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Characterization of antibacterial compounds produced by psychrotrophic *Alcaligenes faecalis* HTP6 isolated from Passu Glacier, Pakistan

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Abstract

Low temperature microorganisms can produce secondary metabolites including anticancer, antiviral and antibacterial compounds. The purpose of the study was to evaluate the possibility of using cold adapted bacteria for production of antibiotics. In the present research work, bacteria were isolated from sediment sample, collected from Passu glacier, using R2A medium . These isolates were screened for their resistance towards various antibiotics, antimicrobial activity against *P. aeruginosa, E. coli, S. aureus, E. faecalis, C. albicans* and *A. fumigatus* and storage stability of crude extract along with cytotoxicity and haemolytic activity. The isolate HTP6 showed best inhibition using spot on lawn test and was selected for further study. The isolate HTP6 was Gram positive, non-pigmented rod, moderate halophilic, with optimum growth at mesophilic range and was identified as *Alcaligenes faecalis* HTP6 on the basis of 16S rRNA gene sequence analysis. The strain showed resistance to clindamycin, cefotaxime and sulfamethoxazole/trimethoprim and was able to inhibit *P. aeruginosa, E. coli, S. aureus, E. faecalis, C. albicans* and *A. fumigatus*. Maximum growth and inhibitory activity of *Alcaligenes faecalis* HTP6 was observed against selected ATCC strains [*Staphylococcus aureus* (ATCC 25923) and *Pseudomonas aeruginosa* (ATCC 27853)] and various clinical isolates (*S. aureus, E. faecalis, Candida albicans* and *Aspergillus fumigatus*) at pH 7 and 30°C, when LB (Luria Bertani) and LB1 (medium supplemented by $FeSO_4$) broth media were used. The crude extract showed good storage and thermal stability at 55°C, and pH stability at 7 along with brine shrimp lethality up to 30%, however, there was no DNA binding and haemolytic activity observed. We can conclude from the study that Alcaligenes faecalis HTP6, isolated from Passu glacier, can be a good candidate for the production of wide spectrum potent thermostable antibiotic with less cytotoxicity.

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Introduction

Low temperature environments are the world's biggest extreme locations, provide harsh conditions for life but still harbour large microbial community. They usually require particular adaptations by the microbial community for its successful colonization and survival (Margesin and Miteva, 2011). Many microorganisms are reported to possess the ability to adapt and even thrive in these harsh conditions of low temperature, low water availability and nutrient deficiency (Feller and Gerday, 2003). The microbes living in harsh conditions like glaciers, harbour many extraordinary characteristics. These characteristics may include tolerating low temperature shocks, high salinity, capability of producing antibiotics, extracellular polysaccharides and different commercially important enzymes (Sajjad *et al*., 2015). The microbes living there evolved different adaptive mechanisms to subside the harmful effects of nature like stress conditions; desiccation, radiation, extreme pH, high osmotic pressure and low nutrient availability (Tehei *et al*., 2005; Morgan-kiss *et al*., 2006; Rodrigues and Tiedje, 2008). Antibiotic production in cold environments, enable microorganisms to reduce interspecies competition in such limited nutrient availability during their life cycle (O'Brien *et al*., 2004). Cold environments are less explored that prompt the scientist's interest due to the probability of new species with potential for valuable antibiotics (Bruntner *et al*., 2005). There are a few reports on antimicrobial compounds from low temperature environment like marine as well as from terrestrial environments. The reports (Bruntner *et al*., 2005; Al-zereini *et al*., 2007; Shekh *et al*., 2011) on secondary antimicrobial compounds from low temperature bacterial isolates are mostly restricted to Polar regions.

The bacteria from genus *Alcaligenes* are relatively less reported for the production of antibiotics or antimicrobial compounds than *Actinomycetes* (Kapley *et al*., 2013). There are two species of genus *Alcaligenes* with genome drafts in NCBI GenBank (Kapley *et al*., 2013) that demonstrate the antimicrobial potential against pathogenic bacterial strains. The genome draft of *Alcaligenes* sp. HPC1271 identified six metabolite synthesizing clusters and suggested amongst them non-ribosomal peptide synthase (NRPS) and dTDP-glucose 4,6-dehydratase showed their role in antibiotic synthesis (Kapley *et al*., 2013). Species of genus *Alcaligenes* has the ability to produce antimicrobial compounds against bacteria, fungi and as well algae (Jayanth, 2001).

The reports showed that *A. faecalis* has the ability to produce broad range antimicrobial compounds against Gram positive/ negative bacteria and fungi (Honda *et al*., 1998; Li *et al*., 2007; Zahir *et al*., 2013). *Alcaligenes* sp. was reported for the production of antibiotic Kalimantacin A, B and C active against MDR Staphylococcal pathogens. *Alcaligenes* sp. HPC1271 synthesized nucleoside antibiotic, the tunicamycin, which was initially reported from Streptomyces (Kapley *et al*., 2013).

To the best of our knowledge there is no published data regarding the antimicrobial activity of microorganisms isolated from Karakoram range. Over all few reports is available regarding the antimicrobial compounds from glaciers' microorganisms. It is the need of time to explore such habitats for microbes having potential to produce novel secondary metabolites including antimicrobial compounds. Therefore, in the current study, bacterial isolates previously characterised from this glacier, were screened for the production of antimicrobial compounds. The aim of the present study was to characterize the psychrotrophic *Alcaligenes faecalis* HTP6 isolated from Passu glacier, Karakoram range, for its polyextremophilic nature of tolerating high salt concentration, varying temperature range, different metal ion concentrations and ability to produce antimicrobial compounds.

Material and methods

Reagents and chemicals

Media, metal salts, methanol, chloroform, H_2SO_4, HCl, NaCl, ethyl acetate and ethanol were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and antibiotic discs were from Liofilchem, (Roseto TE),

Italy.

Sampling site and isolation

Sediment sample was collected from Passu glacier (Karakoram range), Pakistan (36°27.424N to 074°52.010E), following standard protocol described by Yegneswaran *et al.* (1988).

Screening of isolate for antimicrobial activity

Different bacterial strains were isolated from Passu glacier sediment using minimal medium R2A agar. Many bacterial isolates were selected on the basis on colony morphology. All the isolates were screened from antimicrobial activity by spot on lawn assay against bacterial and fungal isolates. Inoculum of test strains was prepared in sterile normal saline and the suspension was adjusted to 0.5 McFarland standards. Then Muller-Hinton agar medium was evenly inoculated with test microorganisms using sterile swab. The isolates were spotted on the lawn and the plates were incubated at 15°C for 3-4 days. The inhibitory zones were measured in mm and mean values were calculated. The isolate HTP6 (*Alcaligenes faecalis*) showed best results and was selected for further optimization and characterization. Microscopic, morphological and physiological characterization of isolate was done according to Garrity *et al.* (2004).

Test microorganisms

Microorganisms used in the current study as test organism included: *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853), *S. aureus* (clinical isolate), *E. faecalis* (clinical isolate), *Candida albicans* (clinical isolate) and *Aspergillus fumigatus* (clinical isolate).

Molecular characterization and phylogenetic analysis

For molecular characterization, the genomic DNA of isolate HTP6 was extracted using Invitrogen™ genomic DNA extraction kit. The DNA was amplified by using universal primers 27F (AGAGTTTGATCMTGGCTCAG) and 1492F (TACGGYTACCTTGTTACGACTT) and sequencing was performed commercially from Macrogen Inc., Seoul, Korea. The obtained sequences were aligned and homology was determined by BLAST search tool in NCBI. Phylogenetic tree was constructed by MEGA 6 software with similar sequences obtained from NCBI GenBank. The sequence of the isolate HTP6 was prepared by sequin and submitted to NCBI GenBank for acquisition of accession number.

Tolerance to sodium chloride (NaCl)

To evaluate the halophilic nature, the isolate HTP6 was grown in LB broth containing different concentrations of NaCl ranging from 2 to 8%.

Temperature range determination

The isolate HTP6 was grown on LB agar medium and incubated at 4, 15, 30, 37 and 45°C for 3 to 7 days and the plates were observed after incubation.

Metal tolerance

To check the minimum inhibitory concentration of heavy metals, the isolate was grown on LB medium containing Cd^{+2}, Cr^{+3}, Hg^{+2}, Fe^{+3}, Ni^{+2}, Ar^{+3} and Zn^{+2} ranging from 5-1600 ppm. The metal ions were supplemented as $CdCl_2.2H_2O$, $CrCl_3$, $HgCl_2$, $FeCl_2$ and $ArCl_3$, $NiCl_3$ and $ZnCl_2$.

Antibacterial susceptibility testing

Antibacterial susceptibility was performed based on the disc diffusion method, following the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2013). A total of 7 antibiotics including colistin sulphate (CT, 50 µg); sulfamethoxazole/trimethoprim (SXT, 23.75/1.25 µg), clindamycin (DA 2 µg), ofloxacin (OFX 5 µg), imipenem (IMI 30 µg), cefotaxime (CTX 30 µg) and nalidixic acid (NA 30 µg) were used for determination of antibiotic resistance.

Effect of growth factors on biomass and metabolite production

Incubation period: The biomass production of the isolate HTP6 was carried out by growing it in 50 mL LB broth in shaking incubator at 15°C. Optical density was determined for biomass production while 2 mL of aliquots were withdrawn at regular intervals of 24

hours for four days and inhibitory activity was checked against *S. aureus, P. aeruginosa*, *C. albicans* and *A. fumigatus*.

Temperature: To check the maximum inhibitory activity and increase in biomass, the isolate HTP6 was grown in LB broth at three different temperatures (4, 15 and 30°C). The optical density was checked at regular intervals of 24 hours, for 5 days the antibacterial and antifungal activity was determined.
pH: The optimal pH for biomass production and inhibitory activity was evaluated by growing the isolate HTP6 in LB broth at varying pH (5-9) and their inhibitory activity was observed against the above mentioned organisms.

Effect of carbon and nitrogen sources on growth and antimicrobial activity
To find the best carbon and nitrogen source, the isolate HTP6 was grown in five different media including (Luria Bertani broth, Nutrient broth, Tryptic soy broth, peptone water, Brain heart infusion and R2A broth) keeping a constant temperature of 15°C. The biomass production was carried out by optical density and inhibitory activity was determined against the test organisms by well diffusion method.

Effect of stress conditions on growth and antimicrobial activity
The effect of stress conditions like addition of metal salt ($FeSO_4$), and dilution of medium composition was evaluated for biomass and antimicrobial metabolite production after 24 hours of interval for 5 days.

Extraction of the compounds
The isolate HTP6 was grown in 500 mL LB broth and incubated for 72 hours at 15°C. After incubation, the supernatant was centrifuged at 10,000 rpm for 30 minutes at 4°C, extracted with ethyl acetate (1:1) and evaporated using Rota vapour. The extracts were weighed and dissolved again in DMSO for bioassays.

Storage and thermal stability of the crude extract
To evaluate the thermal stability, 2 mL of crude extracts were kept at -70°C for three months, while thermal stability was checked by keeping 2 mL of aliquots at 4, 20°C for 24 hours, at 35°C, 60°C for 1 hour and 100°C for 15 minutes. The antibacterial and antifungal activities were determined after incubation time and zone of inhibition was measured.

pH stability: The pH stability was evaluated by adjusting the pH of cell free supernatant from 3 to 10 and incubated for 3 hours. After incubation, pH was again adjusted to neutral and evaluated for antimicrobial activity.

Brine shrimp lethality assay
The cytotoxicity of the crude extract was carried out by brine shrimp assay as previously described by Maridass (2008), using brine shrimp (*Artemia salina)* in Artificial Sea water (34 g/L). After 48 hours of incubation 10 nauplii (larvae) were transferred in a test tube having 5 mL of sea water. Different concentrations of crude extract (50 µL to 200 µL) were transferred to each vial and recorded their cytotoxic activity. Normal saline was used as negative control.

Haemolytic assay
The haemolytic assay was carried out on Muller-Hinton Agar supplemented with 5% human blood. A well was formed, filled with 80 µL of the crude extract and incubated at 37°C for 48 hours.

DNA binding assay
DNA binding assay was carried out by mixing 100 µL of human DNA with 1 mL of crude extract and incubated for 6 hours at 15°C. Crude extract without DNA was used as a control. After incubation, the antibacterial and antifungal activity was carried out and the zones of inhibition were compared with that of the control.

Results

Characteristics of the isolate HTP6
Morphologically, the isolate HTP6 produced small circular, transparent, non-pigmented colonies, while microscopic analysis revealed the isolate HTP6 as

Gram negative, thick rod. Isolate HTP6 showed growth up to 37°C suggesting their psychrotolerant nature, and could grow in the presence of ~8% NaCl showing moderate halophilic characteristics. Interestingly, the isolate HTP6 showed some extent of tolerance to all heavy metals like, cadmium (740 ppm), chromium (760 ppm), arsenic (440 ppm), mercury (100 ppm) and iron (1080 ppm) and zinc (1340). Metal tolerance was observed in order as; Zn > Fe > Cr > Cd > Ar > Hg.

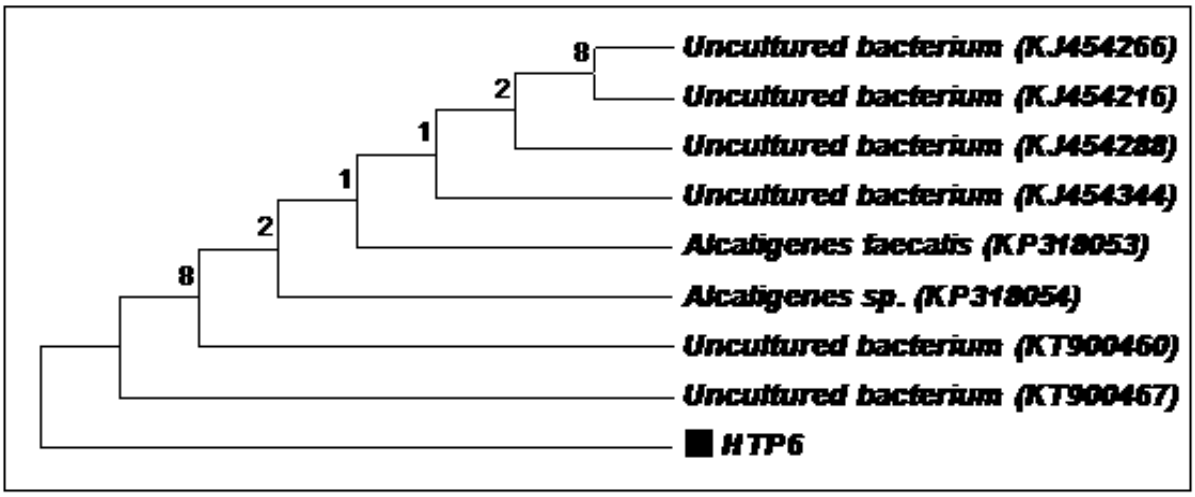

Fig. 1. Molecular phylogenetic analysis of HTP6 (*Alcaligenes faecalis*) by Maximum Likelihood method.

Molecular identification

The BLAST search of 16S rRNA sequence showed that the study isolate HTP6 was 99% similar to *Alcaligenes faecalis*. The phylogenetic tree constructed by MEGA 6 the study isolate clustered into the group of *Alcaligenes faecalis* (Fig.1).

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (1993). The tree with the highest log likelihood (-1084.4799) is shown.

The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree (s) for the heuristic search were obtained automatically by applying Neighbour-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The analysis involved 9 nucleotide sequences. Codon positions included were 1^{st} +2^{nd} +3^{rd} + Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 792 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (2013).

Antibiotic sensitivity assay

The antibiotic resistance was accomplished by disc diffusion method. *Alcaligenes faecalis* HTP6 showed multiple antibiotic resistance to clindamycin (DA), cefotaxime (CTX), and sulfamethoxazole/trimethoprim (SXT), however, the isolate was sensitive to imipenem (IMI), nalidixic acid (NA), ofloxacin (OFX) and colistin sulphate (CT) (Fig. 2).

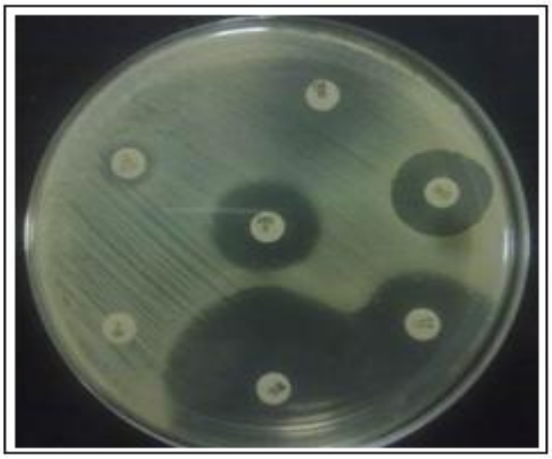

Fig. 2. Antibiotic sensitivity profile of *Alcaligenes faecalis* HTP6, showing variable zones of inhibition against different antibiotics.

Alcaligenes faecalis HTP6 showed broad spectrum activity against both bacterial and fungal pathogens. Maximum activity was observed against multidrug resistant *E. coli* and *S. aureus* (ATCC 25923) followed by *Enterococcus faecalis* (Fig. 3).

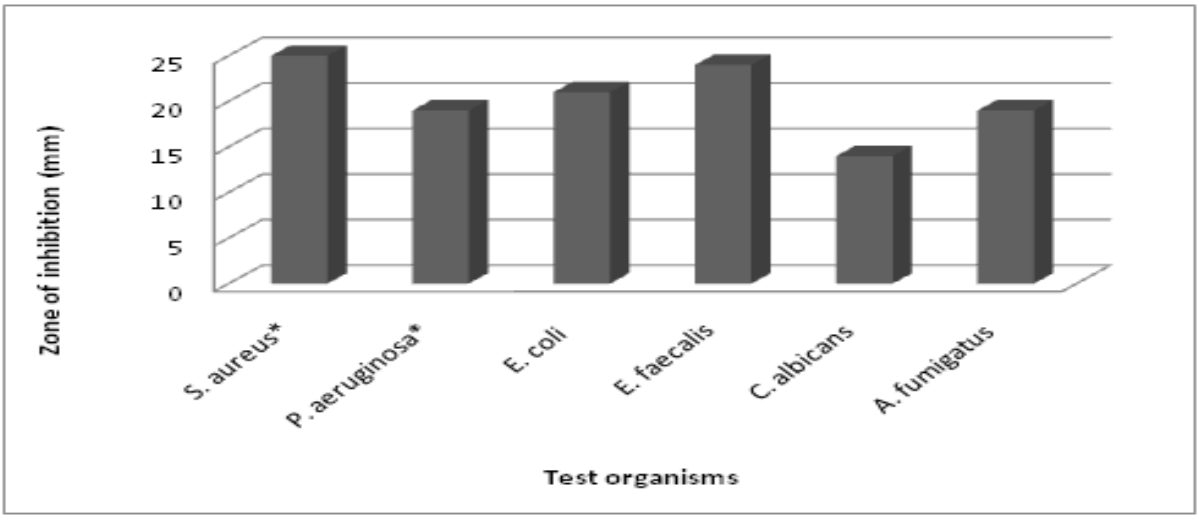

Fig. 3. Zone of inhibition (mm) of *Alcaligenes faecalis* HTP6 against tested ATCC cultures* [*Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853)] and clinically isolated bacterial strains (*S. aureus, E. faecalis, Candida albicans,* and *Aspergillus fumigatus*).

Effect of growth factor on biomass production

Effect of different parameters (incubation period, pH and temperature) on production of biomass was evaluated. *Alcaligenes faecalis* HTP6 showed maximum growth after 72 hours of incubation, and the optimum temperature required for growth was 30°C. The *Alcaligenes faecalis* HTP6 showed optimum growth at pH 7, and best medium for biomass production was found to be LB (Luria Bertani) broth, followed by Brain heart infusion broth. The $FeSO_4$ supplementation was also observed to have a positive impact on growth of the strain (Fig. 4). Maximum activity was observed after 96 hours of incubation at pH 7 and 30°C. The modified culture medium was also observed to increase the production of antimicrobial compounds (Fig. 5).

Extraction and antibacterial activity of crude extract

Ethyl acetate extract (dissolved in DMSO) exhibited maximum activity against *S. aureus, P. aeruginosa, C. albicans, A. fumigatus* and *E. faecalis* (Fig. 7).

Storage, thermal and pH stability of crude extract

The antimicrobial activity of crude cell free supernatant retained at low temperature storage for long time. The extract withstood heating up to 55°C, however, the activity was completely lost at 90 and 120°C. The crude extract showed maximum activity at pH 7 followed by pH 8, and the activity was reduced at pH 10 and pH 5, while the activity was completely lost at pH 3 and pH 4.

Brine shrimp lethality, haemolytic and DNA binding assay

The crude extract of *Alcaligenes faecalis* HTP6 showed no lethal effect on brine shrimps at low concentration (25 µg/ml), however, at higher concentration (200 µg/ml) the crude extract showed 40% lethality. The extract showed no haemolytic activity and DNA binding activity as compared to the control.

FTIR analysis

The FTIR analysis of crude ethyl acetate extract obtained from *Alcaligenes faecalis* HTP6 showed the presence of various functional groups (Fig. 9). Peak in the range of 3311 cm^{-1} represent OH group from carboxylic acid, while, peak obtained at 1117 cm^{-1} and 1290 cm^{-1} represent 'C-O' from alcohol, carboxylic acid and its derivatives. Two peaks at 2854 cm^{-1} and 2924 cm^{-1} represent C-H stretch.

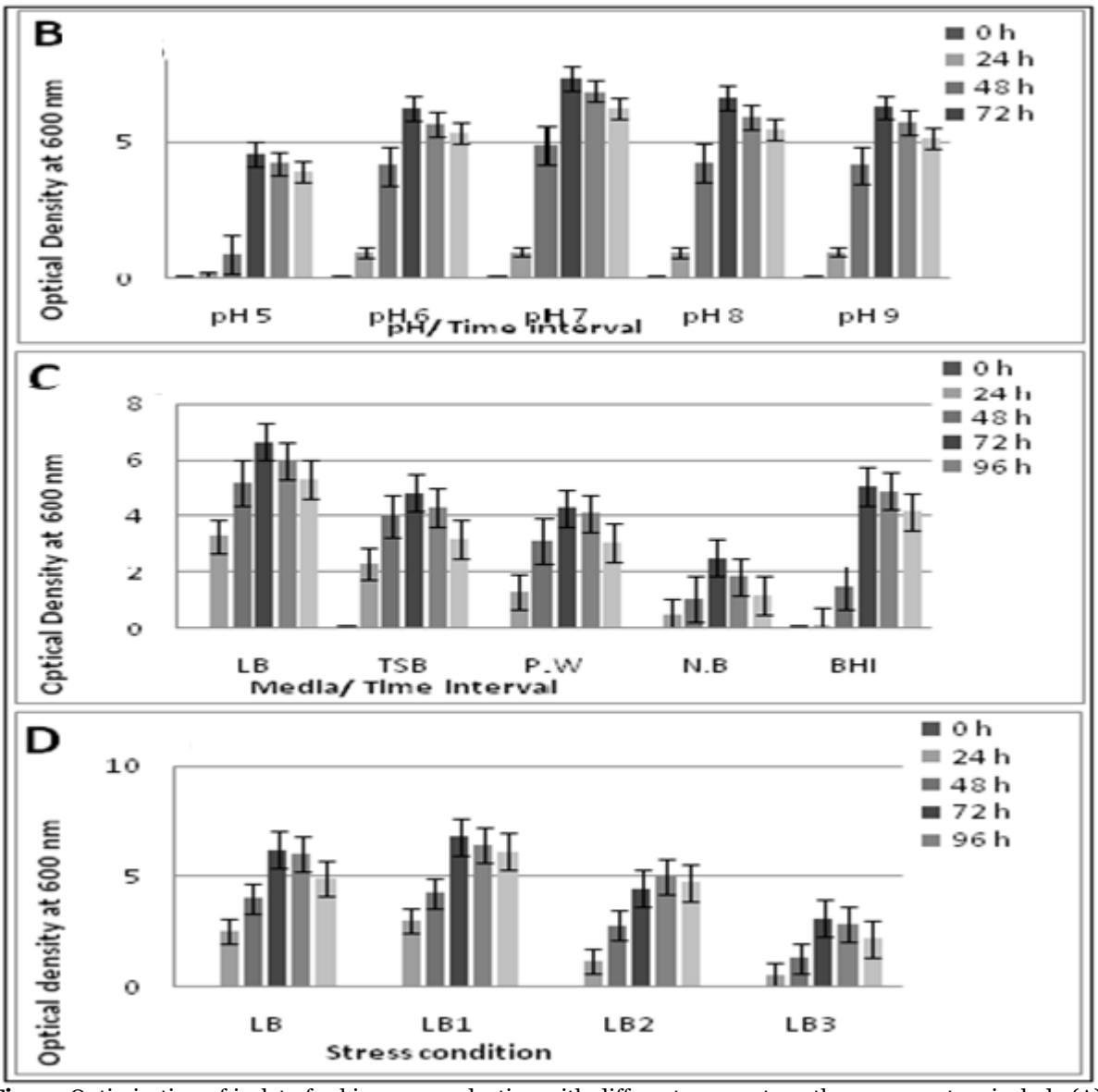

Fig. 4. Optimization of isolate for biomass production with different parameters, these parameters include (A) Optimization of temperature for biomass production. X-axis represent Temperature, Y-axis represent Optical density, (B) Optimization of pH for biomass production. X-axis represents pH and Time interval. Y-axis represents Optical density, (C) Optimization of various synthetic media for biomass production. X-axis represent Media while Y-axis represent Optical density, (D) Optimization of modified media for biomass production (LB normal media, LB1 LB media with addition of $FeSO_4$, LB2 Dilution of LB medium from 1.5 -1% with addition of 0.75% NaCl, LB3 Dilution of LB media from 1.5-0.75% with addition of 1.5% NaCl X-axis represent Media (modified) Y-axis represent optical density.

Peak at 1742 cm^{-1} indicated the presence of C=O from aldehyde, ketone or esters. The presence of alkene (C=C) is confirmed by peak at 1655 cm^{-1}. The presence of nitro compounds (N-O) was confirmed by peak 1538 cm^{-1}. Multiple medium and week bands in the range of 1600-1400 cm^{-1} attributed to aromatic compounds. Arenes may be present indicated by a peak at the range of 699 cm^{-1} and also from pleasant smell of extract. The FTIR analysis shows that the crude extract of *Alcaligenes faecalis* HTP6 constitutes of variety of valuable organic compounds of interest which are required to be purified and characterized. The presence of multiple compounds was also confirmed by various bands on thin layer chromatography (TLC) as shown in Fig. 10.

Discussion

Microbial secondary metabolites are important bioactive compounds for the treatment of infectious diseases. However, the emerging multi and extensive drug resistant microbes are major threats and challenges for the effective management of infections caused by these superbugs (Walsh, 2003; Talbot *et al.*, 2006). Therefore, there is a growing interest to search for better secondary metabolites from unexplored environments. Psychrophilic microorganisms are known as a potential source of antimicrobial metabolites (Sanchez *et al.*, 2009), however data regarding these compounds is uncommon (Ravot *et al.*, 2006).

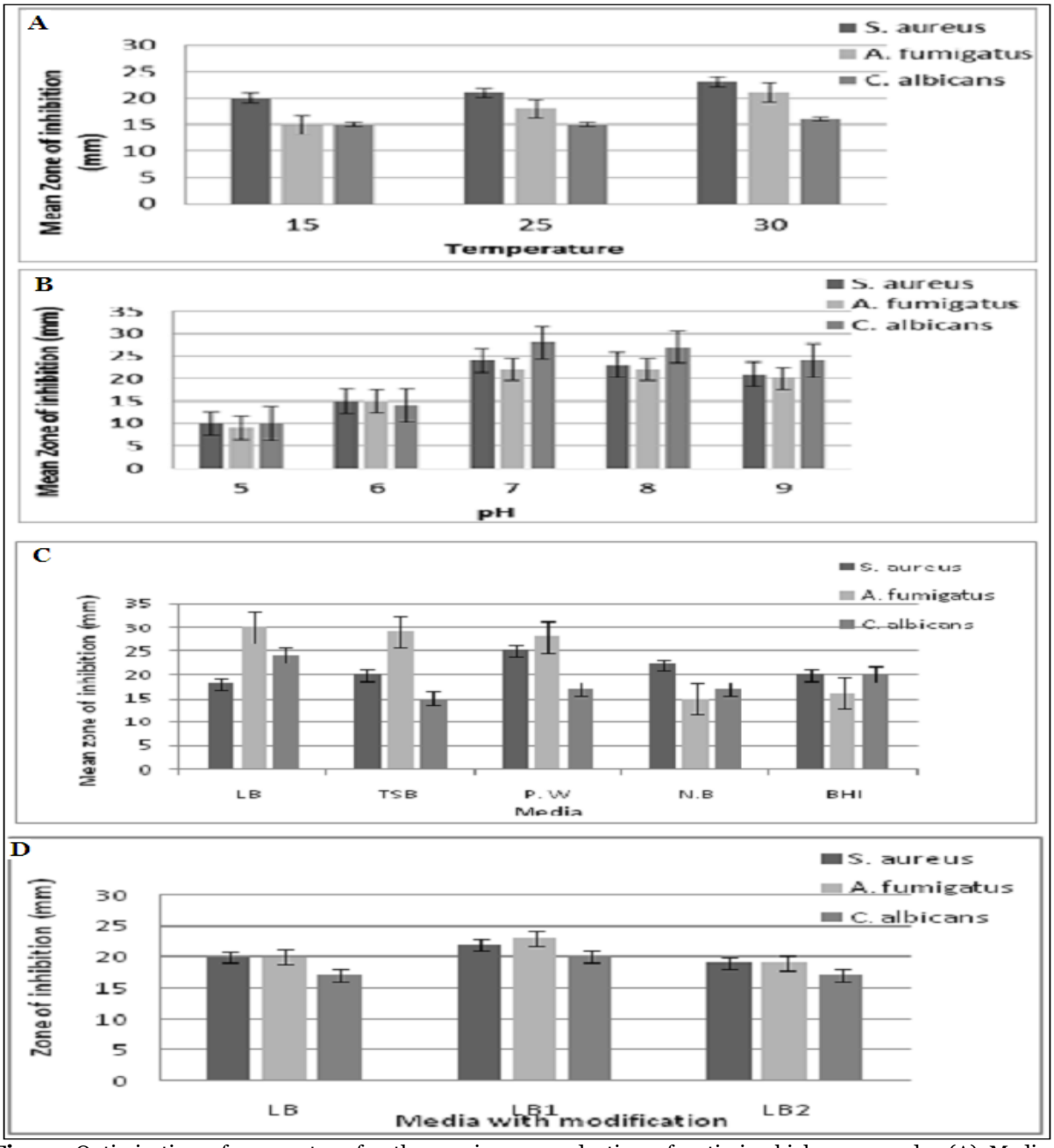

Fig. 5. Optimization of parameters for the maximum production of antimicrobial compounds**.** (A) Media optimization for the production of antimicrobial copounds. X-axis represent media Y-axis represent Zone of inhibation, (B) Temperature optimization for the production of antimicrobial copounds. X-axis represent temperature Y-axis represent Zone of inhibation, (C) Media (Modified) optimization for the production of antimicrobial copounds. X-axis represent media (modifed) Y-axis represent Zone of inhibation, (D) pH optimization for the production of antimicrobial copounds. X-axis represent pH Y-axis represent Zone of inhibation.

In the present study, sediment sample from Passu glacier, Karakoram, was screened for the presence of an efficient producer of broad spectrum antimicrobial compounds against bacterial and fungal pathogens. The studied isolate was polyextremophilic in nature having the ability to tolerate low temperature, high metal concentrations, higher salt concentrations and varying pH.

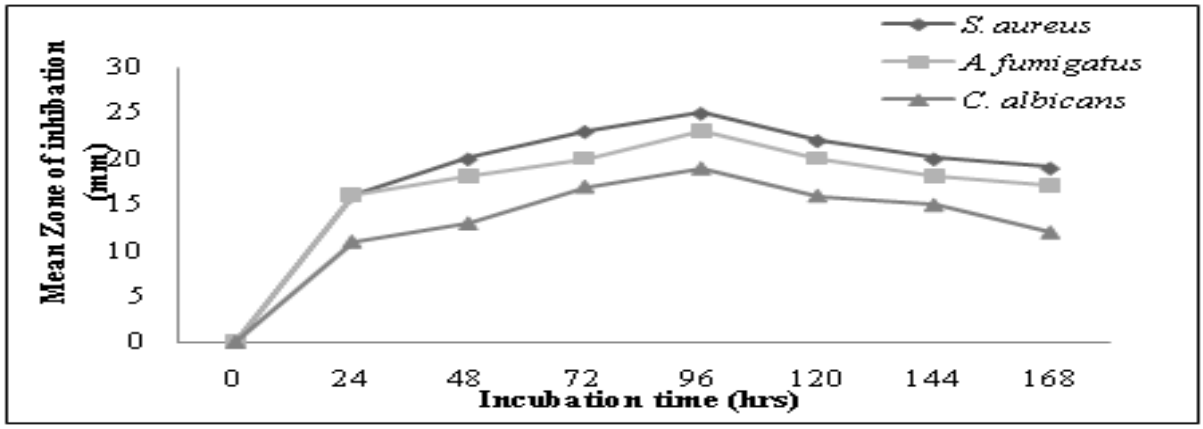

Fig. 6. The effect of incubation time on the antimicrobial compounds activity showed best inhibition at 96 hours of incubation.

The selected isolate, *Alcaligenes faecalis* HTP6 was able to grow at low temperature as well as at mesophilic temperature range. *Alcaligenes faecalis* HTP6 as well as previous reports documented psychrotolerant bacteria with optimum growth at mesophilic range but they can thrive at cold temperature that might be possible due to their greater nutritional adaptability as described by Russell *et al.* (1990). The broad temperature range of *Alcaligenes faecalis* HTP6 indicated that it was Eurypsychrophile (Psychrotroph) in nature according to the definition of Morita (1975). Similar finding was also documented by Hemala *et al.* (2014) who identified the psychrotolerant bacteria with optimum growth at mesophilic temperature. *Alcaligenes faecalis* HTP6 showed moderate tolerance to NaCl concentration.

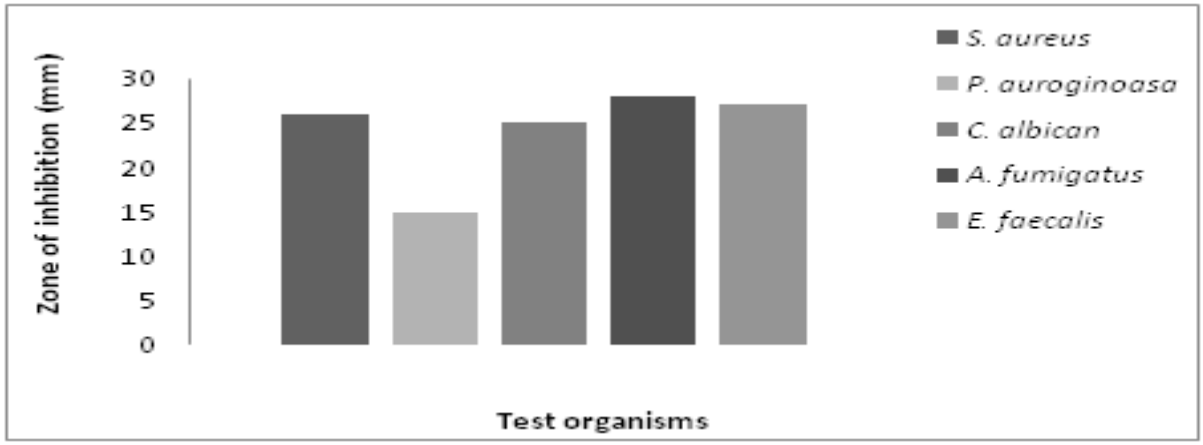

Fig. 7. Zone of inhibition (mm) of crude extract of *Alcaligenes faecalis* HTP6 against the test bacterial and fungal strains. Best inhibition was found against *A. fumigatus* followed by *S. aureus*.

Previous investigations revealed the psychrotrophic bacteria that could tolerate NaCl concentration up to 10%. One of the possible reasons for salt tolerance is the accumulation of solutes to the point where the microbes are growing and have relatively higher temperature than surroundings. In active ecological

environment in glacial habitats, water nuclei forms inside glacier mass, the solutes around that environment diffuse to this active ecological environment and make it hypertonic. The exposure of bacteria to such condition leads to salt tolerance. The salt tolerance of our study isolate may be due to this phenomenon.

Alcaligenes faecalis HTP6 was capable of producing broad spectrum compounds active against bacterial and fungal pathogens. Similar finding revealed the broad spectrum antibacterial and antifungal compounds from low temperature environment (O'Brien *et al.*, 2004; Sanchez 2009; Shekh *et al.*, 2011; Asencio *et al.*, 2014).

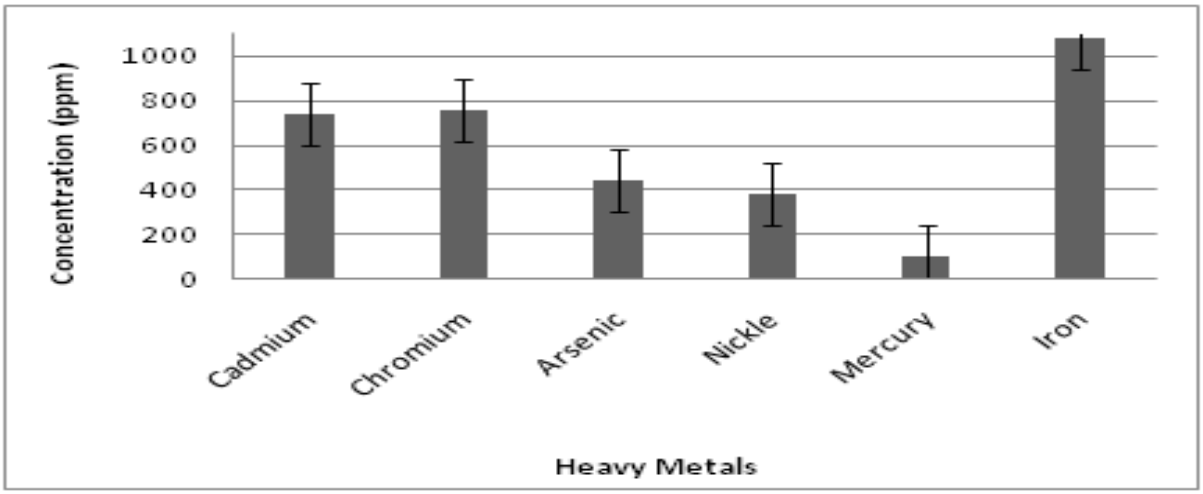

Fig. 8. The tolerance of *Alcaligenes faecalis* HTP6 to different metal ions(ppm) showed best tolerance against Fe^{++}.and least against Hg^{++}.

The *Alcaligenes* sp. is well identified to produce antibacterial and antifungal compounds (Martinez *et al.*, 2006). Several studies (Li *et al.*, 2008), supports our finding that *Alcaligenes faecalis* HTP6 is an efficient producer of antimicrobial metabolites. However, this is the first report of psychrotrophic *Alcaligenes faecalis* from glacier environment of Karakoram range. In addition, this is also the first report on antimicrobial activity, metal and salt tolerance from non-polar glaciers of Pakistan. The antibiotic production in *Alcaligenes faecalis* HTP6 could be due to selective pressure of heavy metals as revealed by the presence of increased level of heavy metals in water and sediment samples of Passu glacier, Pakistan (unpublished data by the authors).

Alcaligenes faecalis HTP6 showed resistance to several classes of antibiotics as well as metals. In non-anthropogenic environment, the resistance among microbial population is intrinsic. Such environment could serve as reservoir for antibiotic resistance genes that can be transmitted to pathogenic bacteria. Our finding was strongly associated by Giudice *et al.* (2013) who determined antibiotic resistance in Antarctic bacteria.

The increased resistance to zinc in *Alcaligenes faecalis* HTP6 was confirms the previous observation (De-Souza *et al.*, 2006; Mangano *et al.*, 2014). This high level of resistance might be described by the fact that zinc is a key micronutrient, which is involved in cellular function including DNA replication, cell activation and division (Mangano *et al.*, 2014).

Alcaligenes faecalis HTP6 showed maximum growth after 72 hours of incubation while the antimicrobial compounds production was observed after 96 hours. *Alcaligenes faecalis* HTP6 showed optimum growth after 72 hours of incubation and was similar to the work reported by Kay and Cheeptham (2013) while the temperature and pH optima was 30°C and pH 7, respectively, which is supported by the findings of

Usha *et al.* (2011) who found the maximum antibiotic production at pH 7 and temperature 30°C. The best medium for growth was LB broth, while decreasing the medium concentration had positive impact on antibiotic production. The antibiotic production was also enhanced by adding $FeSO_4$. In our studies the production of antimicrobial compounds started in idiophase after exhaustion of carbon (Sanchez *et al.*, 2010).

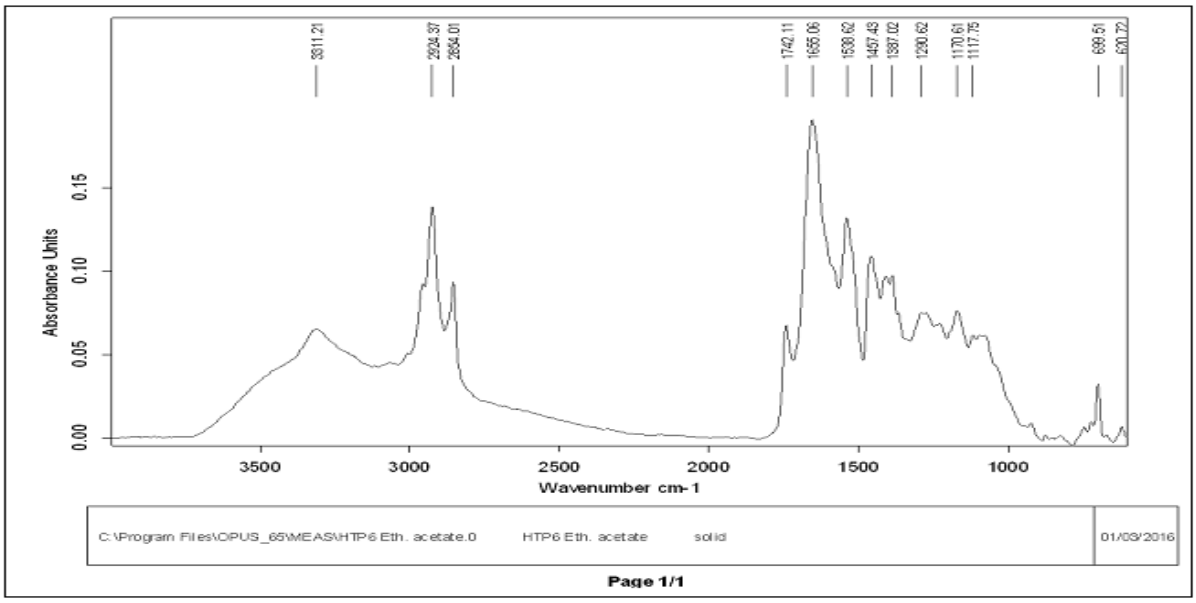

Fig. 9. FTIR analysis of the antimicrobial metabolite produced by *Alcaligenes faecalis* HTP6 showing the presence of various functional groups.

The effect of media on antibiotic production has been revealed by Al-Judaibi (2011). The media used in our study were complex one containing carbon, nitrogen, ammonia phosphate etc. in unknown concentration, and these substances have both positive and negative impact on antibiotic production in culture media (Omura, 1986; Yegneswaran *et al.*, 1988; Ripa, 2009) with enhanced antibiotic production at 1% NaCl which supports our finding. The increased antibiotic production in auxotrophic condition (reducing nutrients contents) in our study could be due to NaCl supplementation.

Regarding the stability to pH, temperature and long term storage, our results were closely related to that reported by Shekh *et al.*, (2011) on antifungal activity of arctic and antarctic bacteria. Similar results regarding storage and thermal stability were observed by Sanchez *et al.* (2009), however, pH stability was different as he observed broad pH ranging from 1-12. Over all the psychrotrophic isolated HTP6 (*Alcaligenes faecalis*) from Passu glacier Pakistan, has tremendous ability of production of antimicrobial compounds at a varying conditions.

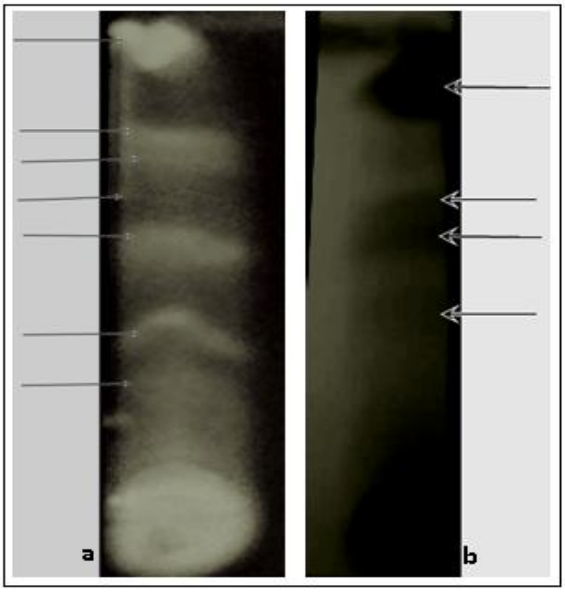

Fig. 10. Multiple bands of the antimicrobial crude extract under (a) UV 365 nm and (b) 254 nm. The arrows showed metabolites of different molecular weight, probably having antimicrobial activity.

The isolate had broad spectrum antagonistic activity against ATCC and clinical gram positive, gram negative and fungal strains. Furthermore purification model testing and formulation will be needed for a successful antibiotic.

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