EFFECT OF VARIOUS CULTURE CONDITIONS ON THE ANTIMICROBIAL ACTIVITY BY BACTERIA FROM PASSU GLACIER, PAKISTAN

RABBANI, I.[1] – AHMAD, T.[1] – MAQSOOD-UR-REHMAN, M.[1] – RAFIQ, M.[2] – NAWAZ, S.[1] – IRFAN, M.[3] – SHAH, A. A.[1] – HASAN, F.[1*]

[1]*Department of Microbiology, Quaid-i-Azam University, Islamabad, Pakistan*

[2]*Department of Microbiology, Balochistan University of Information Technology and Management Sciences, Quetta, Pakistan*

[3]*Department of Oral Biology, College of Dentistry, University of Florida, Gainesville, FL, United States*

[*]*Corresponding author*
e-mail: farihahasan@yahoo.com; phone: +92-519-064-3065

(Received 15th Feb 2021; accepted 10th June 2021)

Abstract. Microorganisms inhabiting cold environments have an incredible potential to produce secondary metabolites including antibacterial, antifungal and antiviral compounds. The aim of the study was to evaluate the possibility of using cold adapted bacteria isolated from Passu glacier for the production of antimicrobial metabolites. Agar well diffusion assay was used to select four best antimicrobial compound producers coded as HTP12, HTP13, HTP36 and LTP10. These strains were identified as *Alcaligenes faecalis*, *Pseudochrobactrum saccharolyticum*, *Alcaligenes pakistanensis* and *Alcaligenes pakistanensis*, respectively, by their phylogenetic analysis. The media, temperature, incubation time and pH were optimized for antimicrobial compound production. The effect of nitrogen sources, carbon sources and salts on antibiotic production was also determined. *Alcaligenes pakistanensis* (LTP10) showed the best antimicrobial activity in Luria Bertani broth, at 25°C, pH 7 and incubation time of 96 hrs against various bacterial ATCC strains and clinical isolates. Yeast extract increased antibiotic activity of *Alcaligenes pakistanensis* (LTP10) while glucose, starch, tryptophan, threonine, L-leucine, arginine, NaCl and KCl decreased the activity. This study concludes that the psychrophilic bacteria are abundant, undiscovered, and good producers of antimicrobial metabolites under optimum conditions. This will lead to the discovery of potent and novel metabolites which could be of medical and industrial importance.
Keywords: *psychrophilic bacteria, Alcaligenes pakistanensis, agar well diffusion assay, antimicrobial metabolites, antibiotic production optimization*

Introduction

The environment which is not suitable and is considered severe for the survival of human beings is referred to as extreme environment and most of the world's extreme environments are low temperature environments. These low temperature environments provide harsh conditions for the survival and growth of organisms but still harbour a large persistent community of microbes. These persistent microbes have adaptations to cope with the challenges of low temperature environments for their survival and successful colonization (Margesin et al., 2002; Furhan, 2020). There are a lot of microorganisms reported that have the ability to adapt and even show best growth and survival in harsh conditions like low or absent oxygen, high salt concentration, less nutrient availability and oxidative stress which occurs due to low temperature environments (D'Amico et al., 2006; Yarzábal, 2016). Cold-loving microbes have adapted several different mechanisms such as production of high amounts of fatty acids, proteins and non-polar carotenoids for excess fluidity of membrane, possess cold adapted enzymes and containing cold acclimation proteins (caps), antifreeze

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2003_20592076

proteins and cryoprotectants that enable them to surmount the adverse effects of low temperature (Morgan-Kiss et al., 2006; Collins and Margesin, 2019). In cold environment low availability of nutrients stimulate the microorganisms to release antimicrobial compounds which empower them to decrease the inter-species competition for nutrients (Yogabaanu et al., 2017; Artini et al., 2019). Such extraordinary characteristics of cold-adapted microorganisms have increased the possibility of using these as a novel source of industrially important antimicrobial compounds (Paun et al., 2021). This attracts the consideration of most scientists because these environments are not extensively explored and there is a maximum chance of novel species with capability of new antibiotics production (Bruntner et al., 2005; Núñez-Montero et al., 2019). A number of reports confirm antimicrobial compounds from cold environment but are mostly restricted to Polar Regions only (Rampelotto et al., 2016). As antibiotic resistance is becoming a worldwide problem, exploring extreme environments like glaciers for antibiotic producing bacteria is really significant.

Antimicrobial metabolites production by microorganisms depends upon availability of specific cultural conditions such as temperature, pH, incubation time, medium composition, carbon sources, nitrogen sources, salts etc. The primary objective of this research was to study the effect of different cultural conditions for maximum production of antimicrobial compounds from bacterial isolates of Passu glacier. There is limited information available concerning the antimicrobial metabolites from microorganisms inhabiting glaciers and rarely from Karakoram mountain range. Thus, in this study, bacterial strains isolated from Passu glacier, were evaluated for antimicrobial compounds production. To investigate such kind of unexplored habitat, for the microorganisms with the ability to produce novel antimicrobial metabolites, is an important need of the time.

Materials and methods

Test organisms

ATCC strains such as *Escherichia coli* (ATCC 25922), *Salmonella enterica* (ATCC 14028), *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853), and *Staphylococcus epidermidis* (ATCC 12228) were used as test organisms in the current study. Similarly, clinical isolates of *Escherichia coli, Psuedomonas aeruginosa, Staphylococcus aureus, Salmonella enterica, Candida albicans* and *Candida krusei* (collected from Pakistan Institute of Medical Sciences hospital) were also used as test organisms.

Screening of isolates for antimicrobial activity

A total of 28 bacterial isolates from Passu glacier with antibiotic activity were available at Applied Environmental and Geomicrobiology Lab, Quaid-i-Azam University, Islamabad. Out of these, 11 isolates (HTP9, HTP12, HTP13, HTP14, HTP19, HTP27, HTP36, HTP37, HTP38, LTP8 and LTP10) were selected on basis of their high antimicrobial activity. Spot-on-lawn and agar well diffusion assays were used for the confirmation of their antimicrobial potential.

Spot-on-lawn method

This method was used for screening of antibacterial compound producing bacterial isolates against ATCC bacterial strains. Sterile normal saline was used for the preparation of

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2003_20592076

inoculum of test strains using 0.5 McFarland standards. Bacterial cells suspensions in normal saline was dipped by sterile cotton swab and streaked uniformly over the plate surface containing Mueller-Hinton agar (MHA) to pledge uniform circulation of the inoculum. The plates were left undisturbed for one hour in the laminar flow hood and the bacterial cultures were spotted on MHA plates inoculated with test microorganisms. The plates were kept at 15°C for 3 days and then at 37°C for 24 hours. The appearance of clear zone of inhibition around spots of isolated strains verified the antibacterial activity.

Agar well diffusion assay

Isolates HTP12, HTP13, HTP36 and LTP10 were selected on the basis of their best antimicrobial activity as shown by spot-on-lawn method. These isolates were inoculated in Luria-Bertani broth and incubated at 15°C for 7 days. After every 24 hrs, samples were taken from the broth and subjected to centrifugation. The agar well diffusion assay was used for analysis of the cell free supernatant, so obtained (Hwanhlem et al., 2017).

Identification of isolates

DNA extraction

The DNA of isolated strains was extracted chemically by phenol-chloroform method (Wright et al., 2017). Gel electrophoresis confirmed the extraction of DNA.

Polymerase chain reaction

The samples of isolated DNA were then amplified by polymerase chain reaction using 16SrRNA primer. Primary denaturation temperature was 96°C for 5 minutes followed by 32 cycles of denaturation, annealing and extension. The temperatures for denaturation, annealing and extension were 94°C for 2 minutes, 55°C for 1 minute and 72°C for 1.5 minutes respectively. The final extension was carried out at 72°C for 10 minutes.

Phylogenetic analysis

ClustalW program executed in MEGA 4.0 was used to study Phylogenetic analysis of isolates (Mahtab et al., 2019). The phylogenetic analysis revealed the sequences of DNA and the analogous sequences were downloaded from National Center for Biotechnology Information (NCBI). After aligning all the sequences, Neighbor Joining method in MEGA4.0 was used for creating phylogenetic tree. The significance of the created tree was studied by Bootstrap analysis (1000 replicate).

Optimum growth conditions of isolates

Effect of incubation period on growth

The isolated strains were incubated in Luria-Bertani broth at 15°C for 6 days to determine their optimum incubation period by taking their absorbance (OD) at 600 nm after every 24 hours using UV/Visible spectrophotometer.

Effect of temperature on growth

The isolated strains were grown at different temperatures in Luria-Bertani broth (i.e., 5, 15, 25 and 35°C) to determine their optimum growth temperature by taking their absorbance (OD) after every 24 hours using UV/Visible spectrophotometer.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2003_20592076

Effect of pH on growth

The isolated strains were incubated at different pH (i.e., pH 5.0, 7.0 and 9.0) to determine their optimum growth pH by taking their absorbance (OD) at 600 nm after every 24 hours using UV/Visible spectrophotometer.

Optimization of antimicrobial activity

Luria-Bertani broth was used for preparation of inoculum and incubated at 15ºC. After every 24 hrs, samples were taken from the broth for a total of 120 hours. The production of antimicrobial compounds in culture medium was determined by agar well diffusion assay (Hwanhlem et al., 2017). The antimicrobial activity of the four isolates were determined in three parallel experiments.

Incubation time

The incubation time effect on antimicrobial compound production was confirmed by taking samples after every 24 hours for 120 hours.

Incubation temperature

The optimum antibiotic production by selected isolates was studied at different temperatures i.e. 5, 15, 25 and 35ºC.

Incubation pH

The antibiotic activity of the selected isolates was determined at different pH values i.e., 4.0, 5.0 and 9.0.

Medium selection

To determine the optimum production of antibacterial compounds by selected isolates, Nutrient broth (NB), Tryptic soya broth (TSB) and Luria Bertani (LB) broth were used as culture media.

Effect of carbon sources

To evaluate the carbon source effect on the production of antimicrobial metabolites, the selected isolates were grown in Luria-Bertani media containing additional glucose and starch in 1 and 2% concentration.

Effect of nitrogen sources

Luria-Bertani broth containing additional tryptophan, threonine, L-leucine, arginine and yeast extract in 1% concentration was used to evaluate the effect of nitrogen sources on the production of antimicrobial metabolites.

Effect of salts

To determine the effect of salts on the production of antimicrobial compounds, the selected isolates were inoculated in Luria-Bertani media containing additional NaCl (2 and 3%) and KCl (1 and 2%).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2003_20592076

Antibacterial and antifungal activity against clinical isolates

Alcaligenes pakistanensis (LTP10) was found to be the best antimicrobial compounds producer and was inoculated in Luria-bertani broth and incubated at 25ºC at pH 7 for 96 hours. After 96 hours, the antibacterial and antifungal activity of cell free supernatant of *Alcaligenes pakistanensis* (LTP10) was evaluated against clinical isolates of *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Salmonella enterica, Candida albicans* and *Candida krusei,* that were collected from Pakistan Institute of Medical Sciences hospital (PIMS), by agar well diffusion technique.

Antibiotic sensitivity test of isolates

Antibiotics sensitivity of selected isolates *Alcaligenes faecalis* (HTP12), *Pseudochrobactrum saccharolyticum* (HTP13), *Alcaligenes pakistanensis* (HTP36) and *Alcaligenes pakistanensis* (LTP10) against various available antibiotics discs in the market was analyzed by Kirby-Bauer disc diffusion method according to the guidelines of the CLSI (clinical and laboratory standards institute) 2013. The plates were transferred to incubator kept at 15°C for 72 hrs. Zone of inhibition was observed and noted after 24 hours of incubation.

Statistical analysis

The difference in activity at different culture conditions was analyzed statistically by measuring their standard deviation and p-value. The p-value was calculated using T.Test in Microsoft Excel 365 to determine the statistical significance of the differences in zone of inhibition under different culture conditions.

Results

In the present study, a total of eleven bacterial isolates coded as HTP12, HTP13, HTP14, HTP19, HTP27, HTP36, HTP37, HTP38, LTP8 and LTP10 from Passu glacier were selected. On the basis of their antimicrobial activity, it was found that LTP10 showed better activity.

Screening of bacterial isolates for antimicrobial activity

The selected strains were screened for antagonistic activity by using spot-on-lawn and agar well diffusion assay against ATCC test strains. After 72 hrs of incubation, the antimicrobial activity was confirmed by the zone of inhibition around the spots of isolates as shown in *Table 1*.

Out of eleven isolated strains, four isolates HTP12, HTP13, HTP36 and LTP10 showed maximum zone of inhibition against test strains. These 4 strains were again evaluated by agar well diffusion assay and their zone of inhibition was measured in mm as shown in *Fig. 1*.

Identification of isolates by phylogenetic analysis

The result of BLAST of total 4 strains show that there are 3 different strains having 90% or more than 90% identity with their respective specie. The isolated strains were identified as *Alcaligenes faecalis* (KF641844), *Pseudochrobactrum saccharolyticum* (KX977558) and *Alcaligenes pakistanensis* (AB968096) as shown in *Table 2 and Fig. 2*.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2003_20592076

Table 1. *Antimicrobial activity of isolates against ATCC bacterial strains by spot-on-lawn assay*

Isolates	*Staphylococcus aureus*	*Bacillus subtilis*	*Staphylococcus epidermidis*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Salmonella enterica*
HTP9	+	-	-	+	-	-
HTP12	+++	++	+	++	++	+
HTP13	++	++	+	+	+	-
HTP14	++	++	+	+++	++	+
HTP19	++	-	+	+++	++	-
HTP27	-	++	-	-	-	+
HTP36	++	++	+	++	+++	-
HTP37	+	-	+	+	-	-
HTP38	+	-	-	++	-	+
LTP8	++	+++	++	++	+++	-
LTP10	+++	+++	++	++	++	++

Inhibition zone: +++ = strong (> 15 mm), ++ = moderate (10-15 mm), + = low (5-9 mm), - = no activity

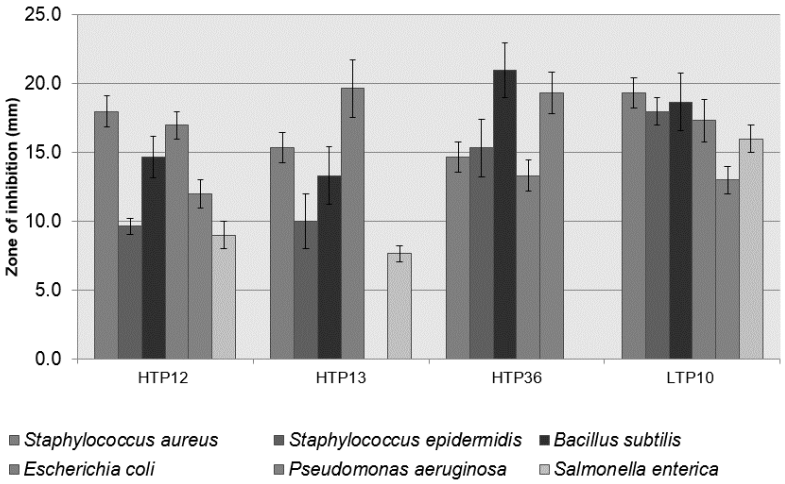

Figure 1. *Zone of inhibition (mm) of isolates against ATCC test strains by agar well diffusion assay after 72 hours. Error bars represent standard deviation (SD) of triplicate measurements of zone of inhibition (mm)*

Table 2. *Identification of isolates by their phylogenetic analysis*

Isolates	Homologous species	Groups	Identity %age
HTP 12	*Alcaligenes faecalis* (KF641844)	Proteobacteria Betaproteobacteria Burkholderiales Alcaligenaceae	99.80
HTP13	*Pseudochrobactrum saccharolyticum* (KX977558)	Proteobacteria Alpha Proteobacteria Rhizobiales Brucellaceae Pseudochrobactrum	99.02
HTP36	*Alcaligenes pakistanensis* (AB968096)	Proteobacteria Betaproteobacteria Burkholderiales Alcaligenaceae	100.00
LTP 10	*Alcaligenes pakistanensis* (AB968096)	Proteobacteria Betaproteobacteria Burkholderiales Alcaligenaceae	99.68

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2003_20592076

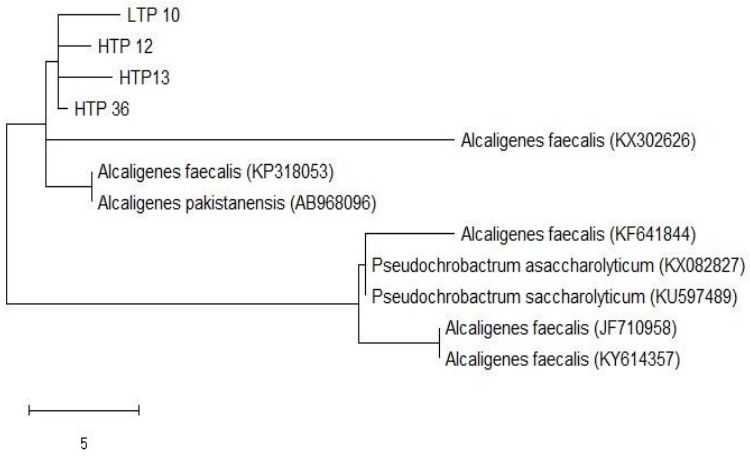

Figure 2. *Phylogenetic relation of study samples with other similar strains downloaded from NCBI*

Optimum growth conditions of isolates

The optimum incubation period for growth shown by selected isolates *Alcaligenes faecalis* (HTP12), *Pseudochrobactrum saccharolyticum* (HTP13), *Alcaligenes pakistanensis* (HTP36) and *Alcaligenes pakistanensis* (LTP10) was found to be 72 hours. The three isolates *Alcaligenes faecalis* (HTP12), *Pseudochrobactrum saccharolyticum* (HTP13), *Alcaligenes pakistanensis* (HTP36) revealed maximum growth at temperature of 25ºC and pH 7.0 while isolate *Alcaligenes pakistanensis* (LTP10) has shown maximum growth at 15 ºC and pH 7.0 as shown in *Figs. 3, 4, 5*.

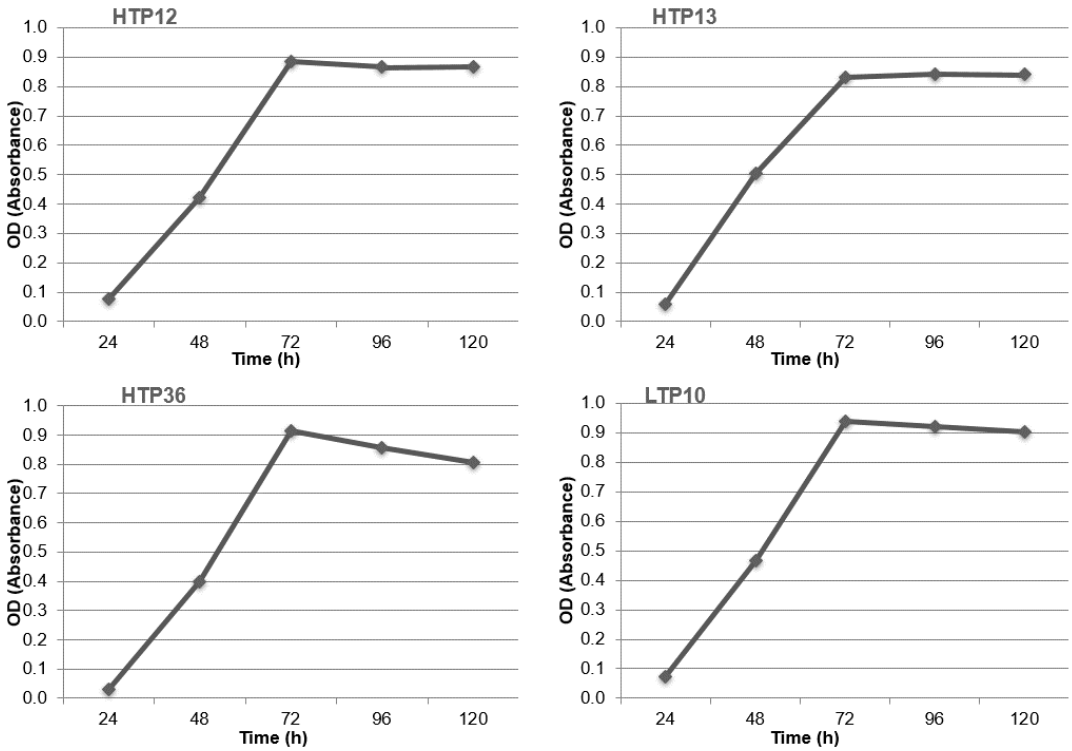

Figure 3. *Effect of incubation period on growth of isolates*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2003_20592076

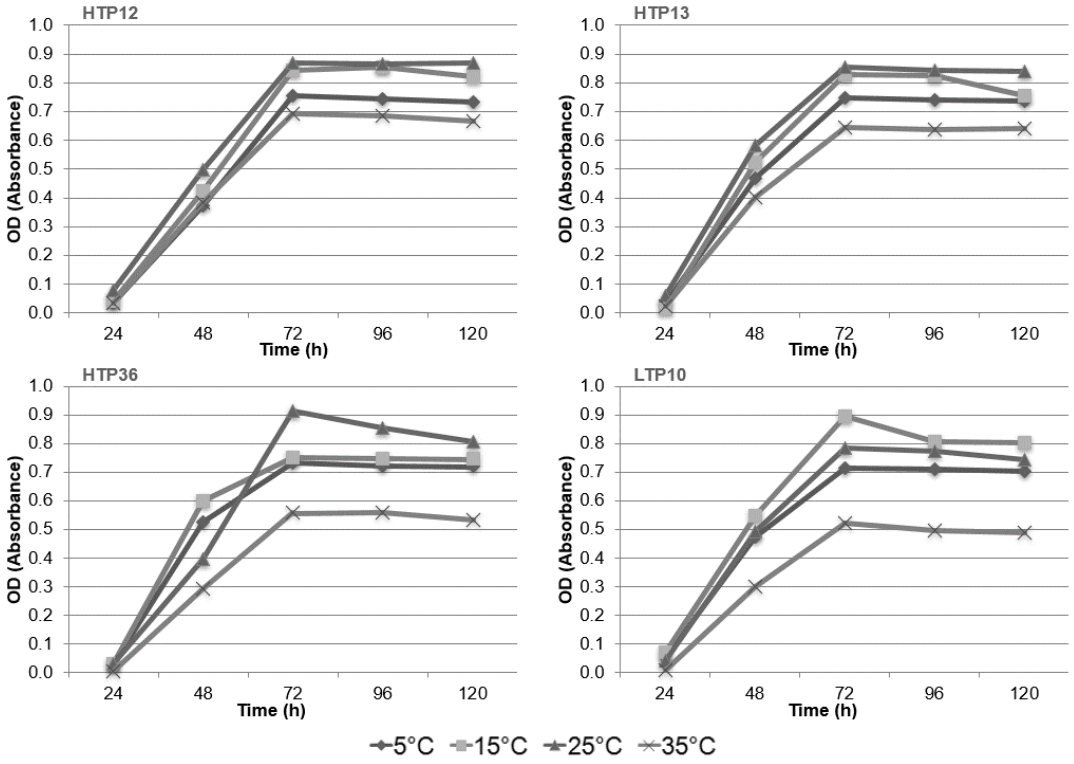

Figure 4. *Effect of temperature on growth of isolates*

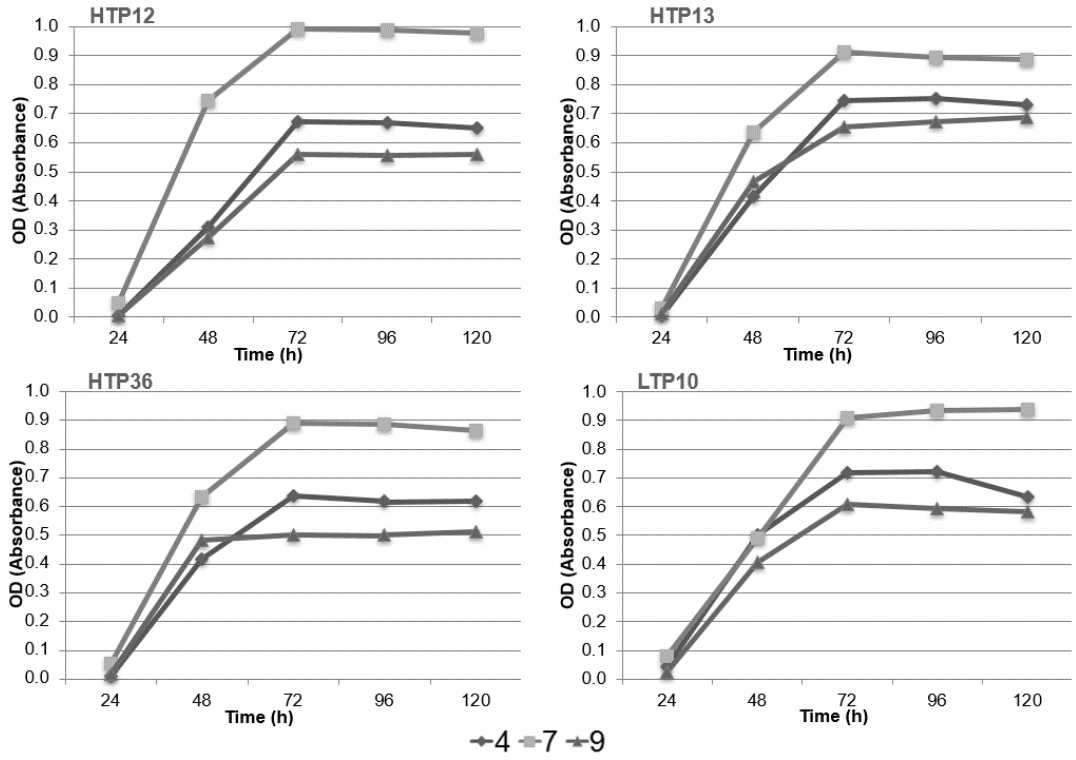

Figure 5. *Effect of pH on growth of isolates*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2003_20592076

Optimization of antimicrobial compound production

The production of antibacterial compounds by the isolated strains was confirmed by agar well diffusion assay. The best activity was shown by *Alcaligenes pakistanensis* (LTP10). Different cultural conditions like selection of growth media, incubation time, temperature, pH and addition of carbon sources, nitrogen sources and salts were considered.

Effect of time of incubation

All the four isolates have demonstrated maximum antimicrobial activity after 96 hours of incubation. The difference in antimicrobial activity at different incubation time was significant statistically ($P < 0.05$). The maximum antimicrobial activity of *Alcaligenes pakistanensis* (LTP10) and *Alcaligenes pakistanensis* (HTP36) was found against *Escherichia coli* (ATCC 25922) and *Bacillus subtilis* (ATCC 6633) respectively with 20±1 mm zone of inhibition as shown in *Fig. 6*.

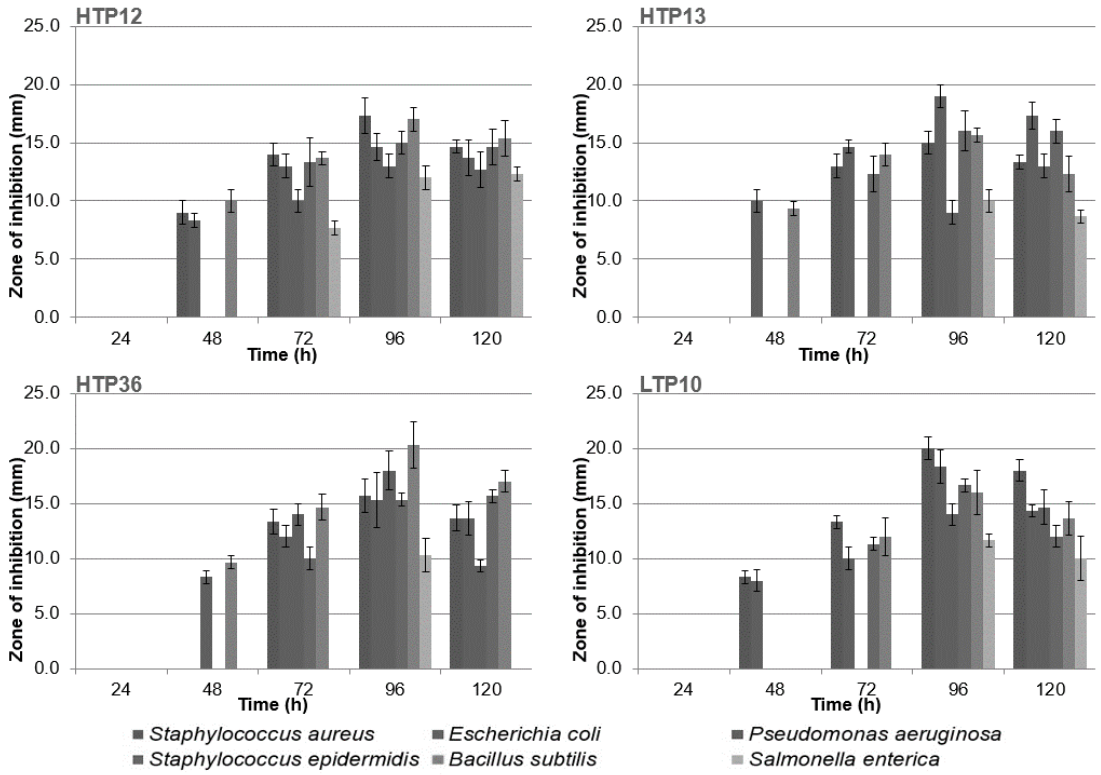

Figure 6. *Zone of inhibition (mm) of isolates against test strains at various incubation periods. Error bars represent standard deviation (SD) of triplicate measurements of zone of inhibition (mm). $P < 0.05$*

Effect of culture medium

Nutrient broth (NB), tryptic soya broth (TSB) and Luria-Bertani (LB) broth were used to evaluate maximum antimicrobial activity of selected isolates. The difference in antimicrobial activity of all the four isolates in different culture media was significant statistically ($P < 0.05$). The cell free supernatants of all the four isolates from Luria-

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2003_20592076

bertani broth have confirmed maximum activity against test organisms. The best activity was shown by *Alcaligenes pakistanensis* (LTP10) against *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25922) with zone of inhibition of 20.7±1.5 mm and 21±1 mm respectively as shown in *Fig. 7*.

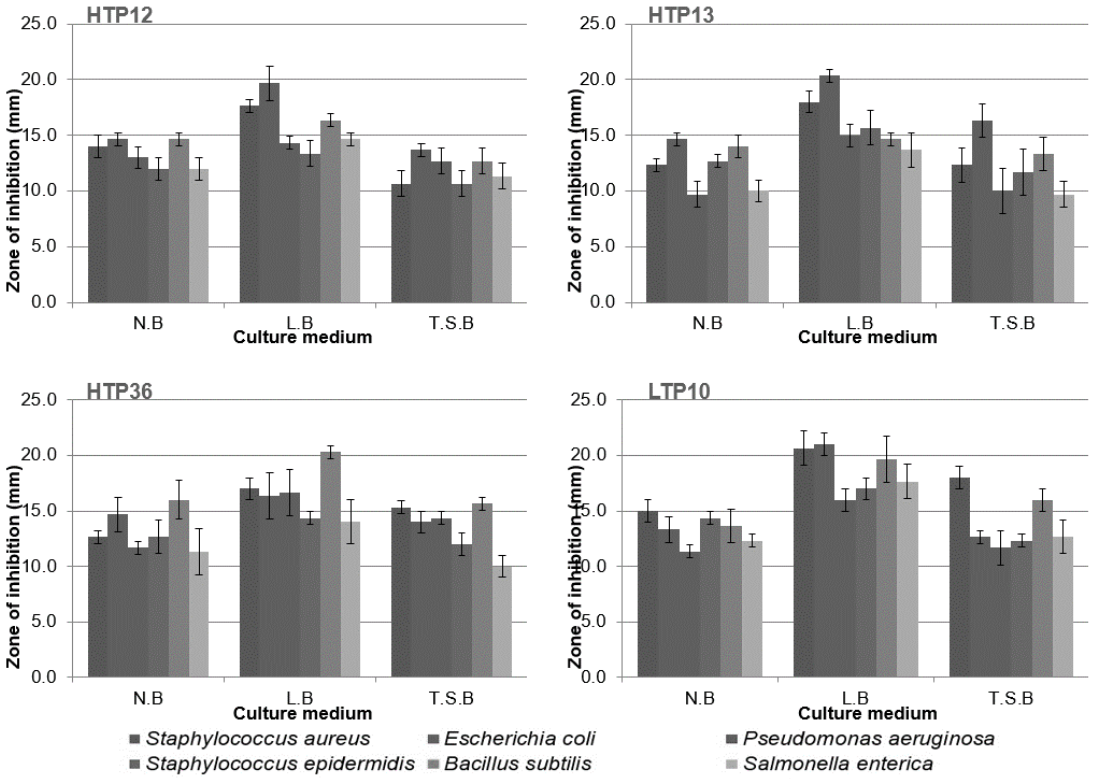

Figure 7. *Zone of inhibition (mm) of isolates against test strains in different culture media. Error bars represent standard deviation (SD) of triplicate measurements of zone of inhibition (mm). $P < 0.05$*

Effect of temperature

The difference in antimicrobial activity of all the four isolates at different temperature was significant statistically ($P < 0.05$). The isolated strains have shown maximum antimicrobial activity at 25°C after 96 hours of incubation in Luria-Bertani broth. The maximum zone of inhibition of 20.3±2.1 mm was shown by *Alcaligenes pakistanensis* (LTP10) against *Staphylococcus aureus* (ATCC 25923) as shown in *Fig. 8*.

Effect of pH

The difference in antimicrobial activity at different pH was significant statistically ($P < 0.05$). All the four isolates have confirmed maximum antimicrobial activity at pH 7. The maximum antimicrobial activity was shown by *Alcaligenes pakistanensis* (LTP10) against *Staphylococcus aureus* (ATCC 25923) with 23.3±0.6 mm zone of inhibition (*Fig. 9*).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2003_20592076

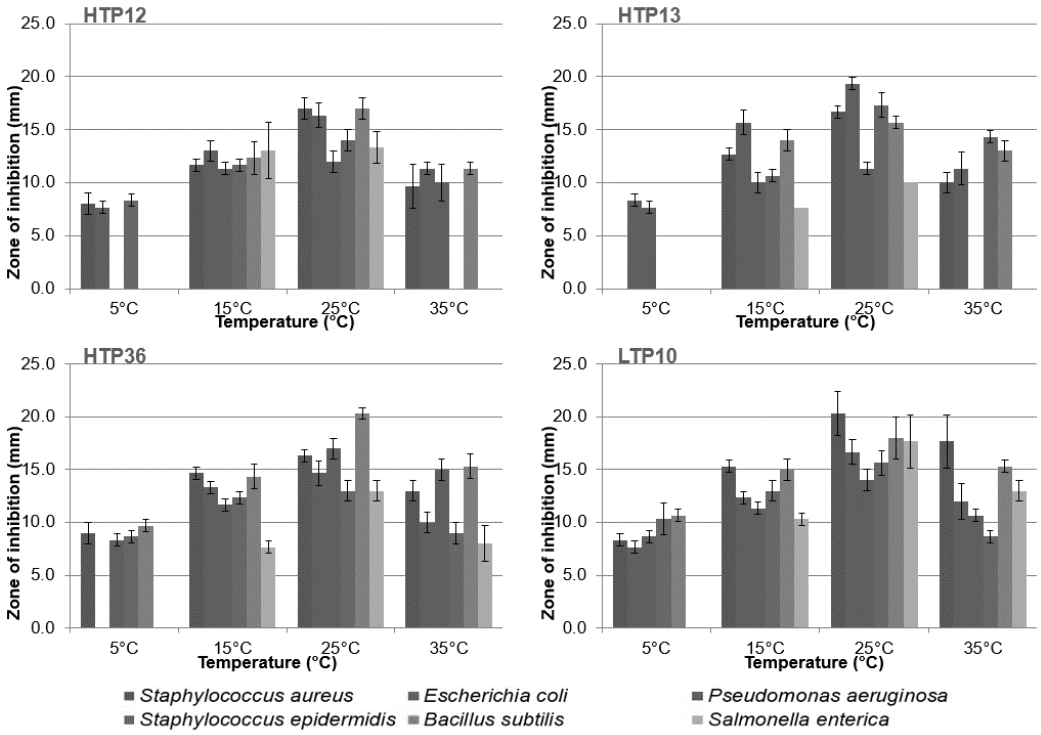

Figure 8. *Zone of inhibition (mm) of isolates against test strains at different temperatures. Error bars represent standard deviation (SD) of triplicate measurements of zone of inhibition (mm). P < 0.05*

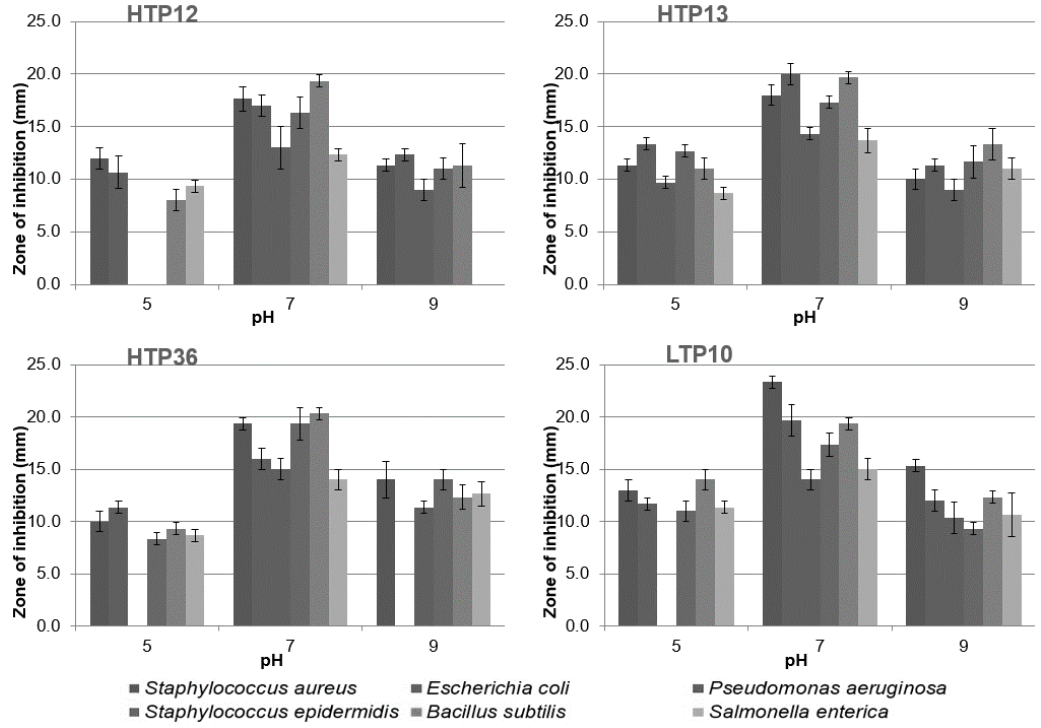

Figure 9. *Zone of inhibition (mm) of isolates against test strains at different pH. Error bars represent standard deviation (SD) of triplicate measurements of zone of inhibition (mm). P < 0.05*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2003_20592076

Effect of carbon sources

The addition of carbon sources to culture media found to have negative effect on the antimicrobial activity of isolated strains. The antibacterial activity in terms of zones of inhibition was decreased by 2 to 8 mm against *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25922) by addition of glucose and starch in 1% and 2% concentrations as given *Table 3*.

Table 3. *Effect of carbon sources on antibiotic activity of isolates*

Isolates	Zone of inhibition (Mean value in mm ± SD) of isolates in against *S. aureus* & *E. Coli*									
	Staphylococcus aureus					*Escherichia coli*				
	*Control	Glucose (1%)	Glucose (2%)	Starch (1%)	Starch (2%)	*Control	Glucose (1%)	Glucose (2%)	Starch (1%)	Starch (2%)
HTP12	15 ± 1.5	13 ± 1.3	10 ± 1.2	14 ± 0.6	13 ± 0.7	18 ± 1.5	13 ± 1.2	15 ± 0.5	15 ± 0.5	14 ± 1.0
HTP13	17 ± 2.0	14 ± 0.7	11 ± 1.5	15 ± 1.7	14 ± 1.8	20 ± 1.3	15 ± 2.0	16 ± 1.4	17 ± 0.3	16 ± 1.2
HTP36	16 ± 1.0	14 ± 0.6	12 ± 0.5	13 ± 1.3	13 ± 2.0	14 ± 0.5	12 ± 0.5	10 ± 1.5	14 ± 0.6	12 ± 0.8
LTP10	21 ± 1.3	16 ± 1.0	15 ± 0.3	15 ± 1.2	16 ± 1.0	21 ± 0.3	17 ± 0.8	16 ± 0.3	18 ± 0.4	13 ± 0.6

*Control = Isolate culture in Luria-Bertani broth

Effect of nitrogen sources

The addition of different nitrogen sources such as amino acids and yeast extract have varying effects on antimicrobial production of isolated strains. Tryptophan, threonine, L-leucine and arginine have decreased the antimicrobial activity of isolates while yeast extract has increased the activity. *Alcaligenes pakistanensis* (LTP10) has shown an increase of 3 mm against *Escherichia coli* (ATCC 25922) with addition of 1% yeast extract. Similarly, antimicrobial activity of *Alcaligenes pakistanensis* (HTP36) against *Staphylococcus aureus* (ATCC 25923) was increased by 2 mm after addition of 1% yeast extract as given in *Table 4*.

Table 4. *Effect of nitrogen sources on antibiotic activity of isolates*

Isolates	Zone of inhibition (Mean value in mm ± SD) of isolates in against *S. aureus* & *E. Coli*					
	Staphylococcus aureus					
	**Control	*Trp	*Thr	*Leu	*Arg	*Y.E.
HTP12	16 ± 0.5	12 ± 1.5	--	11 ± 1.2	13 ± 0.7	16 ± 2.0
HTP13	17 ± 1.2	14 ± 0.5	13 ± 1.5	15 ± 0.6	--	16 ± 1.2
HTP36	18 ± 0.6	13 ± 0.6	14 ± 0.5	12 ± 0.8	10 ± 0.3	20 ± 0.3
LTP10	20 ± 0.3	14 ± 1.2	--	13 ± 1.1	16 ± 1.2	21 ± 0.6
	Escherichia coli					
HTP12	17 ± 1.2	--	15 ± 1.5	14 ± 0.5	10 ± 0.6	15 ± 1.3
HTP13	19 ± 0.5	16 ± 1.0	14 ± 1.5	--	--	21 ± 1.5
HTP36	15 ± 1.7	10 ± 0.5	--	13 ± 1.0	12 ± 0.3	16 ± 1.0
LTP10	19 ± 0.5	--	12 ± 0.5	--	16 ± 0.5	22 ± 1.7

*Trp = Tryptophan, Thr = threonine, Leu = L-leucine, Arg = Arginine, Y.E. = Yeast extract, **Control = Isolate culture in Luria-Bertani broth

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2003_20592076

Effect of salts

Sodium chloride (2% and 3%) and potassium chloride (1% and 2%) were added to culture media separately. High concentration of salts in culture media resulted in lowering or diminishing the antimicrobial activity of isolates as given in *Table 5*.

Table 5. *Effect of salts on antibiotic activity of isolates*

Isolates	Zone of inhibition of isolates in (Mean value in mm ± SD) against *S. aureus* & *E. Coli*					
	Staphylococcus aureus			*Escherichia coli*		
	*Control	NaCl (2%)	NaCl (3%)	Control	KCl (1%)	KCl (2%)
HTP12	17 ± 0.4	13 ± 0.5	10 ± 0.6	15 ± 0.6	10 ± 0.7	--
HTP13	16 ± 0.3	12 ± 0.8	--	16 ± 1.3	12 ± 1.3	9 ± 0.3
HTP36	19 ± 1.3	12 ± 1.0	--	15 ± 0.5	11 ± 0.5	8 ± 0.6
LTP10	21 ± 1.6	15 ± 1.2	10 ± 1.3	20 ± 0.5	14 ± 1.7	--

*Control = Isolate culture in Luria-Bertani broth containing 1 % NaCl (w/v)

Antibacterial and antifungal activity of Alcaligenes pakistanensis against clinical isolates

The best antibiotic producer strain *Alcaligenes pakistanensis* (LTP10) was evaluated for its antimicrobial activity against clinical bacterial and fungal isolates. It was found that the cell free supernatant of culture medium of *Alcaligenes pakistanensis* (LTP10) after 96 hours of incubation at the optimized conditions has shown good activity against clinical isolates of *Staphylococcus aureus, Escherichia coli, Psuedomonas aeruginosa, Salmonella enterica* and *Candida albicans* while no activity was confirmed against *Candida krusei* as shown in *Fig. 10*.

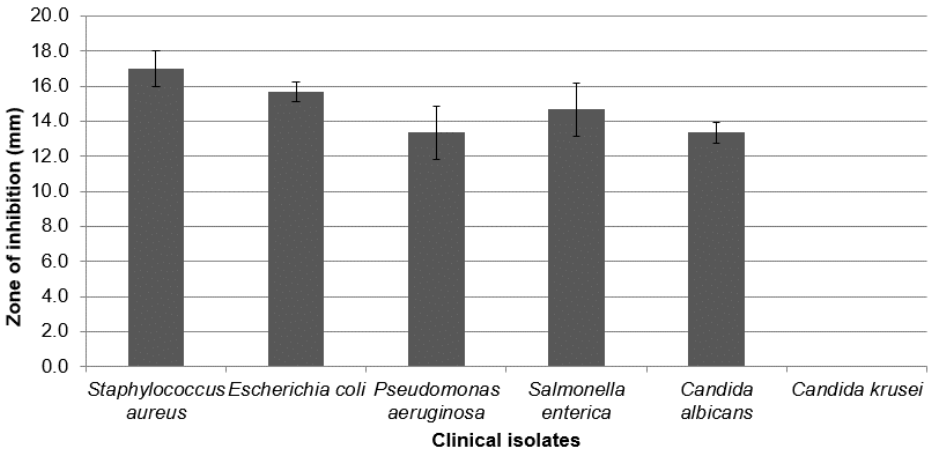

Figure 10. *Zone of inhibition (mm) of CFS of Alcaligenes pakistanensis (LTP10) against clinical isolates. The bars represent standard deviation (SD) of triplicate measurements of zone of inhibition (mm)*

Antibiotic sensitivity test of isolates

Antibiotic discs were used for testing sensitivity of isolated strains. The isolate *Alcaligenes pakistanensis* (LTP10) was resistant to penicillin, monobactams, cephalosporins, quinolones, metronidazole, carbapenem, macrolide, oxazolidinone and

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2003_20592076

glycopeptide while sensitive to ceftaroline, imipenem, cefepime, meropenem, and ticarcillin as given in *Table 6*.

Table 6. *Sensitivity of selected isolates against antibiotic discs*

Isolated strains	Resistant to antibiotics	Sensitive to antibiotics
Alcaligenes faecalis HTP12	Penicillin G Cephalosporin Quinolones Monobactams Carbapenem Ertapenem	Ticarcillin Nalidixic acid Meropenem Piperacillin
Pseudochrobactrum saccharolyticum HTP13	Penicillin Macrolide Cephalosporins Quinolones Oxazolidinone Glycopeptide	Ceftaroline Imipenem Nalidixic acid Ertapenem Piperacillin
Alcaligenes pakistanensis HTP36	Penicillin Monobactams Cephalosporins Quinolones Metronidazole Macrolide Oxazolidinone Glycopeptide	Ceftaroline Imipenem Cefepime Meropenem Ticarcillin Carbapenem
Alcaligenes pakistanensis LTP10	Penicillin Monobactams Cephalosporins Quinolones Metronidazole Carbapenem Macrolide Oxazolidinone Glycopeptide	Ceftaroline Imipenem Cefepime Meropenem Ticarcillin

Discussion

Microbial secondary metabolites are the source of vital bioactive compounds which can be used for the cure of microbial diseases. However, main threat to clinical settings and hospitals is the emergence of multidrug resistant microorganisms (Dantas et al., 2008). That's why researcher and scientists have an increasing curiosity to discover novel antimicrobial compounds from unexplored area which can solve problem of drug resistance up to certain limit (Selvameenal et al., 2009). According to Sanchez et al. (2009) psychrophilic microorganisms are known as a possible source of novel antimicrobial metabolites.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2003_20592076

In the present work, the bacterial strains, having the ability to produce secondary metabolites isolated from Passu glaciers were monitored to produce antimicrobial metabolites by using agar well diffusion assay (Wefky et al., 2009; Abd-Elnaby et al., 2016). The reason for selecting isolates from Passu glacier was to find out antimicrobial metabolites producing microorganisms in that unexplored area. The temperature of that region is less than 0°C, and at such a low temperature, the microorganisms that are present are psychrophiles and psychrotrophs and suffer some harsh conditions such as nutrient deficiency, oxidative stress, high salt concentration, competition and stressful environment, which stimulate the genes responsible for the production of antimicrobial metabolites. Bruntner et al. (2005) reported a novel antibiotic Frigocyclinone produced by a *Streptomyces griseus* strain isolated from Antarctica. These antmicrobial metabolites help the producer organisms to compete and survive in such environment (Oyedele and Ogunbanwo, 2014).

A total of four bacterial isolates were selected among antibiotic producing isolates from Passu glacier. These isolates were coded as HTP12, HTP13, HTP36 and LTP10 and were identified as *Alcaligenes faecalis*, *Pseudochrobactrum saccharolyticum*, *Alcaligenes pakistanensis* and *Alcaligenes pakistanensis* respectively, through phylogenetic analysis. Among these bacterial strains, *Alcaligenes pakistanensis* (LTP10) showed greater activity and maximum zone of inhibition. *Alcaligenes faecalis* (HTP6) was found to be active 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*) (Rafiq et al., 2016). Fandi et al. (2014) reported antimicrobial activities in terms of zones of inhibition (mm) of some thermophiles, isolated from Jordan hot springs, against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* and fungal pathogenic strains.

The isolated strains were incubated at various pH, temperature and incubation periods in different culture media to determine optimum pH, temperature and incubation period for their growth. The selected strains were found to grow at low temperature of 5°C up to maximum of 35°C with maximum growth at 15-25°C in Luria-Bertani broth. These were grouped as psychrotrophs which are cold-loving organisms, having an optimal temperature for growth at above 15°C (Yuan et al., 2017). Margesin et al. (1994) reported that psychrophilic bacteria have shown best growth at around 15°C after 3 days of incubation. The isolates showed maximum growth at neutral pH 7 after 72 hours of incubation period. Heather and Vanderzant (1957) reported that psychrophiles showed maximum growth at environment having neutral pH and temperature around 15°C.

The production of secondary metabolites by microbes is dependent on different physical factors. In the current research work, the effect of different parameters such as pH, temperature, time of incubation and culture media were also considered for production of active metabolites. The maximum antibiotic production by isolates was noted at 25°C and pH 7 after 96 hours of incubation. There was decline in antimicrobials production as temperature increases and pH increases or decreases. Awais et al. (2007) and Gebreel et al. (2008) confirmed that bacitracin was produced by *Bacillus* sp. at temperature range of 25°C to 30°C and pH 7. Oyedele and Ogunbanwo (2014) reported that *Bacillus subtilis* showed maximum antagonistic activity after 96 hrs of incubation.

An important factor in designing of successful laboratory experiments is medium formulation. The components of culture media must contain the elements required for the growth and secondary metabolites production (Abd-Elnaby et al., 2016). The isolated strains were inoculated in LB broth along with extra sources of carbon such as glucose

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2003_20592076

(1% and 2%) and starch (1% and 2%) separately and incubated for 96 hours at 25°C and pH 7. The cell free supernatant from culture broth of *Alcaligenes pakistanensis* (LTP10) with 1-2% glucose and starch has shown a decrease in zone of inhibition by 2-5 mm against *Staphylococcus aureus* and *Escherichia coli*. The effect of nitrogen sources was also studied on the antibiotic production of isolates. Nitrogen sources such as tryptophan, threonine, L-leucine, arginine and yeast extract were added separately to LB broth which was inoculated with isolates and incubated for 96 hours at 25°C and pH 7. The activity of *Alcaligenes pakistanensis* (LTP10) incubated in LB broth with 1 % yeast extract was increased by 3 mm against *Escherichia coli* while that of *Alcaligenes pakistanensis* HTP36 increased by 2 mm against *Staphylococcus aureus*. Rinker and Kelly (2000) reported the effects of different sources of carbon and nitrogen on the growth and production of antimicrobial metabolites by *Thermococcus* sp.

The isolates used in this study have shown resistance to a number of antibiotic classes. *Alcaligenes pakistanensis* (LTP10) was resistant to greater number of antibiotics. To protect themselves from their own antibiotics, antibiotic producing bacteria restrain different complicated mechanisms. The major mechanisms of self-defense in antibiotic producing microorganisms include antibiotic modification, antibiotic target modification, antibiotic efflux, antibiotic target bypass, antibiotic target protection and antibiotic sequestration by special proteins (Peterson and Kaur, 2018). An interesting fact is that the genes responsible for self-resistance are nearly all the time crowded together with the genes for biosynthesis of antibiotics, and their expression is co-regulated (Mak et al., 2014). Thus resistance to a number of antibiotics by *Alcaligenes pakistanensis* (LTP10) ensures its greater potential of antibiotic production.

Conclusion

This research work was designed to search for microorganisms from Passu glacier capable of producing antimicrobial metabolites and optimizing conditions for maximum antimicrobial compound production by isolates. The findings of this study recommends that the antimicrobial compounds produced by the isolates of Passu glaciers should be characterized and identified by analytical techniques like HPLC, LCMS and NMR in order to find their nature. It is also recommended that extreme environments like glaciers, deep seas, oceans, deserts, hot springs, marine environments etc should be explored to find out microorganisms having the ability to produce novel antimicrobial compounds.

REFERENCES

[1] Abd-Elnaby, H., Abo-elala, G., Abdel-raouf, U., Abd-elwahab, A., Hamed, M. (2016): Antibacterial and anticancer activity of marine *Streptomyces parvus*: optimization and application. – Biotechnology & Biotechnological Equipment 30: 180-191.

[2] Artini, M., Papa, R., Vrenna, R., Lauro, C., Ricciardelli, A., Casillo, A., Corsaro, M. M., Tutino, M. L., Parrilli, E., Selan, L. (2019): Cold-adapted bacterial extracts as a source of anti-infective and antimicrobial compounds against *Staphylococcus aureus*. – Future Microbiology 14(16): 1369-1382.

[3] Awais, M., Shah, A. A., Hameed, A., Hasan, F. (2007): Isolation, identification and optimization of bacitracin produced by *Bacillus* sp. – Pakistan Journal of Botany 39(4): 1303.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2003_20592076

[4] Bruntner, C., Binder, T., Pathom-Aree, W. (2005): Frigocyclinone, a novel angucyclinone antibiotic produced by a *Streptomyces griseus* strain from Antarctica. – Journal of Antibiotics 58: 346-349.
[5] Collins, T., Margesin, R. (2019): Psychrophilic lifestyles: mechanisms of adaptation and biotechnological tools. – Applied Microbiology and Biotechnology 103: 2857-2871.
[6] D'Amico, S., Collins, T., Marx, J. C., Feller, G., Gerday, C. (2006): Psychrophilic microorganisms: challenges for life. – EMBO Reports 7: 385-389.
[7] Dantas, G., Sommer, M. O., Oluwasegun, R. D., Church, G. M. (2008): Bacteria subsisting on antibiotics. – Science 320: 100-103.
[8] Fandi, K., Al-Muaikel, N., Al-momani, F. (2014): Antimicrobial activities of some thermophiles isolated from Jordan hot springs. – International Journal of Chemical, Environmental and Biological Sciences 2(1): 57-60.
[9] Furhan, J. (2020): Adaptation, production, and biotechnological potential of cold-adapted proteases from psychrophiles and psychrotrophs: recent overview. – Journal of Genetic Engineering and Biotechnology 18: 36.
[10] Gebreel, H. M., El-Mehalawy, A. A., El-Kholy, I. M., Rifaat, H. M., Humid, A. A. (2008): Antimicrobial activities of certain bacteria isolated from Egyptian soil against pathogenic fungi. – Research Journal of Agriculture and Biological Sciences 4(4): 331-339.
[11] Heather, C., Vanderzant, C. (1957): Effects of temperature and time of incubating and pH of plating medium on enumerating heat-treated psychrophilic bacteria. – Journal of Dairy Science 40: 1079-1086.
[12] Hwanhlem, N., Ivanova, T., Haertlé, T., Jaffrès, E., Dousset, X. (2017): Inhibition of food-spoilage and foodborne pathogenic bacteria by a nisin Z-producing Lactococcus lactis subsp. lactis KT2W2L. – LWT - Food Science and Technology 82: 170-175.
[13] Mahtab, M., Khan, F., Azama, M., Rizvi, M., Sultana, A., Shuklaa, I., Almatroudi, A. (2019): Molecular characterization and phylogenetic analysis of human pathogenic Leptospira species circulating in a tertiary care hospital of Western Uttar Pradesh in India. – Pathogens and Global Health 113(6): 275-281.
[14] Mak, S., Xu, Y., Nodwell, J. R. (2014): The expression of antibiotic resistance genes in antibiotic-producing bacteria. – Molecular Microbiology 93: 391-402.
[15] Margesin, R., Schinner, F. (1994): Properties of cold-adapted microorganisms and their potential role in biotechnology. – Journal of Biotechnology 33: 1-14.
[16] Margesin, R., Feller, G., Gerday, C., Russell, N. J. (2002): Cold-adapted microorganisms: adaptation strategies and biotechnological potential. – Encyclopedia of Environmental Microbiology 2: 871-885.
[17] Morgan-Kiss, R. M., Priscu, J. C., Pocock, T., Gudynaite-Savitch, L., Huner, N. P. (2006): Adaptation and acclimation of photosynthetic microorganisms to permanently cold environments. – Microbiology and Molecular Biology Reviews 70: 222-252.
[18] Núñez-Montero, K., Lamilla, C., Abanto, M., Maruyama, F., Jorquera, M. A., Santos, A., Martinez-Urtaza, J., Barrientos, L. (2019): Antarctic *Streptomyces fildesensis* So13.3 strain as a promising source for antimicrobials discovery. – Scientific Reports 9: 7488.
[19] Oyedele, A. O., Ogunbanwo, T. S. (2014): Antifungal activities of bacillus subtilis isolated from some condiments and soil. – African Journal of Microbiology Research 8: 1841-1849.
[20] Paun, V. I., Lavin, P., Chifiriuc, M. C., Purcarea, C. (2021): First report on antibiotic resistance and antimicrobial activity of bacterial isolates from 13,000-year old cave ice core. – Scientific Reports 11: 514.
[21] Peterson, E., Kaur, P. (2018): Antibiotic Resistance Mechanisms in Bacteria: Relationships Between Resistance Determinants of Antibiotic Producers, Environmental Bacteria, and Clinical Pathogens. – Frontiers in Microbiology 9: 1-21.
[22] Rafiq, M., Hayat, M., Hassan, N., Ibrar, M., Haleem, A., Rehman, M., Ahmad, F., Shah, A. A., Hasan, F. (2016): Characterization of antibacterial compounds produced by psychrotrophic *Alcaligenes faecalis* HTP6 isolated from Passu Glacier, Pakistan. – International Journal of Biosciences 8: 122-35.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2003_20592076

[23] Rampelotto, P. H. (2016): Biotechnology of extremophiles. – Springer, Brazil.
[24] Rinker, K. D., Kelly, R. M. (2000): Effect of carbon and nitrogen sources on growth dynamics and exopolysaccharide production for the hyperthermophilic archaeon *Thermococcus litoralis* and bacterium *Thermotoga maritima*. – Biotechnology and Bioengineering 69: 537-547.
[25] Sanchez, L. A., Gómez, F. F., Delgado, O. D. (2009): Cold-adapted microorganisms as a source of new antimicrobials. – Extremophiles 13: 111-120.
[26] Selvameenal, L., Radhakrishnan, M., Balagurunathan, R. (2009): Antibiotic pigment from desert soil actinomycetes; biological activity, purification and chemical screening. – Indian Journal of Pharmaceutical Sciences 71: 499.
[27] Wefky, S. H., Abou-Elela, G. M., El-Bestawy, E. (2009): Optimization of fermentation conditions for bioactive compounds production by marine bacterium *Enterococcus faecium*. – Journal of Applied Sciences Research 5(10): 1445-1454.
[28] Wright, M. H., Adelskov, J., Greene, A. C. (2017): Bacterial DNA Extraction Using Individual Enzymes and Phenol/Chloroform Separation. – Journal of Microbiology & Biology Education 18(2): 48.
[29] Yarzábal, L. A. (2016): Antarctic Psychrophilic Microorganisms and Biotechnology: History, Current Trends, Applications, and Challenges. – In: Castro-Sowinski, S. (ed.) Microbial Models: From Environmental to Industrial Sustainability. Microorganisms for Sustainability 1. Springer, Singapore.
[30] Yogabaanu, U., Weber, J. F., Convey, P., Rizman-Idid, M., Alias, S. A. (2017): Antimicrobial properties and the influence of temperature on secondary metabolite production in cold environment soil fungi. – Polar Science 14: 60-67.
[31] Yuan, L., Sadiq, F. A., Liu, T., Flint, S., Chen, J., Yang, H., Gu, J., Zhang, G., He, G. (2017): Psychrotrophic bacterial populations in Chinese raw dairy milk. – LWT 84: 409-418.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2003_20592076