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DIVERSITY OF INDUSTRIALLY IMPORTANT HYDROLYTIC ENZYMES EXPLORED IN BACTERIA FROM AQUATIC ENVIRONMENT

***Saima Razzaq*[1], *Rimsha Dilshad*[2], *Rida Batool*[3], *Nazia Jamil*[4]**

[1] ORCID: 0009-0009-3992-1249
[2] ORCID: 0009-0008-2158-6991
[3] ORCID: 0000-0001-6113-1563
[4] ORCID: 0000-0003-4252-7954
Institute of Microbiology and Molecular Genetics
University of the Punjab, Quaid-e-Azam Campus, Lahore, Pakistan

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Abstract

Aquatic bacteria are famous for the production of bioactive metabolites and commercially important extracellular enzymes. Water samples of Arabian Sea (Karachi) and Ravi River (Lahore), Pakistan were analyzed for hydrolytic enzymes producing bacteria. A total of 23 bacterial strains were isolated and their *α*-amylase, DNase, gelatinase, *L*-glutaminase, pectinase, lecithinase and protease production potential was estimated. Following screening, 11 strains (48%) showed amylase activity whereas DNase and gelatinase was produced by 5 strains (22%) and 18 strains (78%), respectively. *L*-glutaminase and pectinase production was shown by 17 isolates (74%) while lecithinase and protease activity was only exhibited by 2 (13%) and 1 strain (4%), respectively. Ribotyping of five selected (S5, S6, H4, H5 and R8) isolates revealed their similarity to *B. tequilensis, B. pumilus, B. flexus*, *B. sonorensis* and *B. subtilis*. These isolates also showed multiple heavy metal and antibiotic resistance. These indigenous *Bacillus* spp. possess great potential to be utilized as commercial strains provided with optimum growth conditions. Extracellular enzymes of these bacterial strains can be used in different industries, agriculture and bioremediation purposes to replace synthetic components.

Introduction

Enzymes are biological catalysts that control the cellular metabolism of all organisms. Different types of enzymes can be obtained from plants, animals, algae, fungi and bacteria. They may be extracellular (exoenzymes) or intracellular (endoenzymes). Extracellular enzymes are secreted

Address: Rida Batool, University of the Punjab, Quaid-e-Azam Campus, Lahore 54590, Pakistan, phone: (+92) 42 35952811, e-mail: rida.mmg@pu.edu.pk

outside the living cell and act on the complex compounds and break them into simpler ones that are then absorbed by cell. Organisms obtain energy as well as play a major role in the cycling of organic matter with the help of extracellular enzymes. While, intracellular enzymes act inside the cell and carry out the essential biochemical reactions in the cell by forming new compounds or through assimilation.

Bacteria are considered as source for the isolation of both intracellular and extracellular enzymes. Commercially important extracellular enzymes produced by bacteria are amylases, gelatinases, proteases, pectinases, glutaminases, lipaseses, DNases, cellulases, coagulases, kinases, hemolysins, pullulanases, xylanases etc. They have various applications in different fields as additives, food and chemical industries and in biomedical research (PANDEY et al. 2000). Industrial processes are carried out under specific conditions which may not be feasible for enzymes' function. So, the enzymes that are capable of working at different conditions as temperature and pH values are of great importance for industries. Aquatic bacteria are source of such enzymes as they are able to survive under high pressure and salt conditions (DALMASO et al. 2015).

Aquatic environment has novel microorganisms which can produce a variety of viable secondary metabolites. Bacteria specifically *Bacillus* spp. have gained particular interest because of their crucial role in matter cycling in aquatic environments (TALLUR et al. 2016). Marine bacteria are more prominent due to production of thermo-stable extracellular enzymes and bioactive compounds while, less is known about freshwater bacteria (DEVI et al. 2008). It is important to explore the potential of aquatic bacteria to produce metabolic products and their particular role in the ecosystem.

Extracellular hydrolytic enzymes have a lot of applications in food and chemical industry and in biomedical sciences (SÁNCHEZ-PORRO et al. 2003). Aquatic extracellular enzymes may be same as their terrestrial counterparts or have completely distinctive properties (DEBASHISH et al. 2005).

Bacillus are ubiquitous in aquatic and terrestrial environments and consist of phylogenetically and phenotypically diverse species (BAL et al. 2009). Mostly extracellular hydrolytic enzymes producing bacterial species belong to the genus *Bacillus*. There are many reports on marine and freshwater bacterial diversity from different coastal areas of the world (NAWAZ and AHMED 2011). Mostly *Bacillus* systematic studies have focused on the terrestrial species, although marine *Bacilli* also have great potential for antibiotics, cyclic acylpeptides, and glucanases (OGUNTOYINBO 2007).

Materials and Methods

Sampling

Marine sample was collected from Arabian Sea, Karachi (24°51′36″N and 67°00′36″E) in June, 2017 while, fresh water was from Ravi River, Lahore (31°15′–31°45′ N and 74°01′–74°39′ E) in August, 2017 in sterilized screw capped bottles. Characteristics i.e., color, odor, temperature and pH of samples were recorded.

Isolation and purification of bacterial strains

To isolate bacterial strains, samples were serially diluted and spread on Nutrient-agar plates and incubated overnight at 37ºC. Total 23 morphologically different bacterial isolates were selected and further purified by quadrant streaking on Nutrient-agar (CAPPUCCINO and SHERMAN 2008).

Screening for extracellular hydrolytic enzyme production

Selected bacterial strains were screened for different extracellular hydrolytic enzymes as amylase, cellulase, DNase, gelatinase, *L*-glutaminse, lecithinase, pectinase, protease and tannase. Amylase activity was checked on starch agar and zones of clearance were observed by starch hydrolysis (SHANMUGASUNDARAM et al. 2015). Cellulase activity was determined on cellulose agar containing Congo-red as indicator (PATAGUNDI et al. 2014). Extracellular DNase activity was observed on DNase agar supplemented with methyl green as pH indicator (SMITH et al. 1969). Extracellular lecithinase production was determined on egg-yolk agar (SHARAFetal. 2014). Gelatinase production was checked in deep gelatin tubes supplemented with 12% gelatin (GERHARDT et al. 1994). *L*-glutaminase production was screened on Zobell's agar media containing *L*-glutamine as enzyme substrate and phenol red as pH indicator (SINHA and NIGAM 2016). The YEP medium supplemented with 2% agar was used to screen pectinase production and visualized zones of clearance through iodine flooding (SOARES et al. 1999). Protease activity was determined on milk Agar (ALNAHDI 2012). The tannase activity was tested on nutrient agar plates containing 2% tannic acid (KUMAR et al. 2010).

Morphological and biochemical characterization

Five bacterial isolates (S5, S6, H4, H5, R8) selected on the basis of maximum production of extracellular enzymes, were characterized morphologically and biochemically (CAPPUCCINO and SHERMAN 2008, HOLT et al. 1989).

Minimum inhibitory concentration (MIC)

MIC of different heavy metals (chromium-Cr, cobalt-Co, lead-Pb, nickel-Ni and zinc-Zn) and antibiotics (ampicillin-AMP, chloramphenicol-CHL, penicillin-PEN, tetracycline-TET and streptomycin-STP) was determined on Nutrient-agar media by progressively increasing the concentration of selected heavy metals and antibiotics by 50 µg/ml each time, until the bacterial strains were unable to give visible growth (SAMBROOK and RUSSELL 2001).

Growth kinetics

For growth kinetics, bacterial isolates were inoculated in Nutrient-broth (pH-7) and incubated at 37°C in shaking incubator (150 rpm) for 3 days. Samples were drawn aseptically and optical density (600 nm) was measured after every 3-hour interval (ZWIETERING et al. 1990).

Effect of different environmental conditions on bacterial growth

Impact of different temperatures (28°C, 37°C and 50°C) and pH (5, 7 and 9) on the growth of extracellular enzymes producing bacterial strains was determined. Effect of different carbon (fructose, glucose, galactose, maltose and sucrose) and nitrogen (ammonium chloride, beef extract, glycine, KNO_3 and peptone) sources on bacterial growth was also checked. For this purpose, bacterial cultures were inoculated in the Mineral salt medium (SHIRANE and HATTA 1987) and incubated at optimized conditions. Samples were drawn out aseptically after every three hours to measure optical density at 600 nm.

Exopolysaccharide (EPS) production

To determine EPS production, 100 ml Nutrient-broth was separately inoculated with respective bacterial strain and incubation was done at 37°C up to 48–72 hours at 150 rpm. Then, cultures were centrifuged and EPS production was determined by cold ethanol precipitation method (SHAHNAVAZ et al. 2015).

Slime production assay

To check slime production ability of *Bacillus* strains, they were streaked on Congo-red agar media and incubated at 37°C for 24 hours. Appearance of black colonies showed slime production (AN and FRIEDMAN, 1997).

Qualitative assay for biofilm formation

Biofilm formation was determined by staining method. Inoculum was given in Nutrient-broth and incubated for 24-hours at 37°C. Media in the test tubes was removed out and stained them with 0.01% crystal violet and let it stand for 20 minutes. Extra stain was washed and dried the test tubes. Biofilm formation was indicated by appearance of purple ring at bottom and walls of tubes (HASSAN et al. 2011).

16S rRNA gene sequencing of selected *Bacillus* strains

Five selected pure bacterial strains were sent to 1st Base Company and identified by 16S rRNA sequencing using Sanger dideoxy method. Thus, unassembled Chromas files were obtained and sequences were manually refined by cross checking them with raw data and assembled by Cap3. Then, refined sequences were analyzed at the BLASTn site (http://www.ncbi.nlm.nih.gov/BLAST, accessed on May 2018) and identified on the basis of maximum similarity index. The assembled sequences were submitted to NCBI GenBank to get accession numbers.

Results

Sample characteristics

Marine water was of grey color, odorless and temperature of sampling site was 28°C. Whereas, fresh water sample was of brown color, had muddy smell and temperature of sampling site was 25°C. The pH of both samples was 7.

Isolation and purification

Total 23 bacterial strains were isolated from marine (S1, S2, S3, S4, S5, S6, S7, S8, W1, W2, W3) and fresh water (H1, H2, H4, H5, H6, H7, H8, R1, R3, R4, R7, R8) samples.

Screening for extracellular hydrolytic enzyme production

Isolated bacterial strains showed positive results for extracellular production of various hydrolytic enzymes such as amylase, DNase, gelatinase, *L*-glutaminase, lecithinase, pectinase and protease as shown in Table 1 and Figure 1. Five bacterial strains (S5, S6, H4, H5, R8) were selected due to most diverse extracellular enzyme activities detected by mentioned enzyme assays.

Table 1

Extracellular enzyme producing activities of isolated bacterial strains

Sr. no	Bacterial strains	Amylase	Cellulose	DNase	Gelatinase	*L*-glutaminase	Lecithinase	Pectinase	Protease	Tannase
1	S1	+	–	–	+	+	–	–	–	–
2	S2	+	–	+	+	+	–	–	–	–
3	S3	–	–	+	+	+	–	–	–	–
4	S4	+	–	+	+	+	–	–	–	–
5	S5	+	–	–	+	+	–	+	–	–
6	S6	+	–	+	+	+	+	+	–	–
7	S7	–	–	–	+	–	–	+	–	–
8	S8	–	–	–	+	–	–	+	–	–
9	W1	–	–	–	–	+	–	+	–	–
10	W2	–	–	–	+	+	–	+	–	–
11	W3	–	–	–	–	+	–	+	+	–
12	H1	–	–	–	+	–	–	+	–	–
13	H2	–	–	+	+	–	+	+	–	–
14	H4	+	–	–	+	+	–	+	–	–
15	H5	+	–	–	+	+	–	+	–	–
16	H6	+	–	–	–	–	–	–	–	–
17	H7	–	–	–	–	+	–	+	–	–

cont. Table 1

18	H8	–	–	–	–	–	–	+	–	–
19	R1	+	–	–	+	+	–	+	–	–
20	R3	–	–	–	+	+	–	+	–	–
21	R4	–	–	–	+	+	–	–	–	–
22	R7	+	–	–	+	+	–	+	–	–
23	R8	+	–	+	+	+	–	+	–	–

+ = positive; – = negative

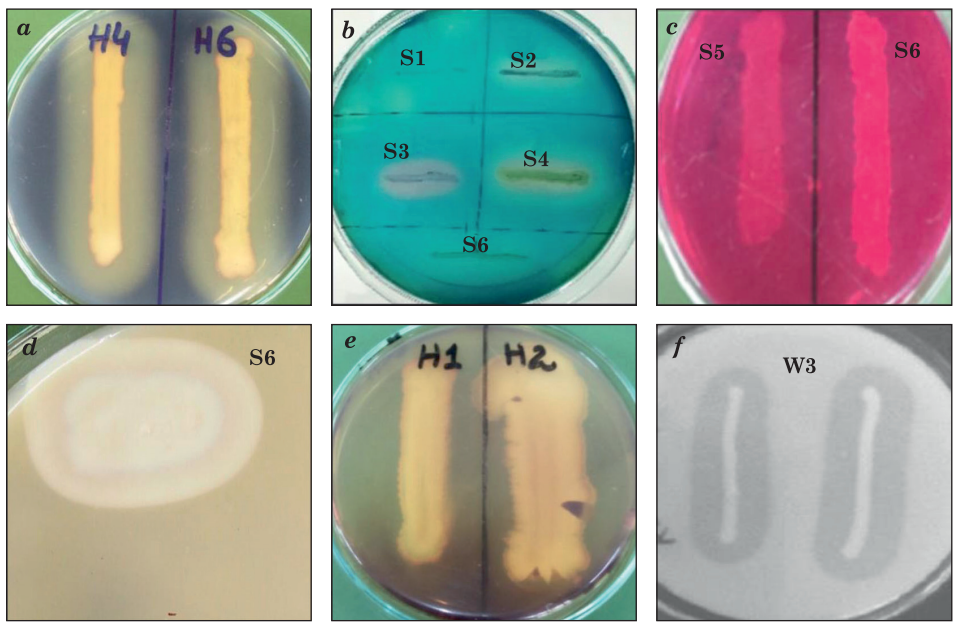

Fig. 1. Positive results of extracellular hydrolytic enzymes production by bacterial strains *a* – amylase activity; *b* – DNAse activity; *c* – *L*-glutaminase activity; *d* – lecithinase activity; *e* – pectinase activity; *f* – protease activity

Morphological and biochemical characterization

Selected bacterial strains were characterized morphologically and biochemically as shown in Table 2.

Table 2

Cultural, morphological and biochemical characteristics of hydrolytic enzyme producing bacterial strains

Characteristics	S5	S6	H4	H5	R8
	cultural and morphological characteristics				
Colony morphology	moderate, white, circular, entire, flat, transparent	small, yellow, circular, entire, convex, opaque	small, yellow, circular, entire, flat, opaque	large, white, irregular, undulate, convex, opaque	large, white, circular, entire, convex, translucent
Vegetative cells	rod	rod	rod	rod	rod
Motility	motile	motile	motile	motile	motile
Gram reaction	+	+	+	+	+
Endospores	+	+	+	+	+
Biochemical characteristics					
Catalase test	++	++	+	+++	+
Oxidase test	++	–	+	++	+++
Mannitol salt agar test	–	+++	+	+++	++
Oxidative/ fermentation glucose test	aerobe	aerobe	aerobe	facultative aerobe	facultative aerobe
Nitrate reduction test	+++	+++	–	++	+
Citrate utilization test	+	–	+	+	+
Voges-Proskauer (VP) test	+++	+	–	++	+++
Methyl red test	–	–	–	+	+
Pigment test	-	-	-	-	-
Novobiocin sensitivity test	+	+	+++	+	++

+ = positive; *++/ +++ = slightly positive/ strongly positive*; *– = negative*

Minimum inhibitory concentration (MIC)

Bacterial strains exhibited multiple heavy metals and antibiotics resistance. They were most resistant to Pb (200 µg/µl MIC) and less resistant to Cr, Ni, Co and Zn (100 µg/MIC) – Table 3. While, in case of tested antibiotics, maximum resistance was shown to TET (200 µg/ml MIC) and for STP, Amp, CHL, and PEN, MIC value was 100 µg/ml (Table 4).

Table 3

Minimum inhibitory concentration MIC for heavy metals shown by the hydrolytic enzyme producing bacterial strains

Bacterial strains	Heavy metals used [µg/ml]									
	chromium (Cr)		nickel (Ni)		zinc (Zn)		cobalt (Co)		lead (Pb)	
	200	100	200	100	200	100	200	100	200	100
S5	–	+	–	+	–	+	–	–	+	+
S6	–	+	–	+	–	+	–	–	+	+
H4	–	+	–	+	–	+	–	–	+	+
H5	–	+	–	+	–	+	–	–	+	+
R8	–	+	–	+	–	+	–	–	+	+

Table 4

Minimum inhibitory concentrations (MIC) for antibiotics shown by the hydrolytic enzyme producing bacterial strains

Bacterial strains	Antibiotics used [µg/ml]									
	penicillin (PEN)		chloramphenicol (CHL)		ampicillin (AMP)		streptomycin (STP)		tetracycline (TET)	
	200	100	200	100	200	100	200	100	200	100
S5	–	+	–	+	–	+	–	–	+	+
S6	–	+	–	+	–	+	–	–	+	+
H4	–	+	–	+	–	+	–	–	+	+
H5	–	+	–	+	–	+	–	–	+	+
R8	–	+	–	+	–	+	–	–	+	+

Growth kinetics

Growth kinetics showed that these aquatic bacteria grow very well under standard growth conditions as temperature 37°C and pH 7. The growth curves demonstrated longer stationary phases showing their stability that was directly related to enzyme production (Figure 2).

Effect of different environmental conditions on bacterial growth

Maximum growth was indicated at 37°C, pH 7, glucose as carbon source and peptone as nitrogen source as shown in Figures 2–6.

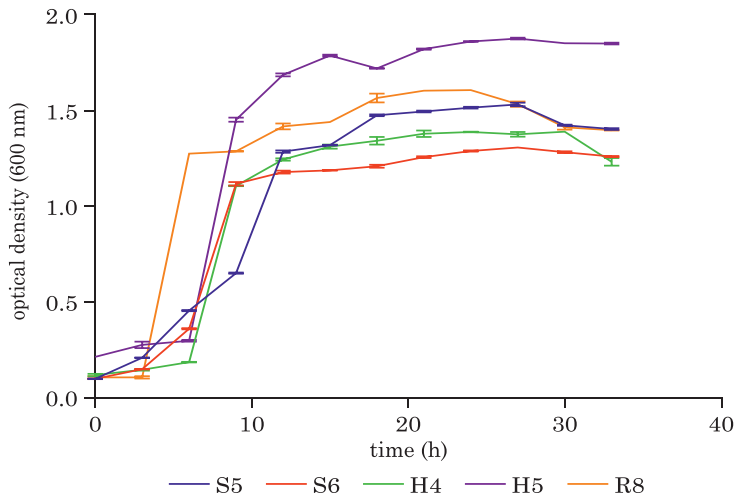

Fig. 2. Growth kinetics of bacterial strains (S5, S6, H4, H5, R8), showed longer stationary phases and stability directly related to enzyme production

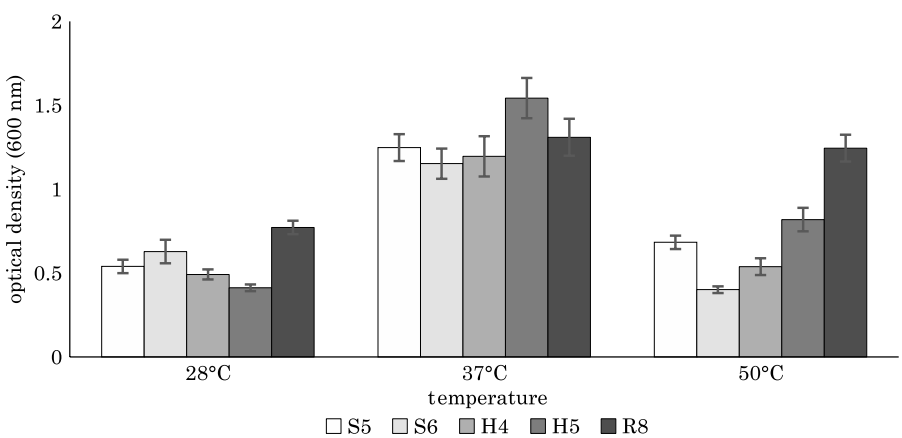

Fig. 3. Effect of variable temperatures on bacterial growth and showed best growth at temperature 37°C

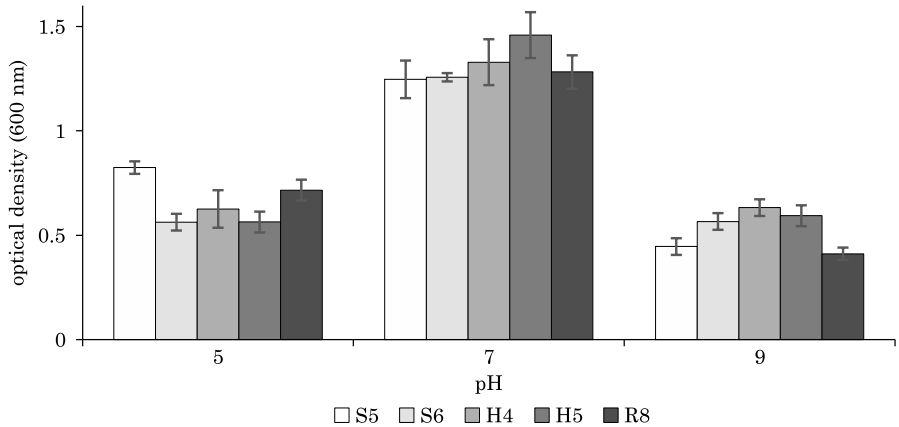

Fig. 4. Effect of variable pH on growth of bacterial strains showed pH 7 as optimum growth pH

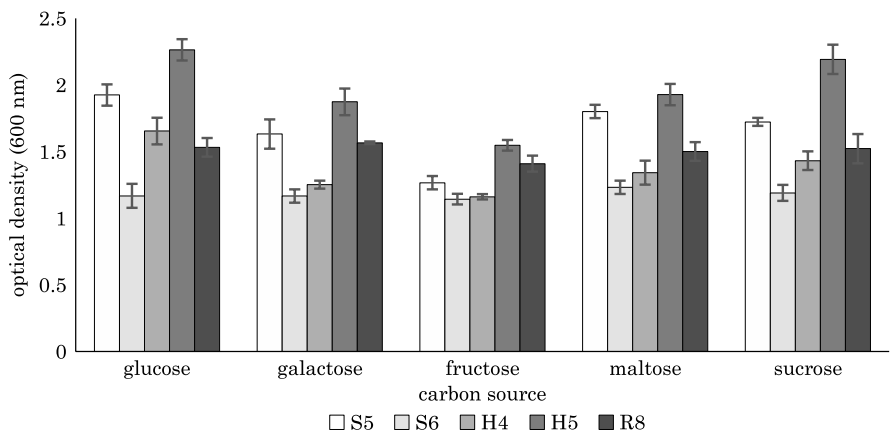

Fig. 5. Effect of different carbon sources on bacterial growth

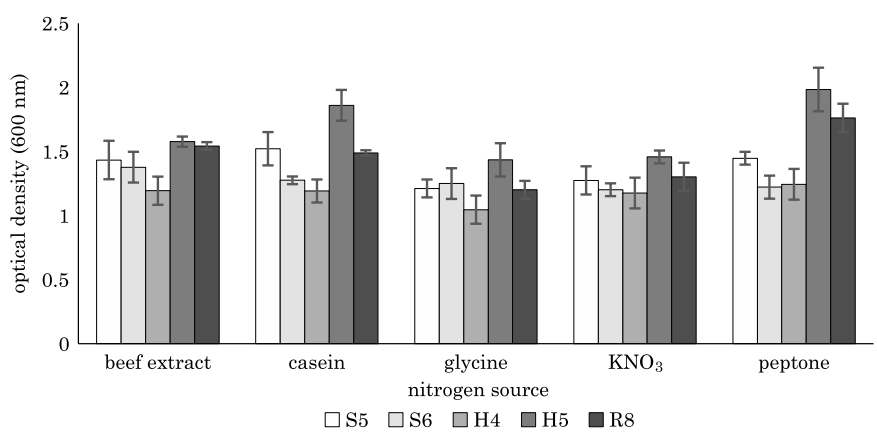

Fig. 6. Effect of different nitrogen sources on bacterial growth

EPS, slime production and biofilm formation

EPS production was demonstrated by S5 (0.08 mg/ml), S6 (0.14 mg/ml), and R8 (0.08 mg/ml). Bacterial strain S5 and S6 also showed slime production potential indicated by appearance of black colored colonies on Congo-red agar (Figure 7*a*). Biofilm formation was also estimated by purple ring formation (Figure 7*b*).

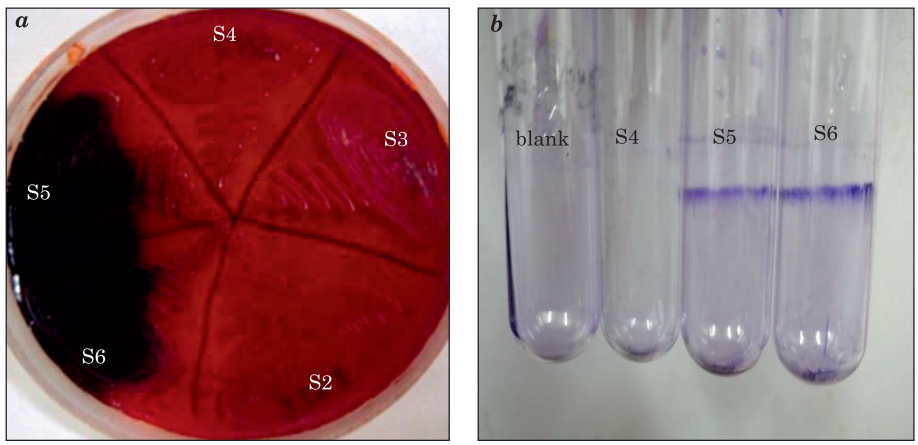

Fig. 7. Black coloration of Congo-red agar revealed slime production by S5 and S6 (*a*) and purple coloration of the walls and at bottom of the test tubes showed biofilm formation by S5 and S6 (*b*)

16S rRNA gene Sequencing

16S rRNA gene sequencing revealed similarity of S5 to *Bacillus tequilensis*, S6 to *B. pumilus*, H4 to *B. flexus*, H5 to *B. sonorensisa* and R8 to *B. subtilis*. These sequences were submitted to NCBI Genbank under the accession numbers MH371775, MH371776, MH371777, MH371778 and MH371779, respectively (Table 1.1, Appendix).

Discussion

Aquatic microbes produce highly thermo-stable extracellular enzymes making themselves different from their terrestrial counterparts with different adaptive characteristics (RAMESH and MATHIVANAN 2009). The main purpose of the current study was isolation and characterization of extracellular hydrolytic enzyme producing bacteria from aquatic environment as bacteria are reported to produce a variety of extracellular enzymes that are manipulated at industrial and commercial scale (KUMAR et al. 2013).

Isolated strains were found to produce amylase, DNAse, gelatinase, *L*-glutaminase, Lecithinase and Pectinase enzymes. Production of these enzymes has also been reported previously by many researchers. Marine and fresh water bacteria have been reported for extracellular amylase production (BAL et al. 2009, SURIBABU et al. 2015). In a past study, more than 72.4% marine isolates showed extracellular DNase activity (DANG et al. 2009). Findings of the present study are supported by several past workers as they reported that gelatinase is produced more frequently by fresh water bacteria than the isolates of soil and plant microenvironments (ALVES et al. 2014). Gelatinase production from marine *Bacillus* spp. was also described (BALAN et al. 2012). Marine and fresh water bacteria have been described for extracellular *L*-glutaminase production (KATIKALA et al. 2009, SALLIS and BURNS 1989). Lecithinase activity was determined in marine bacteria (GAUTHIER 1976). Lecithinase activity has also been reported in several *Bacillus* spp. (MASSOL-DEYA et al. 1995). Isolated *Bacillus* strains showed high potential for the production of extracellular pectinases. In a previous study, 28 bacterial strains from fresh water samples and 16 bacterial strains from sea water were isolated with pectinase activity that strengthen the outcomes of current research (ROHBAN et al. 2009, BAL et al. 2009).

Only one bacterial strain exhibited extracellular protease production whereas no strain showed tannase and cellulase activity. In accordance with this study, another group of researchers has reported that no tannase production potential was observed in the isolates of Arabian Sea whereas, cellulase production was observed in those strains (TALLUR et al. 2016).

These extracellular enzyme producing bacteria showed considerable resistance towards different heavy metals and antibiotics. These results have also been recorded by TALLUR et al. (2016) who reported the isolation of antibiotic resistant bacterial strains from the Arabian Sea simultaneously having extracellular enzymatic activity (TALLUR et al. 2016). Past studies described, that heavy metal resistant bacteria also have antibiotic resistance due to plasmids or transposons (DEVIKA et al. 2013). Various clinical and environmental bacterial cultures also revealed that antibiotic and heavy metal resistance are often closely related (JAFAR et al. 2013).

Enzymes work under optimum pH and temperature values and changes in them may denature their function. Our aquatic bacteria depicted that neutral conditions were best for their growth. Various past workers describe best optimized temperatures for aquatic bacteria in between 37°C to 50°C, and optimum pH was neutral and alkaline. In contrast to this study, researchers have reported optimum temperature between 45°C to 55°C and optimum pH 6.0–7.0 for extracellular hydrolytic

enzymes producing *Bacillus* spp. (SOARES et al. 1999). Previous studies also determined glucose as best carbon source for growth of extracellular enzyme producing bacteria (KRISHNAKUMAR et al. 2011).

Many bacteria are able to excrete extracellular polymeric substances (EPS) (VU et al. 2009). EPS production of microorganisms have fascinated researchers due to their versatile applications and advantages (SHAHNAVAZ et al. 2015). Marine and fresh water have extreme conditions due to contamination from different environmental sources and bacteria produce EPS to protect themselves. EPS production in marine and fresh water bacteria was determined by many workers and they also identified *Bacillus* as good EPS producers (KUMAR et al. 2011).

Exopolysaccharides are involved in bacterial attachment to the substrate and thus, in biofilm formation (COSTERTON et al. 1987). Therefore, EPS producing bacterial strains were further screened for biofilm formation and marine bacteria showed biofilm formation (HASSAN et al. 2011).

Conclusion

Five *Bacillus* marine and fresh water strains with multiple extracellular enzyme activities were identified that can be utilized for therapeutic, industrial, agricultural and bioremediation applications. This study signifies an emerging sight for prevalence of *Bacillus* in Arabian Sea, Karachi and Ravi River, Lahore. There are very limited number of reports about the extracellular enzyme producing *Bacillus* spp. from these specified areas of Pakistan up to our knowledge.

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Appendix

Table 1.1

16S rRNA gene sequencing of hydrolytic enzyme producing bacterial strains

Bacterial strains	Identity	Accession number
S5	*Bacillus tequilensis*	MH371775
S6	*Bacillus pumilus*	MH371776
H4	*Bacillus flexus*	MH371777
H5	*Bacillus sonorensis*	MH371778
R8	*Bacillus subtilis*	MH371779