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Reexamining intra and extracellular metabolites produced by *Pseudomonas aeruginosa*

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ABSTRACT

Objective: To isolate, screen and analyze bacteria from different areas of Pakistan for the production of antimicrobial compounds, zinc solubilization and bioplastic production.
Methods: Isolation and purification was proceeding with streak plate method. Antagonistic assay was completed with well diffusion and thin-layer chromatography. *In vivo* analysis of bioplastic was analyzed with Nile blue fluorescence under UV and Sudan staining.
Results: A total of 18 bacterial strains purified from soil samples while 148 strains form stock cultures were used. Out of 166 only 94 showed antimicrobial activity against each of Gram-positive and Gram-negative; cocci and rods. In case of heavy metal (ZnO and $Zn_3(PO_4)_2{\cdot}4H_2O$) solubilization, 54 strains solubilized ZnO and 23 strains solubilized $Zn_3(PO_4)_2{\cdot}4H_2O$, while 127 strains grown on polyhydroxyalkanoate detection meedia supplemented with Nile blue medium showed bioplastic production by producing fluorescence under UV light. Four bacterial strains (coded as 100, 101, 104 and 111) were selected for further characterization. Induction time assay showed that strains 101, 104, and 111 showed inhibitory activity after 4 h of incubation while strain 100 showed after 8 h. All four strains were tolerable to the maximum concentration of ZnO. Amplified products of both *16S rRNA* and *PhaC* gene fragments of strain 111 were sequenced and submitted to GenBank as accession numbers EU781525 and EU781526.
Conclusions: Bacterial strain *Pseudomonas aeruginosa*-111 has potential to utilize as biofertilize and bioplastic producer.

1. Introduction

The importance of bacteria in industrial and technological field is undeniable especially in medicine, plant growth, food culture, plastics synthesis and bioremediation. The research work was based on three aspects *i.e.* production of antimicrobial compounds, heavy metal solubilization and bioplastic production by bacteria. In different ecological niches, microorganisms struggle for survival by several mechanisms and through evolution form distinctive flora. One of these mechanisms is the production of antimicrobial compounds against competing flora, including pathogenic bacteria[1]. Production of antimicrobial compounds is a common phenomenon among most of the bacteria. Bacteria produce a wide range of antimicrobial compounds, including broad-and narrow spectrum antibiotics, metabolic by-products such as organic acids, lytic agents such as lysozyme, several types of protein exotoxins, and bacteriocins[2]. This natural armory is incredible in its diversity and natural abundance, since some substances are limited to some bacterial groups while others are widespread produced[3].

Mostly studies involving bacterial interactions with heavy metals are associated with resistance, transport, and their functions in metalloenzymes but several aspects, particularly the mechanisms employed to obtain metals and associated nutrients from insoluble sources still remain ambiguous. Sometimes essential nutrients (or metal compounds) such as P and S are present in a microbial habitat only in insoluble form, so microorganisms have to solubilize such compounds before uptake in order to survive[4]. In addition to that, solubilization of insoluble salts by bacteria has many technological applications *e.g.* recovery of metals from low-grade ores (bioleaching or biomining), as biofertilizers (phosphorus biofertilizers)[4]and bioremediation[5]. Insoluble metal forms may be solubilized by protons, chelate ligands, and by oxidation reduction systems at cell surfaces and membranes. These agents

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either set up an ionic gradient or form complexes with metal cations for the acquisition of nutrients[6]. Petrochemical plastic waste is increasing in the environment day by day. In order to cope with this problem, researchers have discovered fully biodegradable plastics, such as polyhydroxyalkanoates (PHAs)[7]. In addition to that, these materials are natural, biocompatible, and renewable[8,9]. Although bioplastics are synthesized by many living organisms but the main producers are plants and bacteria. Unlike plants, bacteria are capable of accumulating PHA to levels as high as 90% (w/v) of the dry cell mass[10]. Bioplastics are the macromolecules (composed of hydroxyl fatty acids) that are synthesized by many Gram-positive and Gram-negative bacteria, cultured under different nutrient and environmental conditions. When the carbon substrate is in excess to other growth limiting nutrients like nitrogen, sulfur, phosphorus or oxygen[11], many microorganisms can accumulate PHAs as intracellular energy yielding and carbon storage granules. These polymers are accumulated in the form of mobile, amorphous, liquid granules of lipids that provide these microorganisms nutrients under stress conditions[12]. The main aim of this study was to screen and analyze different bacterial strains for the production of antimicrobial compounds and to characterize the antimicrobial activity of some selected bacterial isolates, agents solubilizing insoluble metal salts and to check the effect of various factors on solubilizing activity of selected bacterial isolates and of bioplastics production by using phenotypic as well as genotypic approach.

2. Materials and methods

2.1. Isolation, purification and characterization of bacterial strains

Five soil samples were collected from different areas of Lahore, Pakistan. Nutrient medium was used. The incubation time and temperature used were 24–48 h and 37 °C. The bacterial isolates were then characterized morphologically and physiologically by different staining procedures and biochemical tests[14]. The isolated strains were identified following directions of the latest edition of Bergey's manual.

2.2. Screening for antimicrobial activity

The antimicrobial assay was performed by using agar well diffusion method. The plates were incubated for 24 h at 37 °C.

2.3. Induction time assay of antimicrobial compounds

Test was performed with four selected bacterial strains. Each strain was inoculated in 50 mL of nutrient broth and incubated at 37 °C. Optical density and antimicrobial activity were then checked after every 4 h intervals for 24 h. Antimicrobial activity checked against Gram-positive, Gram-negative rods and cocci by agar well diffusion method.

2.4. Effect of heat and proteinase K on antimicrobial activity

The effect of heat on antimicrobial activity of bacterial strains was determined by autoclaving 1 mL of filtered (0.2 μm) supernatant of overnight culture of selected bacterial strains under standard conditions for 15 min. Whereas the effect of proteinase K was determined by adding 3 μL of proteinase K to 1 mL of filtered supernatant of overnight culture of selected bacterial strains and incubating for 1 h at 37 °C. In both cases, the respective supernatant was then checked for antimicrobial activity against the above mentioned bacterial groups along with the untreated supernatant of the same culture.

2.5. Thin layer chromatography (TLC)

TLC was performed to separate the antimicrobial compounds from a mixture of metabolites of selected bacterial strains. Dried ethyl acetate extract of overnight culture of each selected strain as well as of control (simple nutrient both) was spotted on the TLC card (Merck Silica gel 60 F_{254}). The solvent system used was hexane: ethyl acetate in a ratio of 4:1 (v/v). The results were noted with naked eye, under UV light, exposing the TLC card to iodine vapors, and spraying sulfuric acid and potassium permanganate mixture on TLC card and drying it in oven at 60 °C. All the spots that appeared were noted and R_f value was determined for each spot. The antimicrobial activity of each spot was then checked by dipping filter paper discs in silica suspension of each spot and placing them after drying on the surface of nutrient agar carrying each of the four indicator strains separately.

2.6. Selection for solubilization of insoluble metal compounds

Tris-minimal medium was prepared[6]. A 14 mmol/L ZnO and 5 mmol/L $Zn_3(PO_4)_2{\cdot}4H_2O$ was used as insoluble metal salts for solubilization by bacterial strains. Inoculation was carried out using a 10 μL of fresh bacterial culture and incubated at 37 °C for about 7 days. Broth assay was performed to observe pH changes in the media during the process of solubilization. A total of 50 mL of basal Tris-minimal salt medium was supplemented with 5 mmol/L ZnO and 2.5 mmol/L $Zn_3(PO_4)_2{\cdot}4H_2O$ separately in 100 mL Erlenmeyer flasks. The flasks with each salt were then inoculated with 10 μl of an overnight culture of the respective bacterial strains and incubated at 37 °C for 20 days. The pH changes were observed after 7 and 14 days of inoculation.

2.7. Maximum tolerable concentration of insoluble salts assay

In order to find the maximum tolerable concentration of the insoluble zinc salts, 5 different concentrations of each zinc salt incorporated in TMSM were used. ZnO was incorporated in

medium in such a way that the final concentration of zinc was 14, 20, 25, 30, and 35 mmol/L. In case of $Zn_3(PO_4)_2 \cdot 4H_2O$ the final concentration of zinc was made as 5, 10, 15, 20, and 25 mmol/L. A total of 10 µL of each of selected strain culture was inoculated to each concentration of ZnO and $Zn_3(PO_4)_2 \cdot 4H_2O$ and metal solubilization was noted by measuring clear halos formed around bacterial colonies after 7 and 14 days. The pH of the cultures in each case was also determined after 7 and 14 days of inoculation.

2.8. Selection of bioplastic producing bacteria

In vivo analysis of bioplastic production was done by growing all of the bacterial isolates on Nile blue 0.5 µg dye (mL medium)$^{-1}$ supplemented PHA detection medium for 24-48 h at 37 ˚C. The agar plates were exposed to ultraviolet light after appropriate incubation periods to detect *in vivo* accumulation of PHAs[15,16]. The results were also confirmed by performing Sudan black staining and then observing under light microscope[16].

2.9. Amplification of Pha C and 16S rRNA gene

PCR amplification of both types of genes was carried out by using isolated genomic DNA of selected strains[17]as PCR template, Primus96 (PeQLab) thermal cycler and 2X master mix (Fermentas). *PhaC* gene fragment was amplified by using FGen-PhaC (CCGCAATTGAACAAGTTCTACGT) as forward primer, while RGen-PhaC (CGGGAGACGCGTGGTGTCGTTG) as reverse primer. Touch down PCR was carried out by heating lid at 110 ˚C for 10 min and initial denaturation at 95 ˚C for 5 min. This was followed by four rounds of 5 cycles, one round of 25 cycles, and 3 rounds of 15 cycles. All of these cycles involved denaturation at 95 ˚C for 0.5 min, annealing for 1 min and extension at 72 ˚C for 1 min. Annealing temperature was lowered 1 C from 65 ˚C to 62 ˚C with 5 cycles at every temperature followed by 25 cycles at 61 ˚C, 15 cycles at 60 ˚C, 15 cycles at 59 ˚C and 15 cycles at 57 ˚C. Final extension was done at 72 ˚C. Amplification of the *16S rRNA* gene was carried out by using 16S-5 (GCYTAAYACATGCAAGTCGA) as forward primer and 16S-3 (CCCGGGAACGTATTCACCG) as reverse primer. Thermal cycling was undertaken by initially denaturing the DNA at 95 ˚C for 5 min followed by 14 cycles of 94 ˚C for 1 min, 59 ˚C for 30 s and 72 ˚C for 1 min. Annealing temperature was lowered 1 ˚C from 59 ˚C to 52 ˚C, with 14 cycles at every temperature. Last elongation step was carried out at 72 ˚C for 2 min.

2.10. DNA sequencing and sequence analysis

The samples consisting of purified *16S rRNA* and *PhaC* gene framents of strain 111 were sequenced at the Centre of Excellence in Molecular Biology by dideoxy (chain termination) method on applied biosystems DNA sequencer (Applied Biosystems, 3100/ga 3100-1696-013). The sequences were analyzed by using different bioinformatics tools.

3. Resutls

3.1. Selection of bacterial strains

A total of four bacterial strains (100, 101, 104, and 111 identified as *Citrobacter*, *Echerchia*, *Klebsiella* and *Pseudomonas,* respectively) were selected. Percentage frequency of different bacterial species among all of 166 bacterial strains used in this research is given in Figure 1.

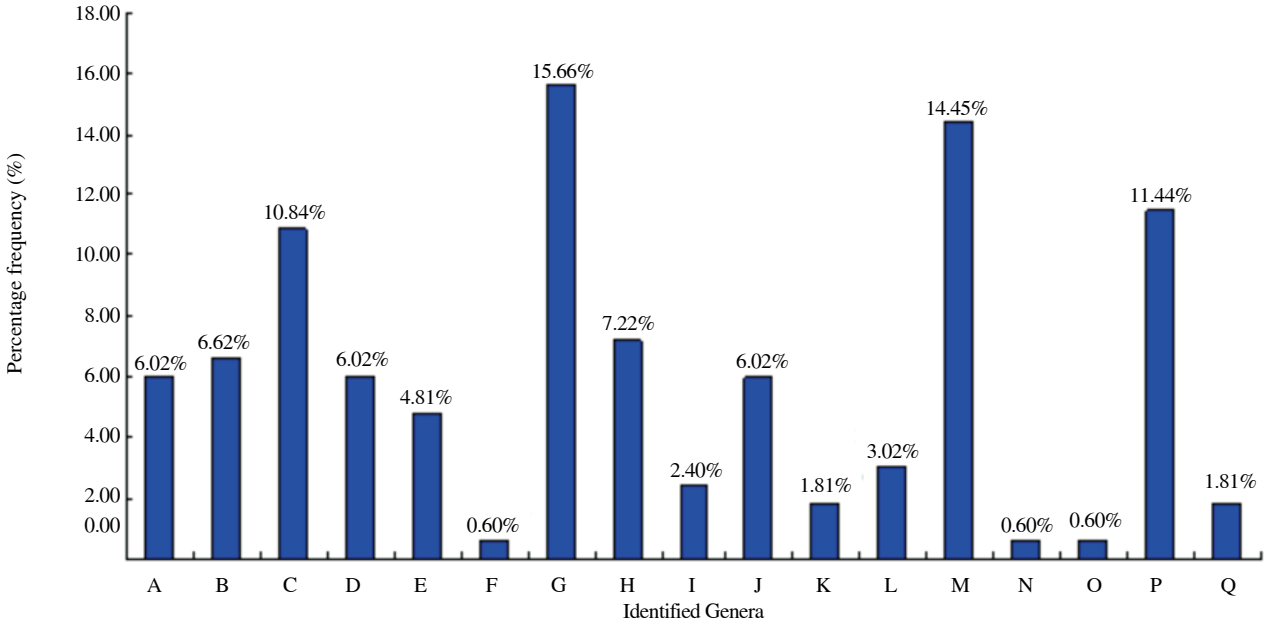

Figure 1. Percentage frequencies of identified bacterial isolates.
A: *Alcaligenes*; B: *Bacillus*; C: *Citrobacter*; D: *Echerchia*; E: *Enterobacter*; F: *Flavobacterium*; G: *Klebsiella*; H: *Lactobacillus*; I: *Listeria*; J: *Micrococcus*; K: *Neisseria*; L: *Planococcus*; M: *Pseudomonas*; N: *Sporolactobacillus*; O: *Sporosarcina*; P: *Staphylococcus*; Q: *Streptococcus.*

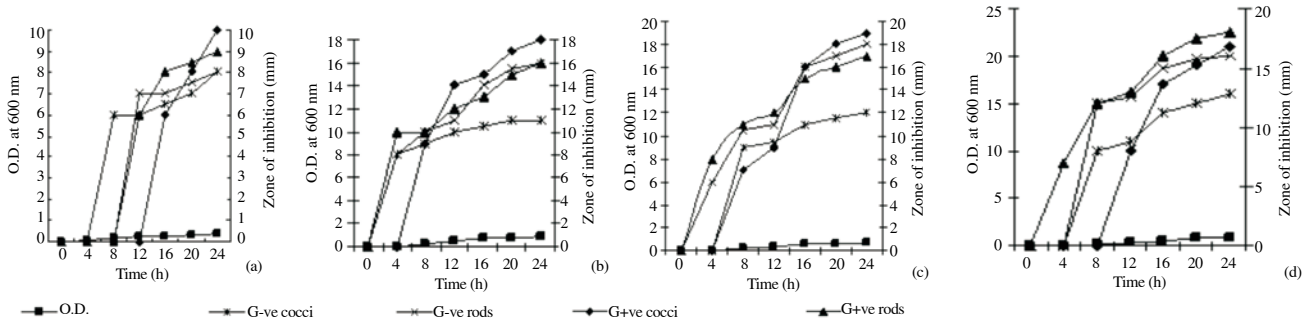

Figure 2. Results of induction time assay of strain 100 (a), 101 (b), 104 (c), 111 (d).
In all the cases, as the growth increased with time, the antibacterial activity also increased giving relatively larger zones of inhibition against all of the four indicators.

3.2. In vitro antimicrobial activity assay

In antimicrobial activity assay, about 45 bacterial strains inhibited Gram-positive cocci, 31 strains inhibited Gram-negative cocci, 67 inhibited Gram-positive rods and 59 inhibited Gram-negative rods. On the whole, 94 out of 166 bacterial isolates were able to inhibit the growth of indicator strains, while 72 remained ineffective. Only four strains coded as 100, 101, 104, and 111 showed inhibitory activities against all of the four indicators used. Zones of inhibition were also measured in each case.

3.3. Induction time assay

Induction time assay of antimicrobial compounds, strain 100 showed inhibitory activity after eight h of incubation when the optical density at 600 nm was only 0.172 (Figure 2a). In this case only Gram-negative cocci were inhibited. Strain 101 started to show inhibitory activity after 4 h of incubation inhibiting all of the three indicator groups except Gram-positive cocci. Here the optical density was only 0.054 (Figure 2b). Strain 104 started to inhibit Gram-positive and negative rods after 4 h of incubation with O.D. being 0.052 (Figure 2c). Strain 111 antimicrobial activity was observed after 4 h with O.D. 0.028 inhibiting only Gram-positive rods (Figure 2d).

3.4. Influence of heat and proteinase K

In these tests it was observed that even after heating for 15 min at 121 °C and treating with proteinase K for an hour, all of the selected isolates were able to inhibit the growth of all the four indicator strains forming relatively smaller zones of inhibition.

3.5. Antagonistic assay of each TLC spot

In TLC, the solvent front was found to be 6.5 cm. In case of strain 100 and 111, no spot was visible with naked eye whereas in case of strain 101 spot numbers 4 and in strain 104, spot number 1, 2, and 3 were visible with naked eye. In TLC of all four strains, all spots could be seen under UV light while spots visible with naked eye in any case became clearer on exposure to iodine vapors. None of the spots became visible when the TLC plate was exposed to H_2SO_4 and $KMnO_4$ solution. In this experiment simple broth was run as control and it showed no spots after TLC (Table 1). All spots of selected strains were able to inhibit the indicator strains some if not all except spot number 2 of strain 104. The zones of inhibition formed in each case were relatively small in size as compared to normal condition.

Table 1
Antimicrobial activity of each spot resulting from TLC.

	Spot No.	R_f Value	AA Against
100	1	0.092	G+ve Cocci
			G-ve Cocci
	2	0.153	G+ve Cocci
			G-ve Cocci
			G+ve Rods
	3	0.20	G-ve Cocci
			G+ve Rods
	4	0.261	G+ve Cocci
	5	0.661	G+ve Cocci
			G+ve Rods
	6	0.80	G+ve Cocci
			G-ve Cocci
			G+ve Rods
			G-ve Rods
101	1	0.107	G+ve Rods
			G-ve Rods
	2	0.215	G-ve Cocci
	3	0.338	G-ve Cocci
			G-ve Rods
	4	0.692	G-ve Rods
	5	0.861	G+ve Rods
104	1	0.138	G-ve Cocci
			G+ve Rods
	2	0.261	NA
	3	0.353	G+ve Cocci
			G-ve Rods
	4	0.446	G-ve Rods
	5	0.723	G+ve Cocci
	6	0.892	G+ve Rods
111	1	0.138	G+ve Cocci
			G+ve Rods
	2	0.215	G+ve Cocci
			G-ve Rods
	3	0.353	G+ve Rods
	4	0.723	G+ve Cocci
	5	0.861	G-ve Cocci
			G+ve Rods
			G-ve Rods

3.6. Characterization of agents instigating solubilization

The degree of solubilization by each isolate was determined by

Table 2

Relationship between metal solubilization and pH changes by selected strains.

Metal salt	Concentration (mmol/L)	Zones of solubilization (mm) and pH changes by selected bacterial isolates											
		100			101			104			111		
		7 days	14 days	pH	7 days	14 days	pH	7 days	14 days	pH	7 days	14 days	pH
ZnO	14	21	24	6.16	32	25	6.26	20	27	6.26	24	33	6.11
	20	20	20	6.23	20	25	6.29	24	24	6.29	24	30	6.23
	25	19	21	6.62	22	25	6.31	23	25	6.31	20	28	6.28
	30	18	21	6.73	7	10	6.33	18	20	6.33	19	25	6.61
	35	18	19	6.81	11	12	6.45	8	19	6.45	14	18	6.84
$Zn_3(PO_4)_2.4H_2O$	5	10	13	5.55	10	11	5.60	12	14	5.60	11	12	5.24
	10	8	10	5.60	9	9	5.98	G-	G-	5.98	G-	G-	5.51
	15	G-	G-	5.98	G-	G-	6.23	G-	G-	6.23	G-	G-	5.60
	20	G-	G-	6.20	G-	G-	6.17	G-	G-	6.17	G-	G-	5.76
	25	G-	G-	6.75	G-	G-	6.50	G-	G-	6.50	G-	G-	6.10

G: Growth but no solubilization.

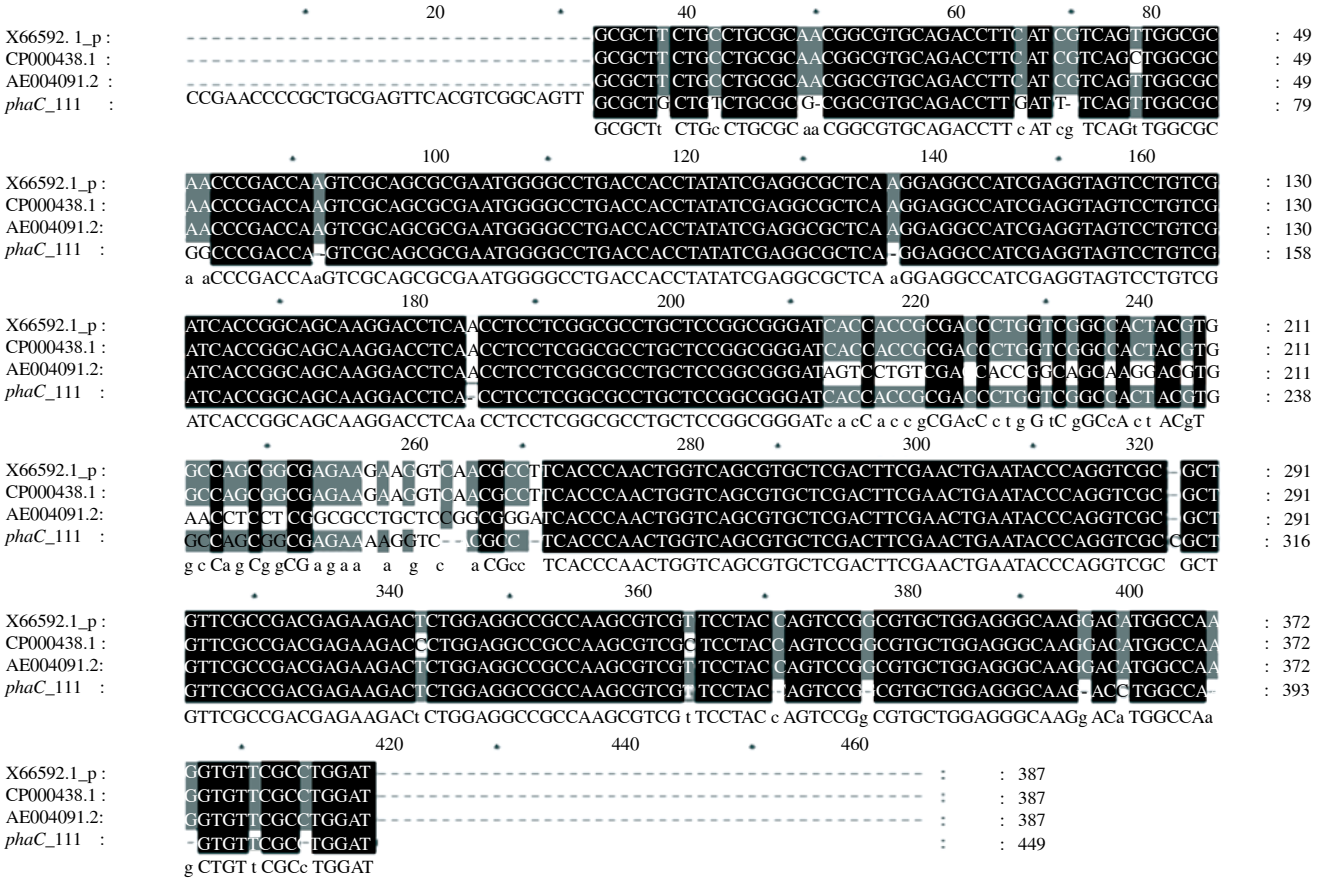

Figure 3. Homology and conserved domains of *PhaC* of 111, with AE004091.2 (*Pseudomonas aeruginosa* PAO1), X66592.1 (PAPHAC1C2 *Pseudomonas aeruginosa*), CP000438.1 (*Pseudomonas aeruginosa* UCBPP-PA14).

measuring the zone of solubilization (Table 2). About 54 isolates solubilized zinc oxide while 23 solubilized zinc phosphate. On the whole 70 bacterial strains out of 166 were able to solubilize metal salts while 96 strains did not solubilize any. It was observed that in all cases of both salts used, the pH decreased periodically. A larger extent of a decrease in pH was observed in case of zinc phosphate by all of the four selected bacterial strains. The results are shown in Table 3. It should be noted that the pH of medium in all cases was found to be 7.0 on day 0. The maximum tolerable concentration assay showed that in case of zinc oxide all of the selected strains not only showed growth but also were able to solubilize all concentrations of zinc oxide. In case of zinc phosphate all of the isolates were able to grow on all of the concentrations used but 10 mmol/L was the highest concentration where only strain 100 and 101 were able to solubilize. The pH of the medium in all the cases was 7.0 on day 0, whereas a gradual decrease in pH of media was observed whether that salt was solubilized or not (Table 2).

3.7. In vivo production of bioplastic

All of 166 bacterial isolates grown on Nile blue supplemented PHA detection medium were observed under UV light. A total of 127 isolates showed luminesce under UV light thus indicating to be PHA positive whereas 39 isolates gave negative results. These results

were confirmed by Sudan black staining. Sudan black stained the intracellular PHA granules black thus confirming our results of PHA production obtained from previous method.

3.8. PCR Amplification of Pha C and 16S rRNA genes

The PCR amplification of 540 bp was successfully performed (Figure 4a). The gel picture shows that the amplified band of our respective gene having a size of 540 bp was only observed in well B and C from strains 104 and 111. Ribotyping of selected strains 104 and 111 (that showed the presence of *PhaC* gene fragment) was done by amplifying 16S rRNA gene and partial gene of size 1.5 kb was amplified (Figure 4b).

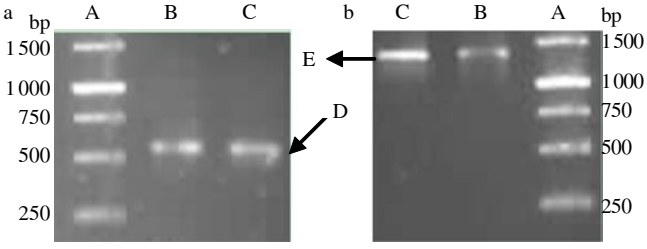

Figure 4. a: Amplified PCR product of *Pha C* gene of strain 104 and 111 in wells B and C, respectively, well A contains gene ruler; b: Amplified PCR product of *16S rRNA* gene of strain 104 and 111 in wells B and C respectively, well A contains gene ruler.
D: Amplified 540 bp *PhaC* gene fragment; E: Amplified *16S rRNA* gene product.

3.9. DNA sequence analysis

Amplified gene fragments *i.e. PhaC* and 16S rRNA of strain 111 were sequenced and obtained accession numbers (EU781525 and EU781526). The sequence of 16S rRNA showed homology to a total of 100 sequence entries from Gene bank. The sequences belonged mostly to *P. aeruginosa* and about 99% homology was observed in each case. The sequence of *PhaC* gene fragment showed homology to *PhaC* of *P. aeruginosa*. Phylogenetic tree of *PhaC* sequence of strain 111 were drawn with *PhaC* sequences of top three blast hit sequences which confirms conserved domains were present in amplified sequences of *PhaC* gene (Figure 5).

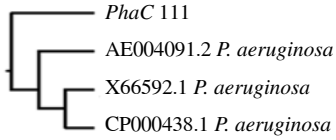

Figure 5. Phylogenetic tree of *PhaC* of 111, with AE004091.2 (*P. aeruginosa* PAO1), X66592.1 (PAPHAC1C2 *P. aeruginosa*), CP000438.1 (*P. aeruginosa* UCBPP-PA14) formed by using CLUSTALW program.

4. Discussion

Natural products and their important functions have long been objects of fascination and utility since they have steered most of the development of different areas of biology and chemistry[18]. Such important products and functions of bacterial source are vastly under study since they have been proven to be useful to humans both economically (*e.g*. medicines of bacterial source) as well as environmentally (*e.g*. bioremediation and biodegradation). The aim of this study was to isolate, screen and analyze bacteria from different areas of Pakistan for the production of antimicrobial compounds, zinc solubilization and bioplastic production. A total of 166 bacterial strains were used in this research.

Production of antimicrobial compounds seems to be a general phenomenon for most bacteria[19]. The search for new antimicrobial agents is a field of utmost importance. Considering this fact, we screened the bacterial isolates for the production of antimicrobial compounds. It was observed that the most inhibited indicator group was of Gram-positive rods mostly involved the formation of larger sized zones of inhibition *i.e*. 15 mm or greater that 15mm with maximum being 28mm (formed by strain 89). Motta *et al.* in 2004[3] also reported that the only inhibited indicator group was of Gram-positive rods that also formed highest zones of inhibition (17 mm). It was found that mostly the antimicrobial compounds were produced by Gram-negative rods Motta *et al*.[3] suggested that the high proportion of antimicrobial producing strains may be associated with an ecological role, playing a defensive action to maintain their niche, or enabling the invasion of a strain into an established microbial community.

Production of antimicrobial compound actually starts during the specific course of bacterial life, induction time assay of selected strains was performed. It was found that strain 100 started to show inhibitory activity after 8 h of incubation. In case of strain 101, 104 and 111, the antimicrobial activity started only after 4 h of incubation. All strains showed highest antimicrobial activity after 24 h of incubation. In all of the four strains, optical density was also noted to check the effect of growth on antimicrobial activity. It was found that in both cases i.e. time and optical density, as these two parameters increased, the measurement of the zones of inhibition also increased thus showing an increase in antimicrobial activity. Uzair *et al*., 2006[21] suggested the same thing that degree of production of antibacterial compound by *Pseudomonas* strain increased with increasing culture age with maximum zone size observed after 72 h of growth.

Antimicrobial activity of selected strains was further characterized by observing the effects of heat and proteinase K. In case of heat effect, the antimicrobial activity was shown by all four strains but its intensity was a bit decreased as indicated by the formation of smaller zones of inhibition. None of the strains under inspection totally lost its antimicrobial activity. As far as the effect of proteinase K on antimicrobial activity of selected strains is concerned, antimicrobial activity was a bit retarded indicated by the formation of smaller zones of inhibition as compared to normal conditions but it was not totally lost. In both cases, strains 104 and 111 were more capable of forming larger zones of inhibition as compared to strains 100 and 101. These results are in agreement to Uzair *et al*., 2006[21]

suggested the thermo resistance of the inhibitory metabolite. They also suggested that enzymes treated supernatant was found to be resistant to enzymes thus indicating antibiotic metabolite is not protein in nature. In addition to that, proteolytic enzyme had a lesser effect on antimicrobial activity of selected bacterial strains as compared to heat effect that was evident by the formation of relatively larger zones of inhibition.

In order to separate different antimicrobial compounds from the filtered supernatant, TLC was carried out with ethyl acetate extract of each selected strain. It was found that only spot number 2 of strain 104 having R_f value 0.261 had no inhibitory effect against any of the indicators. Spot number 6 of strain 100 having R_f value 0.80 had the maximum activity inhibiting all of the four indicators. Since in each strain, different spots inhibited the growth of indicators differently but not all four indicators at the same time and since each of these four selected strains were able to inhibit all of the four indicator organisms so it can be assumed that synergistic effect of all spots occurring in each case results in the formation of larger sized zones of inhibition and a complete antimicrobial action of each strain. In addition to that TLC was performed in case of each strain with its ethyl acetate extract, so most of the antimicrobial compounds in our test strain were ethyl acetate soluble and of low polarity. Frequently, microorganisms need to solubilize insoluble metal compounds present in the natural environment before uptaking and utilizing essential metals *e.g*. P and S. In addition to that, these microbial activities are also important to humans[22]. In this study, to qualitatively estimate solubilization activity of each isolate, zones of solubilization were also measured. It was observed that a total of 70 bacterial isolates were able to produce halo-forming colonies on both zinc oxide and zinc phosphate supplemented medium, when glucose was incorporated in the medium. Of the halo-forming colonies, 77.14% were able to solubilize ZnO, and 32.85% solubilized $Zn_3(PO_4)_2.4H_2O$. It was observed that zinc oxide was solubilized forming larger zones of inhibition as compared to zinc phosphate. In fungal systems, it has been reported that ZnO has a good buffering capacity that neutralizes protons close to the plasma membrane H+-ATPase where they are generated[23]. Mujahid *et al*.[25], the availability of a specific carbon source *i.e*. glucose is an essential requirement for solubilization to be visualized.

In order to analyze the pH changes occurring in the media during zinc solubilization, the four selected bacterial strains that were able to solubilize both insoluble metal salts were subjected to broth assay. The pH decreased in case of all four strains and two insoluble metals salts used as the corresponding to the time of incubation. Morley *et al*.[26] and Sirohi *et al*.[4] have reported that production of H+ and organic acids seem to be the most significant mechanisms for heterotrophic metal solubilization although some contribution may also arise from excretion of other metabolites, siderophores and CO_2 from respiration, the significance of all these processes being variable and dependent on the organisms and the growth conditions. Karamushka *et al*.[27] suggested that the proton translocating ATPase of the plasma membrane generates the electrochemical gradients that are required for the acquisition of nutrients by active efflux of protons into the external medium. Maximum tolerable concentration of insoluble zinc salts can be related directly to zinc tolerance, the more a strain is zinc tolerant, the more will it be able to solubilize the insoluble zinc salt because zinc becomes toxic at high concentrations. Saravanan *et al*., 2004[28]has suggested that unless the cultures tolerate a higher level of zinc, its solubilization may not continue. Alhasawi[29]reported that the production of protein-rich, zinc-binding moieties by *Pseudomonas fluorescens* ATCC 15325 accounted for a mechanism of zinc tolerance in this strain.

Subsequently four selected strains were able to produce antimicrobial compounds as well as agents causing zinc solubilization and Podolak *et al*.[30] suggested that the antimicrobial effect can be due to organic acids that cause a reduction in pH, so it can be assumed that the organic acids that are acting as antimicrobial agents on one side can act as agents causing zinc solubilization on another because as mentioned above acid production is an important phenomenon in metal solubilization.

Bioplastics are lipid in nature that are accumulated as storage materials (in the form of mobile amorphous, liquid granules); allowing microbial survival under stress conditions[12]. For bioplastics screening test, all 166 bacterial isolates grown on Nile blue supplemented PHA detection medium were observed under UV light. A total of 76.50% isolates were found to be PHA positive. These results were confirmed by Sudan black staining. Spiekermann *et al*.[15] reported that the Nile blue A and Nile red dyes could be applied at very low concentrations to agar plates to stain colonies with cells containing PHAs or other lipophilic storage compounds which do not negatively affect the growth of the cells. In this study most of the bacterial isolates were PHA positive suggesting that these bacteria originate from lipid rich environments. Bacterial bioplastics or PHAs are chromosomally encoded by PhaCBA operon[19]. For checking the presence of this operon in our selected strains, isolated genomic DNA was used to amplify the *PhaC* gene fragment through PCR. As a result, only two (104 and 111) out of four selected bacterial strains were found to be PHA positive at genotypic level. Therefore, for identification, their *16S rRNA* cistrons were amplified.

Since strain 111 showed better results, therefore its purified PCR product of both *PhaC* and *16S rRNA* gene fragments was sequenced. The sequence of *16S rRNA* showed homology to a total of 100 sequence entries from Gene bank, most of them belonging to *P. aeruginosa* with about 99% homology in each case. The sequence of *PhaC* gene fragment showed homology to a total of 5 sequence entries from Gene bank with all sequences belonging to *P. aeruginosa*. The sequencing result of *16S rRNA* and *PhaC* gene fragments corresponded to each other, confirming our results that strain 111 is *P. aeruginosa*. On checking the allignment of *PhaC* gene sequence of our strain (111) with that of other homologous strains as matched by BLAST, it was observed that all the bacterial strains have a common ancestor.

Conflict of interest statement

We declare that we have no conflict of interest.

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