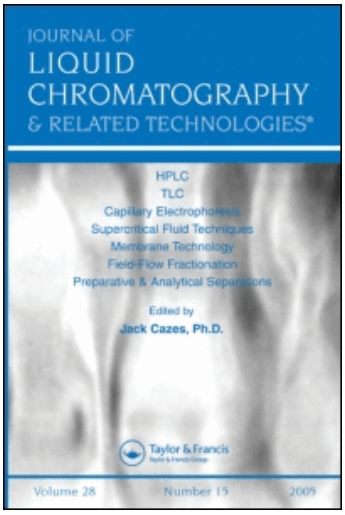

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Simple and Rapid Isolation of a Novel Antibiotic from *Bacillus subtilis* Mz-7

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Simple and Rapid Isolation of a Novel Antibiotic from *Bacillus subtilis* Mz-7

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Abstract: The difficulties surrounding comparative methods in natural byproduct screening such as the novelty of product, the availability of standards, and the number of trials that can be performed for identification present real challenges to all investigators. A novel antibiotic was isolated and purified from the supernatant of a wild type of *Bacillus subtilis* Mz-7 by chloroform precipitation only. The product was checked by TLC and HPLC and proved to be of high purity. Techniques applied in this paper are conveniently reproducible and successfully simplified to isolate and purify an antibiotic, which has the potential to counteract the rise in microbial resistance.

Keywords: Isolation, Purification, Bacteria, Novel, Screen, Antibiotic, HPLC, TLC

INTRODUCTION

Many types of antibiotics are produced by a wide assortment of microorganisms. Over 8000 antibiotics are known to exist and hundreds more are discovered yearly; however, few prove to be commercially useful.[1] With the concern that pathogenic bacteria are quickly becoming resistant to commonly used therapeutic agents,[2,3] the search for new antibiotics is becoming increasingly important.[4] The problem of bacterial resistance to antibiotics is exacerbated by the downward trend in antibacterial discovery and development.[5,6] While several strategies exist for the discovery of antibiotics, the majority of the work still consists of

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screening naturally occurring sources, most commonly soil, which is the major repository of microorganisms that produce antibiotics capable of inhibiting or killing the growth of other microorganisms. In the search for new antibiotics, the genus *Bacillus* is an excellent place to look. *Bacillus* species produce a large number of peptide antibiotics representing at least 25 different basic chemical structures.[7] Novel *Bacillus specie* exhibiting antibacterial activity was isolated from Pakistan with a spectrum of activity, which covers multiple resistance clinical isolates and was identified genotypically on the basis of 16S rRNA to be a novel strain of *Bacillus subtilis* Mz-7. The cell-free culture supernatant of this bacterium demonstrates marked antibacterial activity, suggesting that the bacteria produce some type of extracellular inhibitory compound. The isolation, purification, detection methods, and the related techniques of this antimicrobial agent are notably direct and simple, and therefore presented in this paper.

EXPERIMENTAL

Reagents

All solvents used in this study were of HPLC grade. Diethyl ether, chloroform, ethanol, and methanol were purchased from Sigma Chemicals. Water was prepared in M-Pure water system (USA).

Apparatus

Sykam HPLC system (Germany) was used; the system was equipped with a model (S 1122) pump, an UV/VIS detector model (S 3210), and peak simple data system Model (203). A double beam spectrophotometer Cecil 7200 was used.

Strains and Culture Conditions

The bacterium was isolated from a soil sample near a drainage bank, Lahore, Pakistan. The bacterium was identified by 16S rRNA, the amplified gene was sequenced, which was analyzed by NCBI web site (www.ncbi.nlm.nih.gov/blast/Blast.cgi) with GenBank accession no. DQ327713 that shows a 97% homology with *Bacillus subtilis* HJ11. Test bacteria were the collection of our laboratory, while the eight *Klebsiella spp.* were received from Children Hospital, Lahore, Pakistan. All strains were maintained in L-agar slants[8] at 4°C.

Antibiotic Assay

Samples of culture supernatant containing the antibiotic were assayed for activity using an agar-well diffusion assay.[9] Fifty μL of *Bacillus fusiformis* liquid culture of 1.0 OD_{600} was spread onto the surface of a petri dish containing L-agar,[8] using a sterile bent glass rod. A well was made in the center of the plate using a No. 3 cork borer, and a 50 μL liquid sample was transferred into the well. The sample was allowed to diffuse into the agar, and the plate was inverted and incubated at 37°C until a lawn of the indicator bacteria appeared on the plate (approximately 8–10 h).

Spectrum of Activity

The following 17 different bacteria species were tested for their sensitivity to the antibiotic using the agar-well diffusion assay: *Escherichia coli* C600, *Escherichia coli* DH5α1, *Bacillus subtilis* PY49, *Bacillus subtilis* 92, *Bacillus subtilis* 29, *Bacillus cereus* F, *Bacillus cereus* 92, *Bacillus fusiformis*, *Ochrobacterum intermedium*, and 8 clinical isolates of *Klebsiella spp*. Aliquots of an overnight culture of the bacteria being assayed for sensitivity to the antibiotic were added to sterile 1% (w/v) peptone water blanks, to yield a bacterial suspension with a final optical density (600 nm) of 1.0. Fifty microliters of the bacterial suspension was spread onto an agar plate and used in the agar-well diffusion assay as previously described.

Mode of Action

Culture of *Bacillus subtilis* Mz-7 was inoculated on a side of a L-agar plate and the zone of inhibition was monitored by streaking the test bacterium on vertical lines. On the surface of inhibitory zone, fresh inoculum from an overnight culture of the same test bacteria was streaked carefully. The plate was left at room temperature where a small amount was taken from it at regular intervals.

Bactericidal Assay

The obtained culture filtrates were checked for Gram positive activity with *B. fusiformis* as test organism: 50 μL of bacterial suspension of 1.0 $OD_{(600)}$ prepared from an over night culture of the test organism was added to three 100 mL flasks containing 10 mL L-broth. The three preparations were two replicas and one control. Exponential phase was achieved after approximately 8 h of incubation at 37°Cand 100 rpm. After a proper dilution (1 : 100 000), one mL of filtered sterilized bacteria supernatant containing the antibiotic, was

mixed to each dilution. From each mixture 10 μL was plated on L-agar plates and the number of colonies was noted. The killing percentage was determined from the formula: Killing % = (CFU Control-CFU Sample) × 100/CFU Control.

Isolation and Purification of Antimicrobial Substance

The bacterium was inoculated on L-agar plate at 37°C over night, a loop full of bacteria was resuspended in autoclaved distilled water, the Optical density was adjusted to 1 at 600 nm. One mL from the later suspension was aseptically added to 100 mL L-Broth (1000 mL capacity flask) and placed in a rotary shaker at 150 rpm for 72 h. To the supernatant, obtained from centrifugation at 20000 × g for 15 min, an equal volume of chloroform was added in a separating funnel, shaken well, and left to clear turbidity. A thin layer of antimicrobial substances was formed at the interface that was collected. All the three phases were tested for activity. In purification, the precipitate was redissolved in water and reprecipitated by chloroform several times. Finally, homogeneity was checked on TLC silica plates (Merck Co.) and by HPLC.

TLC Analysis

The chloroform solution of the precipitate was subjected to thin-layer chromatography (TLC) with $CHCl_3$-methanol-Diethyl ether (10:70:20) as a development solvent. The bioassay was performed with *Bacillus fusiformis* as the test organism. TLC plates were placed in bioassay plates and overlaid with Muller-Hinton agar (0.5% [wt/vol]; Merck), which had been seeded with *B. fusiformis*. The inhibitory zones were observed after 10 h of incubation at 37°C.

Spectrophotometeric Analysis

The solution of purified antibiotic, centrifuged bacterial supernatants, and medium Luria-Bertani Broth[8] were scanned against M-pure water from 190 to 900 nm in quartz cells, at a rate of 100 nm per min, to determine λ max for different components.

HPLC Analysis

The analysis was performed on an ODS (5 μm, 250 × 6 mm), ThermoHypersil-Keystone, column. The cell free supernatant was passed through 0.2 μm filters and 20 μL were injected into the system, mobile phase was methanol at a flow rate of 1 mL per minute. The eluted components were detected at 220 nm. The same procedure was repeated for the purified biologically

Table 1. Spectrum of activity: The zones of inhibition ranged from non-existent (−) to a diameter of >2.0 cm (++++)

Test organism	Activity expressed by diameter of inhibitory zone
Escherichia coli C600	−
Escherichi coli DH5α1	−
Bacillus subtilis PY79	+++
Bacillus subtilis 92	++++
Bacillus subtilis 29	+++
Bacillus cereus F	+
Bacillus cereus 92	++
Bacillus fusiformis	++++
Ochrobacterum intermedium	−
*Klebsiella spp.*1	+
*Klebsiella spp.*2	++
*Klebsiella spp.*3	+
*Klebsiella spp.*4	+
*Klebsiella spp.*5	+
*Klebsiella spp.*6	++
*Klebsiella spp.*7	++
*Klebsiella spp.*8	++

active product and comparison was made to determine the purity and to identify peak of interest from the original bacterial supernatant.

RESULTS AND DISCUSSION

Spectrum of Activity, Mode of Action and Bactericidal Assay

The wide and selective spectra exhibited by this novel antibiotic were demonstrated by using several bacterial stains and a number of clinical isolates. This active substance could inhibit the growth Gram positive bacteria, while all the *E. coli* strains showed negligible inhibition, however. In the case of multi-drug resistance clinical isolates of *Klebsiella spp.*, the inhibition was noted (Table 1).

Table 2. Bactericidal assay; filter sterilized supernatant of *B. subtilis* strain Mz-7 was added to $1:10^5$ dilutions of test strain, the reduction of colony forming unit (CFU) indicates the mode of activity

Antibacterial strain	Test strain	Control CFU	Replica 1 CFU	Replica 2 CFU	Killing %	Result
B. subtilis Mz-7	*B. fusiformis*	400	15	20	96.25	Cidal

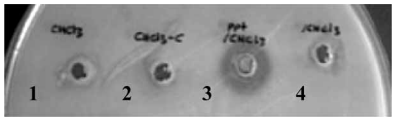

Figure 1. Fifty μL of different extraction phases: 1. control chloroform, 2. extraction chloroform, 3. precipitate, and 4. treated supernatant.

The inoculum taken from an indicator strain streaked on the zone of inhibition did not grow again, indicating that the mode of action of the produced product was bactericidal in nature. The killing percentage/efficiency was also determined to be as high as 96.25% (Table 2).

Isolation and Purification of Antimicrobial Compound

The over all experimental work was more or less more dependent on the solubility profile of active substance. The solubility profile was determined by correlating the diameter of zone of inhibitions with the amount that can be extracted by different solvents; hence, the more is the solubility of the product the more is the diameter of zone of inhibition, considering that an equal volume of a single batch of bacterial supernatant containing the antibiotic was treated with the same volume of different solvents. By treating the supernatant with chloroform an interface was developed, which was further investigated and was determined to be the inhibitory product, as shown in Fig. 1. The precipitate from the interface was washed thoroughly with double distilled water then freeze dried. This isolation procedure of this particular active product proved to be the simplest of a large number of trials, where the efficiency and quantity was maintained. More over, it demonstrated a high purity level.

TLC Analysis

The TLC experiment was designed to confirm the selective isolation of the active product. A single uniform hallow disk of no growth was noted on the

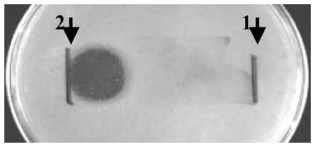

Figure 2. TLC sheet laid on Muller-Hinton agar (0.5%) seeded with *Bacillus fusiformis.* Arrow one indicates the base line, arrow two indicates front line. A spot of active antibiotic showing symmetrical inhibition.

Table 3. Determination of absorption maxima by comparing medium, supernatant of bacterial incubation and product to double distill water (DDW)

L-broth against DDW	Supernatant against DDW	DDW+ product against DDW
Peaks recorded (nm) (Auto scale, Speed = 450 nm/min,1:100 dilution)		
197.00, 209.00, 259.00, 356.00, 379.50.	194.60, 204.80, 209.00, 362.20, 386.60.	195.00 204.22
New peak ranges	205.00 ± 5.00 nm	

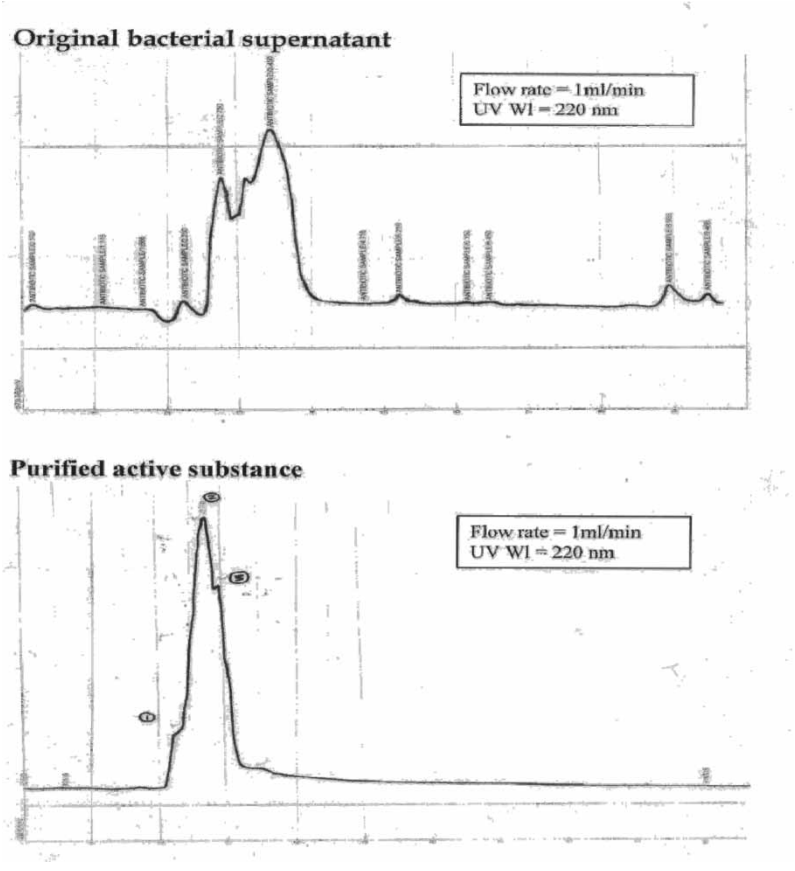

Figure 3. Comparison between HPLC profiles of the culture supernatant and the purified antibiotic, a single peak determines the degree of homogeneity, as well as the peak of interest (indicated by arrow).

development of the freeze dried active product with different solvents. This uniform shape, Fig. 2, clearly indicates successful isolation by the above chloroform precipitation method.

Spectrophotometeric and HPLC Analysis

New peaks were screened by a double beam spectrophotometer in which the active products maximum absorption was traced by using the original supernatant and the isolated active product. Table 3 shows the different results obtained, indicating that the isolated product has λ max of 205 nm. The comparative analysis provided by HPLC, with the original supernatant and the purified substance, further verified the efficiency of isolation, as it resulted in a single peak with three components, which was contaminant free and sufficiently purified for any investigation (Fig. 3).

CONCLUSION

The isolation of the antibiotic was successfully performed using relatively simple methods. Different detection techniques determined that chloroform precipitation was clearly selective to recover the antibiotic from the culture of *Bacillus subtilis* Mz-7. This newly developed method could be profitably used in further research on this effective antibiotic and in small scale production too.

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