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Full Length Research Paper

Screening of antibiotic sensitivity, antibacterial and enzymatic activities of microbes isolated from ex-tin mining lake

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Water sample from ex-tin mining lake was collected and cultured on nutrient agar (NA). Morphology characteristics revealed that isolates WL1 and WL4 were Gram-negative cocci and WL3 was Gram-positive cocci. Meanwhile, WL2 and WL5 were Gram-positive bacilli and spore formers. These isolates were screened for their antibiotic sensitivity against 17 types of antibiotics using Kirby-Bauer method. Antibacterial activity against 11 types of indicator bacteria were also tested using agar well diffusion method. The antibiotic susceptibility test demonstrated that all five isolates had high degree of inhibition to five types antibiotics namely amikacin, kanamicin, gentamicin, norfloxacin and tetracycline. Gram-negative cocci WL4 is a multiple resistant isolate that resistant to 10 types of antibiotics. The Gram-positive bacilli WL2 had antibacterial activity against Gram-negative *ESCHERICHIA COLI*. Enzymatic assay showed that all isolates were proteolytic, lipolytic and phosphatase enzyme producers. Amylolytic activity was absent in all isolates. Protease, leucine arylamidase, alkaline and acid phosphatases, phospholipase esterase and esterase lipase were found in all isolates. The preliminary survey demonstrated that the water environment in the vicinity of Campus Lake is a reservoir of microbial populations. The present work concerns a screening of a few selected isolates with some significance importance in the application of medical and industry fields. In conclusion, the multi resistance isolates were not suitable for industry application due to some facts that it might produce toxic compounds and cause infection. The antimicrobial substance and enzymes produced by the potential isolates could be further screened for clinical application and mass production in industry. However, they must be evaluated for their safety and toxicity to human, animals and the environment.

Key words: Nutrient agar (NA), Kirby-Bauer, antibacterial assay, antibiotic susceptibility test, enzymatic assay.

INTRODUCTION

According to Madsen (2008), the lake is a site of tremendous microbial activity and the microorganisms play an important role on nutritional chains as well as maintaining the biological balance of earth life. Mining lake is rich in mineral and suitable for the growth of microorganism thus creating of new lentic habitat for variety of microorganisms. However, mining lake is still poorly explored as there is lack research done on the mining lakes (Shamshuddin et al., 1986).

The new emerging pathogen with increasing antibiotic resistance, along with the susceptibility of immunocompromised to common diseases has become an alarming problem worldwide. Many diseases have become difficult to treat due to the introduction of a new class antibiotic that is efficacious and safe which leads to widespread use and thus development of resistance (Christopher, 2003). A variety of antibiotic resistance strains were discovered by the work done by Alvero (1987) and Lobova et al. (2002). Therefore, the antibiotic sensitivity

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Abbreviations: NA, Nutrient agar; **P10,** penicillin; **AMP10,** ampicillin; **VA30,** vancomycin; **B10,** bacitracin; **E15,** erythromycin; **C30,** chloramphenicol; **F300,** nitrofurantoin; **AK30,** amikacin; **K30,** kanamycin; **N10,** neomycin; **S10,** streptomycin; **CN10,** gentamycin; **TE30,** tetracycline; **NA30,** nalidixic acid; **NOR100,** norfloxacin, **RL100,** sulphanethoxazole; **W5,** trimethoprim.

test was carried out on the isolates of UTAR Lake to test the efficacy of certain drugs against microbes using the Kirby-Bauer method.

To date, increasing in bacterial resistance to traditionally used antimicrobials, an appropriate antimicrobial agent is hardly to select. In the current scenario, microorganisms are still a dominant source for antimicrobial compounds. Thus, various continuous research studies were carried out to detect antimicrobial agent producing strains and discovered the new one such as the bacteriocin that kill a range of Gram-positive and Gram-negative bacteria (Fariha et al., 2009).

Mudryk and Podgorskar (2006) had drawn the attention to the ability of bacteria from lake to synthesise various extracellular enzymes. Proteases of microbial origin possess considerable industrial potential due to their biochemical diversity and wide applications in food industries, medicinal formulations, detergents and waste treatment (Saurabh, 2007). At present, the largest part of the market is occupied by the alkaline proteases and the major proportions of these are derived from *Bacillus* species. Abundant lipolytic bacteria also found in the lake where they produce the enzyme phospholipase. Phospholipase plays key roles in bread making, egg yolk industry and refinement of vegetable oils degumming. Their use in industrial processes depends on their ability to clone and express the genes in microbial hosts with commercially attractive amounts (Maria et al., 2007). Dastager et al. (2009) noted that α-amylase is important in many industrial processes and constitute 25% of the enzyme market. Amylases act by hydrolysing the starch molecules into polymers composed of glucose units (Sindhu et al., 1997). The API ZYM kit has been used by many researchers to elucidate the extracellular enzymatic activity of bacterial strains. Mudryk and Podgorskar (2006) had shown that neustonic and planktonic bacteria carried out the enzymatic activity and degradation of organic macromolecules in an Estuarine Lake.

The objectives of this study are to explore the microorganism from ex-tin mining Lake of Kampar and screened for their antibiotic sensitivity, antibacterial activities and enzymatic activities such as phospholipase, protease, α-amylase enzyme assays in conjunction with the importance and purposes to screen microbial activities involving their contribution to various fields of application in industry.

MATERIALS AND METHODS

Source of sample

Water sample was collected with a sterile spatula from the ex-tin mining UTAR Lake, Kampar and kept in a sterile universal bottle.

Isolation

The water sample was serially diluted with phosphate buffer solution (PBS) composition, 137 mM NaCl; 2.7 mM KCl; 4.3 mM Na_2HPO_4; 1.47 mM KH_2PO_4, adjust to a final pH of 7.4 to obtain $1:10^6$. After this, the diluted sample was spread evenly on NA surface. The plates were then incubated overnight at 30°C. Next, five different isolates were selected and streaked on prepared NA plates. The five isolates were obtained and designated as isolate WL1, 2, 3, 4 and 5.

Morphological and biochemical examination of the respective isolates

The morphological characteristic of isolates were observed and recorded. The cell shape and arrangements of isolates were determined following the standard procedures of basic stain, Gram stain and endospore stain (James and Natalie, 2008). All isolates were screened for the presence of protease, α-amylase and phospholipase on skim milk agar plates, egg yolk agar plates and starch agar plates as described by James and Natalie (2008). *Escherichia coli* and *Bacillus subtilis* serve as control strain in this study. The API ZYM assay was also carried out accordingly to the manufacture procedure (BioMeriux SA). The API stripes were incubated for 4 h and results were determined by the level of color intensity.

Determination of antibiotic susceptibility (Kirby-Bauer Method)

The isolates were inoculated in nutrient broth (NB) at 30°C. A sterile cotton wool swab dipped into the bacterial suspension was spread evenly on the surface of the NA plates. The inoculated plates were allowed to dry before placing the diffusion discs containing antibiotics. Susceptibility of the isolates to 17 types of antibiotics was performed using the standard Kirby-Bauer method as described by Robert et al. (2009). Commercially available discs (Oxoid, UK) containing penicillin (P10), ampicillin (AMP10), vancomycin (VA30), bacitracin (B10), erythromycin (E15), chloramphenicol (C30), nitrofurantion (F300), amikacin (AK30), kanamycin (K30), neomycin (N10), streptomycin (S10), gentamycin (CN10), tetracycline (TE30), nalidixic acid (NA30), norfloxacin (NOR100), sulphanethoxazole (RL100) and trimethoprim (W5) were placed on the surface of the agar plates and incubated at 30°C for 24 h. The diameters of inhibition zones formed surrounding each isolate were measured inclusive diameter of the discs. Results were expressed as susceptible (≥ 21 mm); intermediate (16 to 20 mm) or resistant (≤15 mm) followed a standard range (Liasi et al., 2009). *E. coli* and *B. subtilis* serve as control strain for these assays. All isolates were tested duplicate for each type of antibiotic.

Screening for antibacterial agent

All isolates were screened for antibacterial activity against 11 types of indicator bacterial species using the agar well diffusion method as described by Ahmed et al. (2008). The indicator bacteria included seven Gram-positive bacteria (*Staphylococcus aureus*, *B. subtilis*, *Bacillus cereus*, *Staphylococcus epidermidis*, *Micrococcus luteus*, *Enterococcus faecalis* and *Bacillus sphaericus*) and four Gram-negative bacteria (*E. coli*, *Pseudomonas aeruginosa*, *Proteus vulgaris* and *Salmonella*). The plates were incubated at 30°C for 24 h. Results were expressed as moderate (6 to 9 mm); strong (10 to 14 mm) or very strong (15 to 18 mm) according to Liasi et al. (2009).

RESULTS AND DISCUSSION

In the present study, there were more than five isolates

Table 1. Morphological characteristic of five isolates from water in UTAR Lake.

Isolate	WL1	WL2	WL3	WL4	WL5
Form of colony	Circular	Circular	Circular	Circular	Circular
Translucency & opacity	Opaque	Opaque	Opaque	Translucent	Opaque
Elevation of colony	Convex	Flat	Flat	Convex	Convex
Margin of colony	Entire	Entire	Entire	Entire	Entire
Surface of colony	Smooth	Smooth	Smooth	Smooth	Smooth
Pigmentation	White	Milky White	Orange	Colourless	Yellow
Cell morphology	Bacilli	Bacilli	Cocci	Cocci	Bacilli
Gram stain reaction	-	+	+	-	+
Spore stain	No	Yes	No	No	Yes

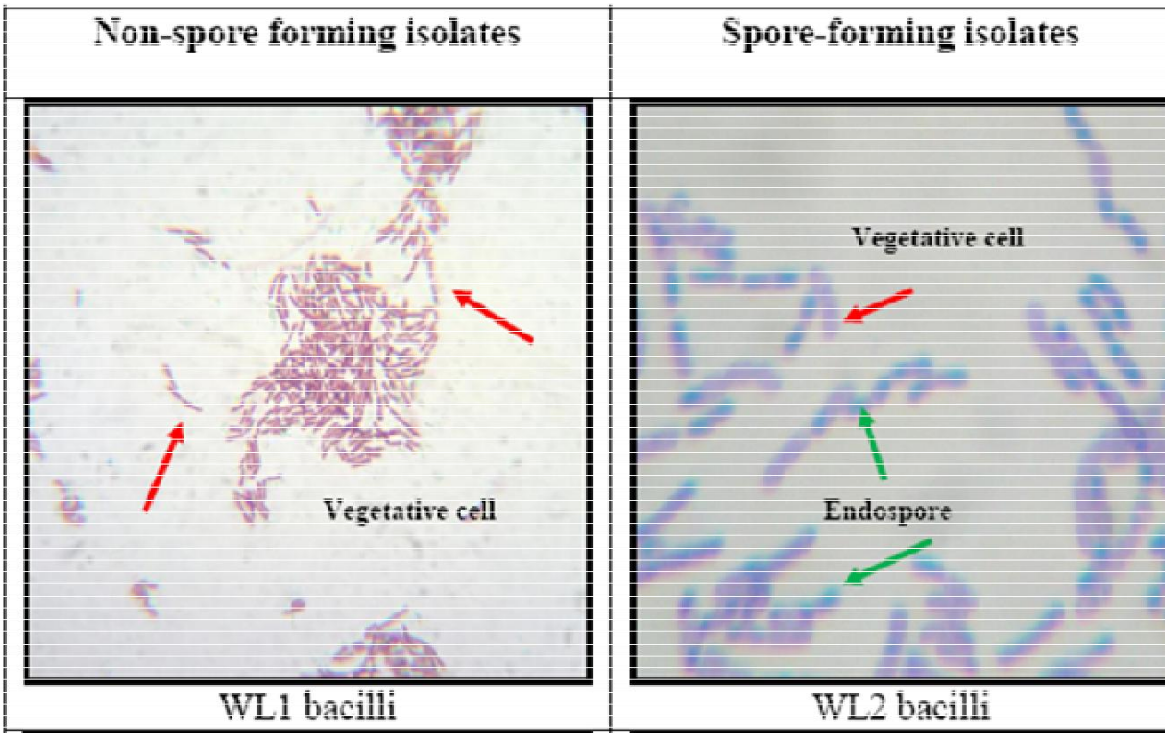

Figure 1. Gram stain and endospore stain of isolates WL1 and WL2.

but these five isolates chosen were of our interest due to the multi resistance features and the ability to excrete extracellular enzymes.

All five isolates were circular, entire margin and smooth surface as tabulated in Table 1. From the microscopic observation, isolate WL1 was Gram-negative bacilli (Figure 1), WL4 was Gram-negative cocci, WL3 was Gram-positive cocci and isolates WL2 and WL5 were Gram-positive bacilli respectively. Isolate WL2 (Figure 1) and WL5 were spore-formers.

The degree of susceptibility of the isolates towards 17 different types of antibiotics was shown in Table 2. Isolates WL1 and 2 were susceptible to all the antibiotics tested. Isolate WL3 was sensitive to 15 types of anti-biotics meanwhile isolate WL5 was sensitive to 16 types of antibiotics. Isolate WL4 only susceptible to seven types of antibiotics. Overall, all isolates were strongly susceptible to five types of antibiotics which include amikacin (AK30), kanamycin (K30), gentamycin (CN10), norfloxacin (NOR10) and tetracycline (TE30). There are 20% of the isolates were resistant to penicillin G (P10), ampicillin (AMP10), streptomycin (S10), chloramphenicol (C30), nitrofurantoin (F300), sulphanethoxazole (RL100) and trimethoprim (W5). *E. coli* and *B. subtilis* serve as control in this study.

Previous study by Mudryk (2002) discovered the

Table 2. Assessment of selected isolates to 17 different types of aantibiotics.

	Diameter of inhibition zone (mm)						
Isolate	**WL 1**	**WL 2**	**WL 3**	**WL 4**	**WL 5**	***E. coli* Control**	***B. subtilis* Control**
Penicillin G (P10)	(35) +	(29) +	(43) +	_	(33) +	(8) +++	_
Ampicillin (AMP10)	(31) +	(30) +	(43) +	_	(40) +	(15) +++	_
Vancomycin (VA30)	(20) ++	(21) +	(21) +	_	(21+	(15) +++	(21) +
Bacitracin (B10)	(22) +	(21) +	(20) ++	_	(21) +	(11) +++	_
Erythromycin (E15)	(28) +	(29) +	(13) +++	(14) +++	(21) +	(15) +++	(10) +++
Chloramphenicol (C30)	(31) +	(30) +	(31) +	_	(30) +	(36) +	(27) +
Nitrofurantoin (F300)	(21) +	(21) +	(21) +	_	(21) +	(22) ++	(17) ++
Amikacin (AK30)	(30) +	(28) +	(27) +	(24) +	(23) +	(27) +	(21) +
Kanamycin (K30)	(25) +	(30)+	(29)+	(26) +	(36) +	(23) +	(21) +
Neomycin (N10)	(21) +	(17) ++	(15) +++	(17) ++	(21) +	(21) +	(15) +++
Streptomycin (S10)	(27) +	(23) +	(21) +	(21) +	_	(26) +	(21) +
Gentamicin (CN10)	(26) +	(24) +	(25)+	(22)+	(28) +	(23) +	(21)+
Tetracycline (TE30)	(25) +	(31)+	(33) +	(21) +	(36) +	(36) +	(25)+
Nalidixic acid (NA30)	(22) +	(28) +	(27) +	(11) +++	(17) ++	(31) +	(23) +
Norfloxacin (NOR10)	(23) +	(21) +	(28) +	(30) +	(21) +	(31) +	(22) +
Sulphamethoxazole (RL100)	(32) +	(36) +	(40) +	(10) +++	(29) +	(21) +	(26) +
Trimethoprim (W5)	(36) +	(32) +	(42) +	_	(30) +	(21) +	(28)+

Degree of susceptibility: + = susceptible/sensitive (≥ 21 mm) ++ = intermediate (16 to 20 mm) +++ = resistant (≤ 15 mm).

presence of multiple antibiotic resistance bacteria inhabiting Lake Gardno. The present study noted that all the isolates except WL4 were susceptible to most of the antibiotics tested. This study found that Gram-negative cocci WL4 had multi-resistance properties which might resulted from their ability to detoxify the antibiotic substances by altering the transport of compound through the bacterial cell membrane or produce specific enzymes that can modify the antibiotics. According to Herwig et al. (1997), these resistance strains might have resistance gene coded in the R plasmid or coded in the transposon. The R plasmid can transfer genetic material among different species in the conjugation and transformation processes. Thus, the isolate WL4 might have the R plasmid that carry four basic mechanisms of resistance which involve inactivation, the creation of substitute metabolic pathways, impermeability of cytoplasmic membranes and alteration in the target site. Further experiments were required to support this hypothesis.

The isolates were tested for their ability to produce inhibitory substances against 11 indicator species (Table 3). Isolate WL2 showed intermediate inhibition against Gram-negative *E. coli* by producing antibacterial agent that form a moderate zone of inhibition with diameter of 6 mm. Isolates WL1, 3, 4 and 5 did not show any sensitivity to all the indicator species.

Some pathogenic strains of *E. coli* can cause enterohemorrhagic gastroenteritis which are serious and required the antibiotic treatment (Gangle, 2005). Thus, the present study had been done in conjunction with the rise of resistance strains and it was found that Gram-positive bacilli WL2 able to produce certain agent that inhibit *E. coli*. This showed that WL2 might be the potential source of antibacterial agent used to treat disease caused by *E. coli*. Similar result was obtained by Kiran et al. (2009) in the study of antimicrobial and cytotoxic activities of Actinomyces in Lonar Lake in which they produce potent metabolite compound against *E. coli*. The antibacterial substance might effectively inhibit Gram-negative *E. coli* through different modes of action such as inhibiting synthesis of specific membrane proteins or stress proteins, arrest of DNA synthesis, breakage of single-strand DNA, interaction with DNA and production of hydrogen peroxide (Park et al., 1998). Fariha et al. (2009) noted that the genus *Bacillus* can produce low molecular weight peptides via the non-ribosomal biosynthetic pathway which involves specific enzymes called peptide synthetases. These peptide antibiotics including bacitracin, polymyxin, gramicidin, tyrocidine, subtilin and bacilysin had a varied range of remarkable biological activities including antimicrobial, antiviral and antitumour activities (Cane et al., 1998). The antibacterial substance produced might belong to the peptide antibiotic which can be extracted and further studied for bacterial diseases management, pharmaceutical exploits and contributed to the research in microbiology, biotechnology and general medicine.

All isolates possess protease activity that can hydrolyse casein (Figure 2 and Table 4). Isolates WL1, 2, 3 and 4 produced phopholipase with the capability to hydrolyse phospholipid that present in the egg yolk agar. They also produce white opaque zone of precipitation on the agar.

Table 3. Inhibitory spectrum of antibacterial-producing isolates on Gram-positive and Gram-negative bacteria.

Indicator species	WL 1	WL 2	WL 3	WL 4	WL 5
Gram-positive					
B. subtilis	ND	ND	ND	ND	ND
B. cereus	ND	ND	ND	ND	ND
B. sphericus	ND	ND	ND	ND	ND
M. luteus	ND	ND	ND	ND	ND
S. aureus	ND	ND	ND	ND	ND
S. epidermidis	ND	ND	ND	ND	ND
E. faecalis	ND	ND	ND	ND	ND
Gram-negative					
E. coli	ND	(6) +	ND	ND	ND
Salmonella	ND	ND	ND	ND	ND
P. aeruginosa	ND	ND	ND	ND	ND
P. vulgaris	ND	ND	ND	ND	ND

Degree of inhibition: + = moderate inhibition zone (6 to 9 mm) ++ = strong inhibition zone (10 to 14mm) +++ = very strong inhibition zone (15 to 18 mm) ND = Non-detectable.

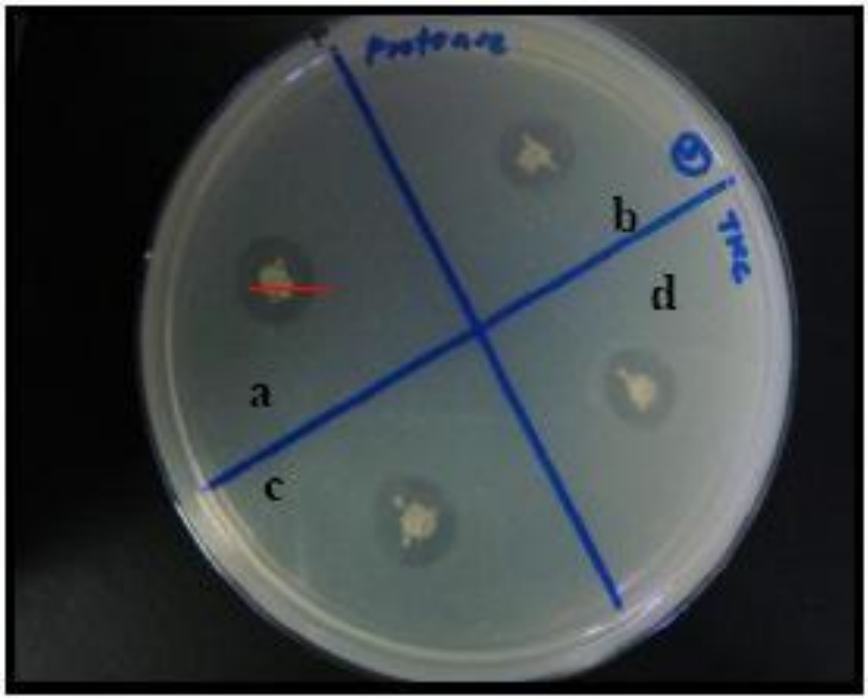

Figure 2. Protease enzyme on skim milk agar plates. Positive results shown by isolate WL1, WL2, WL3, WL4 and WL5 with clear zones formation.

None of the isolates produce extracellular amylase. Extracellular enzyme profile was shown by API-ZYM assay. It was found that all isolates produce alkaline phosphatase (Bph), esterase (C4), esterase lipase (C8), leucine arylamidase (Leu) and acid phosphatase (Aph) as shown in Table 5.

The enzymatic activity had suggested that these five isolates from UTAR Lake were capable of decomposing organic compounds under tropical climatic condition. This outcome was similar to the result reported by (Mudryk and Podgorskar, 2006). All isolates produced protease and 80% of the isolates were phospholipase producers. Proteolytic bacteria constituted a high percentage among the group of microflora (Austin et al., 1977). Vargas et al.

Table 4. Assessment of enzymatic assay.

Isolate	Enzymes		
	Protease	**Phospholipase**	**α-amylase**
WL1	(8) ++	++ (8)	ND
WL2	(8.25) +++	++ (5.75)	ND
WL3	(8.50) +++	++ (4.50)	ND
WL4	(6.50) ++	+++ (10.50)	ND
WL5	(8) ++	ND	ND
E. coli Control	ND	ND	ND
B. subtilis Control	(5) ++	+++ (15)	+++ (10)

Degree of enzymatic activity: + = low (≤ 2 mm) ++ = moderate (>2-8 mm), +++ = high (> 8 mm), ND = Non-detectable.

Table 5. Assessment of API ZYM assay.

Enzyme	WL1	WL2	WL3	WL4	WL5
Control	(0) -	(0) -	(0) -	(0) -	(0) -
Alkaline phosphatase	(1) +	(4) +	(1) +	(1) +	(2) +
Esterase (C 4)	(4) +	(3) +	(2) +	(3) +	(3) +
Esterase lipase (C 8)	(4) +	(3) +	(2) +	(2) +	(2) +
Lipase (C 14)	(1) +	(2) +	(1) +	(1) +	(0) -
Leucine arylamidase	(3) +	(2) +	(1) +	(4) +	(2) +
Valine arylamidase	(1) +	(0) -	(1) +	(0) -	(1) +
Cystine arylamidase	(0) +	(1) +	(1) +	(1) +	(0) -
Trypsin	(0) -	(0) -	(1) +	(1) +	(1) +
α-chymotrypsin	(0) -	(1) +	(1) +	(1) +	(2) +
Acid phosphatase	(1) +	(3) +	(1) +	(2) +	(5) +
Naphthol-AS-BI-phosphohydrolase	(1) +	(2) +	(2) +	(2) +	(4) +
α –galactosidase	(1) +	(2) +	(1) +	(0) -	(2) +
ß-galactosidase	(1) +	(2) +	(1) +	(0) -	(3) +
ß-glucuronidase	(1) +	(2) +	(1) +	(0) -	(0) -
α –glucosidase	(2) +	(0) -	(2) +	(1) +	(2) +
ß-glucosidase	(1) +	(2) +	(0) -	(0) -	(5) +
N-acetyl-ß-glucosaminidase	(1) +	(0) -	(1) +	(1) +	(4) +
α –mannosidase	(1) +	(0) -	(1) +	(1) +	(0) -
α –fucosidase	(0) -	(0) -	(2) +	(0) -	(0) -

Number indicates colour intensity which is proportional to concentration of respective enzyme presence. + = Enzymatic activity was detected, - = Enzymatic activity was non-detected (Mudryk and Podgorskar, 2006).

(2004) had successfully isolated 150 lipolytic enzyme producers from Kenyan alkaline soda lake. Gram-positive WL2 and 5 might belong to *Bacillus* that was mostly extracellular protease producers (Ellaiah et al., 2002). Studies of Martinez et al. (1996) have drawn the attention to the role of lipolytic bacteria in the processes of modification and transformation of lipid compounds in water bodies. Lipids were actively assimilated by bacteria such as the phytoplankton and zooplankton in respiratory processes or in biosynthesis of cellular structures (Albers et al., 1996).

API ZYM assay showed that higher activity of alkaline phosphatase which was comparable to the study conducted by Mudryk and Skorczewski (1998). High level of phosphatase activity was found in UTAR Lake. It mainly appeared as alkaline and acid phosphatases which are able to hydrolyse phosphoric esters (Chrost and Overbeck, 1987). These isolates from lake might have an efficient system of transporting phosphorus into their cell which contributes to its active enzyme activities (Mudryk and Skorczewski, 1998). Furthermore, four isolates out of five in present study were esterase and lipase producers which capable of attacking emulsified mono-, di- and triglycerides to yield the glycerol and fatty

acid residues (Gajewski et al., 1997). According to Jones and Lock (1989), leucine arylamidase is a peptide bond hydrolyzing enzyme and its enzymatic level is a good measure of the proteolytic activity of bacteria and thus the high level of leucine arylamidase synthesized by the isolates from UTAR Lake corresponds to the high level of proteolytic activity of the respective isolates. These were related to the protease enzymatic study which showed that all isolates were good protease producers.

In this study, we have successfully isolated five multiple enzyme producers from UTAR lake. All isolates were proteolytic enzyme producers with high level of protease and leucine enzyme activities. Four isolates were lipolytic enzyme producers. All isolates were susceptible to amikacin, kanamycin, gentamycin, norfloxacin and tetracycline. The Gram-negative cocci (isolate WL4) might be a multiple resistance strain and might be infectious. Gram-negative bacilli WL1 and Gram-positive bacilli WL2 were to be the most suitable for industry application as they do not show any resistance to various types of antibiotics. Moreover, WL2 has antibacterial activity against *E. coli* which made them a potential source for screening of novel antibacterial agent. This pilot study will be followed up by the 16S rRNA gene analysis for phylogenetic analysis. The nucleotide sequence data from 16S ribosomal RNA genes will be used to identify the species and to infer phylogeny for the respective isolates.

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