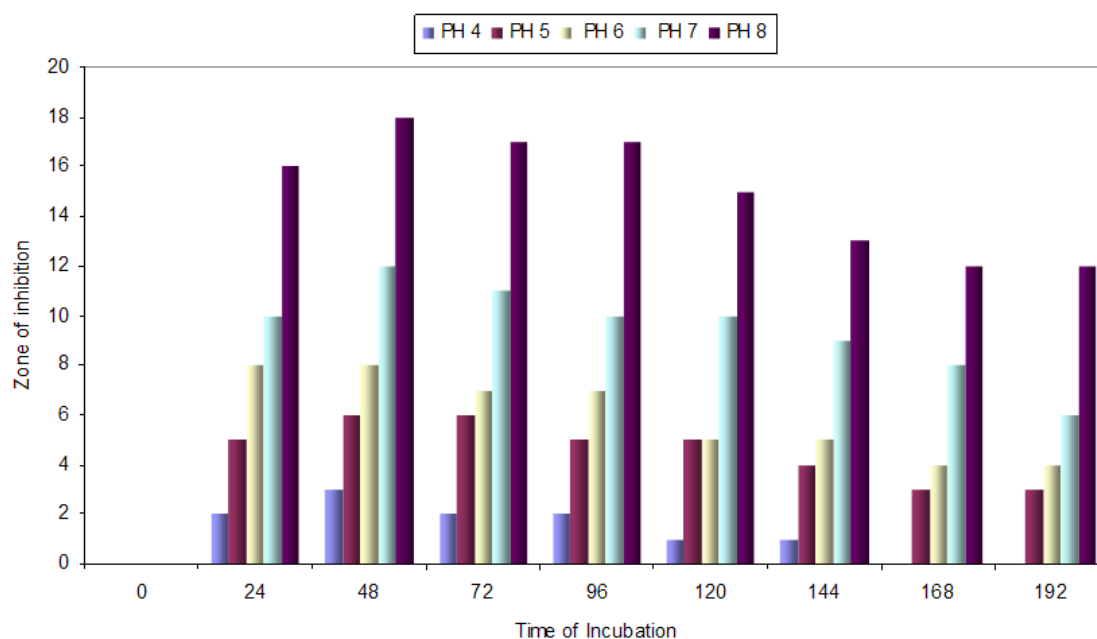


Figure 4. The effect of various pH levels on the zone of inhibition produced by *Bacillus* spp.GU057 against *Staphylococcus aureus*.

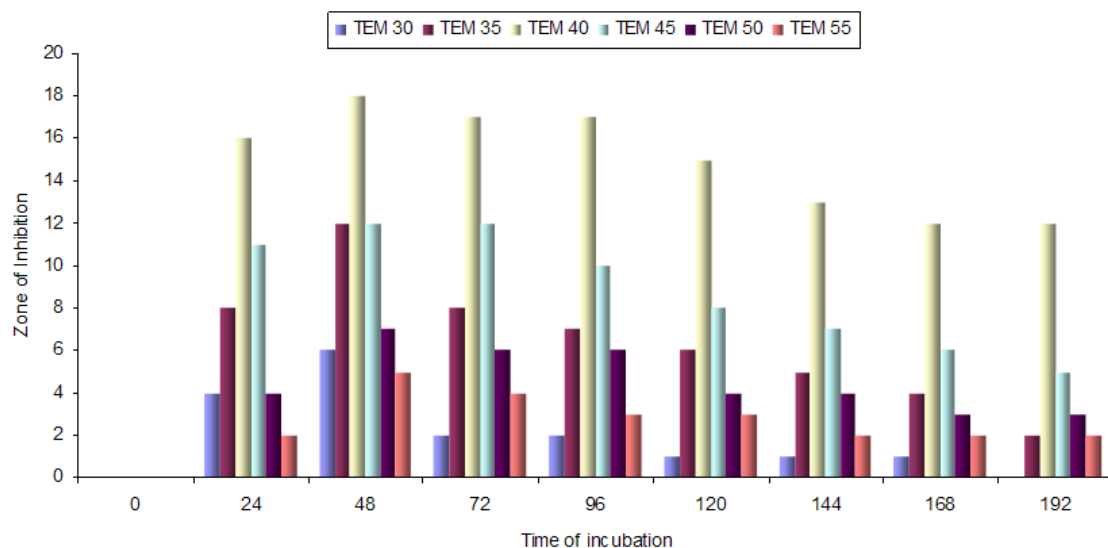


Figure 5. The effect of time of incubation on the zone of inhibition produced by *Bacillus* spp.GU057 against *Staphylococcus aureus*.

DISCUSSION

Soil is a prosperous source of microorganism that produces a wide range of antibiotics including peptide antibiotics (2, 14). The *Bacillus* species are most popular for

producing peptide antibiotic compounds such as polymyxin, colistin, and circulin (15). The development of resistance and lesser safety margins provoked the scientist to search for antimicrobial agents with modified properties and maximum activity. With this background present study was conducted to

assess the antibiotic production from *Bacillus* species, identification of antibiotic and optimization of various growth parameters.

Among all soil samples collected from different areas of District D.I.Khan and Kohat, the strain GU 057 presented widest zones of inhibition against *Staphylococcus aureus* (18mm) followed by *Micrococcus luteus* (13mm) after 48 hours of incubation. This trend remained approximately same till 62 hour, however a gradual decline in the inhibition was observed as the time of incubation increased till 192 hours. This trend is supported by earlier findings (1, 2,16).

Glucose is an essential nutrient required for the growth and production of secondary metabolites including the antibiotics (11). The strain GU 057 presented a steady increase in the zone of inhibition by increasing the concentration of glucose in the production media (16) and maximum activity was reported at 4% glucose concentration. Nevertheless a controlled decline in the activity was observed at higher glucose concentrations as have been described previously (11, 13).

The effect of temperature on the growth and secondary metabolites (antibiotics) synthesis was determined by incubating the strain GU 057 at various temperatures. The strain showed encouraging results at the 40°C after 48hours of incubation. This may be the thermophilic nature of the strain (1, 12). Significantly lower levels of zones of inhibitions were observed at all other temperatures (30, 35, 45, 50 and 55°C).

Similarly the effect of pH on zones of inhibition produced by *Bacillus* strain was not un surprising. The widest inhibitory zone was reported at pH 8 after 48 hours of incubation. This activity is the result of extremophilic nature of the strain (saline soil) as reported earlier (19, 21). Comparatively much lesser inhibition was reported at pH 4, 5, 6 and 7 as the time of incubation was increased from 49 to 192 hours as observed previously (6,12). The results of present study confirm the *Bacillus* sp. GU 057 strain isolated from saline soil, as producer of peptide antibiotic (bactitracin).

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