INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
19–0183/2019/22–5–855–865
DOI: 10.17957/IJAB/15.1141
http://www.fspublishers.org

Full Length Article

Characterization of Recombinant Cold Active Lipase from a Novel *Pseudomonas* spp. MG687270

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Abstract

Bacteria produced maximum thermolabile alkaline lipase at 25°C after 96 h incubation in alkaline conditions. We tested the effect of different salts on its activity and found that $CaCl_2$ stimulated activity by 3766 U, whereas, the $ZnSO_4$ and $FeCl_3$ strongly inhibited the activity. A novel organic solvent stable alkaliphilic lipase enzyme (GenBank: QBA82211) encoding from YLip gene (GenBank ID MH338242), was amplified from psychrotrophic *Pseudomonas* spp. The cloned YLip, expressed in BL21 (λDE3), gave a soluble protein of molecular mass 32-kDa after induction with 1 m*M* IPTG followed by standard purification procedures. It maintained thermal stability from 4 to 60°C and half of the catalytic activity was inhibited subsequently after 60 min of incubation at 60°C. Enhanced lipolytic activity of the lipase segregated from *Pseudomonas* spp. was observed in the presence of organic solvents of dimethyl sulfoxide (DMSO) and ethanol. Present results suggest that YLip as thermolabile lipase had comparatively maximum thermal stability and remarkable resistance toward organic solvents. Overall, this cold active alkaliphilic lipase is a potentially desirable candidate for biotechnological application, because of its stability at low temperature across a range of organic solvents.

Keywords: Psychrotrophic *Pseudomonas* spp.; Organic solvent alkaliphilic lipase; 16s rRNA; Gene cloning; Biochemical characterization; Gilgit-Baltistan

Introduction

Microorganisms existing in extremely cold temperature are considered as psychrophiles having a potential to propagate at a temperature below 20°C, however, other groups are existing like facultative psychrotolerants that having optimum growth temperature in the mesophilic range in spite could grow by close to zero temperature (Joseph *et al.*, 2008). Earlier research suggests of receiving more psychrotolerants microorganisms from cold habitations similar to Antarctica than actual obligate psychrophiles (Vaz *et al*., 2011; Hatha *et al*., 2013; Antony *et al*., 2016). The probabilities of finding psychrophiles and psychrotrophic microorganisms are more noticeable at extreme conditions of the polar environment and could similarly be found in elevated mountains, ocean, alpine soils and glaciers (Maharana and Singh, 2018). Lipases are triacylglycerol hydrolases (E.C.3.1.1.3) performing different chemical reactions such as transesterification, esterification, diverse organic synthesis underneath water-restricted conditions and stereospecific chemical breakdown of active racemic esters (Soni and Madamwar, 2001; Ferrer *et al*., 2005).

Lipases are group of enzymes used in biotechnological techniques and perform chemical transformations of the carboxyl ester bonds in triacylglycerols in the existence of aqueous conditions to generate diacylglycerols, monoacylglycerols, glycerol, and fatty acids and the synthesis of esters compounds in organic solvents (Niaz et al., 2014; Kaur *et al.*, 2016). Lipase active site has a hydrolytic triad comprising of histidine, serine and aspartate residues (Jaeger *et al*., 1999). Lipases secreted from psychrotrophic bacteria have gained significant consideration in biotechnological functions because of their high lipolytic activity at low temperature (Joseph *et al.*, 2008). To operate in low- temperature conditions, cold active lipases enzyme increases in their structure's flexibility comparative to other lipases function at the higher temperature, in a selected area or complete structure of the protein. These differentiations comprise salt bridges and weakened hydrogen bonds within a protein molecule, that

To cite this paper: Ali, Y., B. Ahmad, K.M. Alghamdi, T. Kamal, H.S.H.M. Ali, Y. Anwar, A. Hussain and N.U. Jogezai, 2019. Characterization of recombinant cold active lipase from a novel *Pseudomonas* spp. MG687270. *Intl. J. Agric. Biol*., 22: 855–865

reduces loop extension of amino acid and also the hydrophobic interactions inside protein structure (Joseph *et al.,* 2008). Though, these characteristics have similarly caused cold active bacterial lipases susceptible to a greater extent as compared to their counterpart's lipase enzyme at the warmer temperature that causes denaturation of protein structure by organic solvents, heat, and extreme pH. Cold-adaptive alkaliphilic lipases covering a broad spectrum of biotechnological functions such as applications in detergents formulation, bioremediations, food industries additives, biotransformation and perform important applications in molecular biology and the expression of the heterologous genes in psychrophilic microorganisms to avoid the formation of inclusion elementary bodies (Joseph *et al*., 2008).

Cold adaptive alkaliphilic lipases usage has a huge potential in bacteriological contamination in different industrialized processes and also in terms of lower energy costs (Alquati *et al.,* 2002). While the identification of organic solvent stable bacterial lipase, which was derived from *Pseudomonas aeruginosa* LST-03 (Ogino *et al.,* 1994), numerous bacterial lipases resistant of diverse organic solvent were cloned, expressed and biochemically characterized, the greater number originating in *Bacillus* spp. and *Pseudomonas* spp. (Gupta and Khare, 2009). *Pseudomonas* has been observed as an extraordinary producer of cold active alkaliphilic lipase (Zeng *et al*., 2004; Maharana and Ray, 2014, 2015). Different solvent stable alkaliphilic lipases derived from psychrotrophic bacterium have significant potential as catalysts that increase the reaction rate at low temperatures for the synthesis of the organic compounds of chiral intermediates (Joseph *et al.,* 2008). Only limited research on organic solvent stable alkaliphilic lipases from psychrotrophic bacterium have been reported (Zheng *et al.,* 2011).

The *Pseudomonas* spp. (MG687270) was isolated from surface debris of soil on ice of the mighty Rakaposhi glacier, in Karakorum Range of mountains near Pakistan China border. Strain PAK3 exhibited to be a psychrotroph capable to grow at 10^{o}C but not at 37^{o}C, with an observable optimum temperature range of 10–30^{o}C (Hong *et al.,* 2012). To construct a clone, we used the nucleotide sequence of *P. mandelii* JR-1(Kim *et al.,* 2013) that is closely related to psychrotrophic *Pseudomonas* spp., from amongst completed bacteriological genomes. The Ylip gene has been amplified by utilizing primers based on the non-coding nucleobases region that surrounding the *P. mandelii* JR-1 lipase encoding gene. YLip exhibited high genome sequence resemblance to the solvent stable lipase Lips from *P. mandelii* JQ071496 (Kim *et al.,* 2013) (96% genome sequence identity) and rPFL from *P. fluorescens* (Zhang *et al*., 2009) (80% sequence identity). We discovered that YLip is cold active alkaliphilic lipase that demonstrates stable against organic solvents and has relatively high thermal stability.

Other than isolation of a novel producer strain, the current research was focused on cloning and expression of alkaline hydrolytic lipase YLip gene (MH338242 GenBank ID) from PAK3. To the best of our knowledge, this is the first report of recombinant alkaliphilic lipase obtained from a novel psychrotroph isolated from non-polar glacier.

Materials and Methods

Sampling of Psychrotrophic Bacteria

In order to isolate psychrotolerant bacteria, samples of water and soil were collected from three diverse localities of non-polar glacier: Juglot (latitude 35°41′06″, longitude 74°37′26″), Jutial (latitude 35°54'276″, longitude 74°19'841″) and Rakaposhi glacier (latitude 36°14'368″, longitude 74°26'576″) Gilgit Pakistan, Northern areas of Pakistan. Microbiological prospects like sterility of instruments, personnel and handling of samples were performed according to standard microbiological techniques (Sagar *et al.,* 2013). A total of 20 samples were collected from 10 different sites. Samples of water and soil were collected carefully with intense care and transferred to respective portable ice boxes. The materials included a manual drill, sterile gloves, and sample bags, pH strips, thermometer, GPS, ice cabins, ethanol, methylated spirit, organized petri plates with nutrient agar medium. Geographic coordinates, height, and atmospheric pressure were recorded using a GPS. Dissolved oxygen (DO) was measured by using Portable Dissolved Oxygen Meter. The water samples were obtained in sterile bottles by opening their lids inside the water. The soil samples were collected in sterile bags. All samples were conveyed to the laboratory in intact physical conditions. The soil and water samples were preserved at 4°C.

Isolation and Screening of Cold Active Alkaliphilic Lipase Producing Bacteria

Soil and water samples were subjected to isolation of psychrotrophic bacteria. Glacier soil samples used for bacterial isolation has been completed by formulating dilutions of 2 g of soil to 8 mL of distilled water. For this stock serially, dilution technique has been exploited. Around 100 μL of every dilution and water samples were spread on nutrient agar medium (peptone 0.5%, NaCl 0.5%, yeast extract 0.5%, agar 2% and 9 pH of Tris-HCl buffer). Aerobically the duplicate spread dishes were incubated for 48 h at 10 and 20°C in alkaline conditions at pH 9.

Primary screening has been implemented by spot inoculation of all the alkaliphilic lipase secreting bacterial isolates on TBA medium plates comprising of (peptone, 5 g/l; tributyrin, 10 mL/l; beef extract, 3 g/l, and agar-agar, 20 g/l) exploiting sterilized toothpick and incubated at 20°C. This screening has been performed with the culture filtrates of the selected isolates employing well diffusion technique. Entirely the selected psychrotrophic bacterial isolates were

cultivated in the nutrient broth after that 1 mL of all tested inoculums with 0.6 OD has been inoculated to 200 mL of growth medium and incubated at 180 rpm in a rotatory shaker at 25°C. The bacterial cultures were harvested subsequently after 48 h of incubation and centrifuged at 15000 rpm for 20 min at 4°C and the supernatant has been gathered. Wells in TBA dishes were prepared using sterile cork borer.

The filtrate of bacterial culture of all the isolate in each well has been placed at 200 μL and incubated at 20°C for 48 hrs. Subsequently after the incubation phase, isolates efficient of producing cold active alkaliphilic lipase were investigated on the bases of the zone of clearance: colony size ratio on plates. So, the potential colonies were selected and streaked for culturing pure psychrotrophic bacterial colonies. The psychrotrophic isolates producing zones of clearance were selected and exposed to secondary quantitative screening. Quantitative screening of the selected psychrotrophic bacterial strains was experimented in submerged fermentation for 48 h at 20°C in alkaline conditions at pH 9. After incubation bacterial strain that has the potential to catalyze the maximum substrate of triacylglycerol was selected and stored at -20°C for further analysis. Further processing was done at the chemical engineering department of Texas A&M University, U.S.A.

Amplification and Sequencing of 16S rRNA Genome

Selected bacterial strains were identified on the basis of 16S rRNA sequencing. Genomic DNA of the selected bacteria was extracted from the pure fresh culture of 24 h and PCR amplification of closely the whole length 16S ribosomal RNA fragment of 1500bps has experimented. Isolation of genomic Bacteria DNA extraction was done by Quick-DNA™ Fungal/Bacterial Miniprep Kit. Primers sequence utilized for amplification were 5′-AGAGTTTGATCATGGCTCAGA- 3′ and 5′-GTTACCTTGTTACGACTT-3′ respectively from 8 to 28 and 1493 to 1510, correspondingly, that are segments of 16S ribosomal RNA gene of *Escherichia coli,* and which are also helpful for amplifying 16S rRNA genome from different types of microorganisms.

PCR reaction procedure was employed in a Thermal cycler. Every 50 μL of PCR reaction mixture comprised of: purified genomic DNA of 20 ng, Taq DNA polymerase for elongation at a concentration of 2.5 Units, 10 X concentrated 5 μL volume of Taq polymerase buffer (100 m*M* and 500 m*M* of Tris-HCl and KCl at pH of 8), forward primer and reverse primer of 27F to 1492R each universal primer of 10 pmoles concentration and dNTP of 200 μM and magnesium chloride at a quantity of 2 m*M* was utilized for PCR amplification. Amplification has been directed by exposing the genetic samples to an initial denaturation phase of 3 min at 95℃ and then denaturation step was conducted by 25 PCR cycles for 1 min of at 94℃, annealing at 55℃ for 1 min followed by 1 min for extension step at 72℃. The closing step comprised at 72℃ for 10 min. Amplified PCR products were then purified exploiting with a DNA Clean & Concentrator™-5 and stored in -20°C for further analysis. Further processing was done in the chemical engineering department of Texas A&M University, U.S.A.

Construction and Analysis of Phylogeny

The 16S ribosomal RNA genome of selected solvent stable alkaliphilic lipase yielding psychrotrophic bacteria was sequenced. To acquire the 1500 bps complete nucleotide sequence of the purified amplicon of 16S ribosomal RNA was then referred to the Laboratory of MCLAB California, USA. The genome sequences were aligned with other genome sequences of 16S rRNA deposited in the GenBank database by the usage of BLAST (Altschul *et al.,* 1997) and also Ribosomal Databank Project II for diverse microbial identification. Nucleotide sequences of selected bacteria were aligned in CLUSTAL W database (Thompson *et al.,* 1997). The analyzed alignment was then manually tested and modified. Pairwise evolutionary gaps between diverse microorganisms' genome were processed utilizing the Cantor and Jukes equation applied in the MEGA7 software database with subsequent phylogenetic analysis by neighbor joining algorithm (Tamura *et al*., 2007). The validated sequences were submitted to Genbank for accession number of strain.

Lipase Enzyme Assay

Lipase catalytic activity has been evaluated by exploiting *p*-nitrophenyl palmitate substrate (Sigma, U.S.A.). Substrate reaction mixture was made by the addition of freshly 1st solution that contains *p*-nitrophenyl palmitate with 0.03 g concentration was dissolved in 10 mL of isopropanol with 2nd solution comprised gum Arabic of 100 mg, 100 m*M* $NaCl_2$ and 0.5% Triton X-100 of 0.4 mL was suspended in 90 mL of 100 m*M* Tris-HCl buffer pH 9 was prepared while stirring till completely dissolved. Substrate solution of 9 mL and enzyme solution of 1 mL was kept for 10 minutes at 25°C and the absorbance of released para-nitrophenol quantity was measured at the wavelength of λ = 410 nm. Under the conditions explained molar extinction coefficient of (PNP) was measured from the absorbance at 410 nm of PNP (0.01 to 0.1 μmol/mL) standard solutions (ε410 = 14.653 L/mol/cm). Appropriate controls were prepared for each experimentation. 1 unit of lipase activity has been stated as p-nitrophenol of 1 nmol concentration liberated per minute under the standard assay conditions. The technique of (Lowry *et al.,* 1951) was utilized for the estimation of protein contents taking bovine serum albumin (BSA) as standard.

Production of Thermolabile Lipase Enzyme

Production factors were targeted to analyze the

significance of a specific parameter at a time interval and so standardized conditions demonstrating it later before optimizing the subsequent parameter. Hydrolytic lipase activity was analyzed to estimate the highest yield production after each step. *Pseudomonas* spp. was cultivated in tributyrin nutrient broth reaction mixture (yeast extract (10 g/l), peptone (30 g/l), $CaCl_2$ 0.01%, NaCl (5 g/l) and tributyrin (2 mL) (Joseph *et al*., 2006). The inoculum preparation has been accomplished by incubating the inoculated nutritional media in a refrigerating shaker incubator at 150 rpm at 18°C. For optimization of inoculum age and size, the production medium was inoculated with varying inoculum age 6–48 h and the size of inoculum ranged amongst 1 to 20%. Underneath above-optimized factors, the optimum incubation time was investigated for the maximum production of thermolabile lipase by 100 mL of tributyrin broth was infected with 5 mL inoculum and incubated at 20°C in shaking incubator.

Samples of the crude enzyme were withdrawn at interims of 12 h up to the total time of 120 h and afterward, centrifugation was done at 10,000 rpm for 15 min at 4°C, analyzed supernatant for catalytic activity and estimation of protein. To investigate the consequence of the temperature on thermolabile alkaliphilic lipase from PAK3 production, tributyrin broth inoculated media were kept at a temperature varying from 4–50°C.

To optimize the pH for lipase production, the production medium Tributyrin (TB) nutrient broth was regulated to pH level (6–12 pH) with different buffers (Gupta *et al.,* 2008). To research the consequence of diverse sources of nitrogen, tributyrin production media were utilized as a basal medium at which 1% of each nitrogen source was supplemented to optimize for the maximum production of cold-active alkaline lipase. The optimized pH, temperature and time interim were utilized at every phase for standardizing the influence of supplementary carbon sources. To examine the consequence of distinctive carbon sources, basal media were augmented with 1% of each carbon sources such as maltose, glucose, lactose, and sucrose. Distinctive substrates for the thermolabile alkaliphilic lipase production was experimented by the supplement of Tween 20 and olive oil to broth basal media by substituting the tributyrin as a control. KCl, NaCl, and $MgCl_2$ at a concentration of 1% were supplementary to basal fermentation media to investigate the influence of varying salts on the production of thermolabile solvent resistant alkaliphilic lipase in submerged fermentation by switching $CaCl_2$ as a control.

To study the effect of different metal ions on cold active alkaliphilic lipase production from PAK3, the varying metal ions such as $FeCl_3$, $CaCl_2$, $NaCl_2$, $MgCl_2$, KCl and $ZnSO_4$ were added to the production nutrient media at 1% (w/v) concentration. All experimentations were evaluated for optimum culture conditions and composition of the medium was accomplished by shake flask culture.

Cloning of the Solvent Stable Alkaliphilic Lipase Gene

Genomic DNA was isolated from the *Pseudomonas* spp. PAK3 by using a commercially available Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research). Specific PCR primers were designed using the sequence of gene LipS (JQ071496) capable of encoding the alkaliphilic solvent tolerant lipase of *P. mandelii* JR-1. The Ylip gene was cloned from *Pseudomonas* spp. PAK3 by polymerase chain reaction (PCR) in the following steps.

Firstly, gene was amplified by using the Forward Primers FLG (Forward Lipase Gene) and Reverse Primer RLG (Reverse Lipase Gene) FLG: 5 GCGCATCCATATGTTTGTTTAACTTTAAGAAGGAG AATGTCGCAAGGTTCTGCCACG 3 (Nde1 position highlighted and the N- terminal fragment of Ylip gene is given in bold-face style). The reverse amplification was primed using RLG: 5 GCGCATCCTCGAGTTACACGCCAGCCCCTTTCAAT C 3 (Xho1 locale is highlighted and the section of C-terminal of Ylip gene is given in bold-face style). The final product of PCR after elution from an agarose gel was purified by using commercially available kit (DNA Clean and Concentrator ^{TM} (Zymo Research), then it was ligated into plasmid pET28 DNA vector separated from *E. coli* using the Zyppy^{TM} Plasmid Miniprep kit (Zymo Research). A commercially available kit Mix & Go Transformation & Buffer Set (Zymo Research) was used for the transformation procedure of competent *E. coli* BL21 (λDE3) and recombinant plasmid pET28-YLip. A linker sequence of about 11 amino acids and His_6 sequence 'HHHHHHSSGLVPRGSHM' derived from a plasmid pET28, which was positioned on the N-terminus of Ylip. The construct was confirmed by deoxyribonucleic acid molecule sequencing.

Sequence Analysis

A homology exploration was completed by BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple sequence alignments were by ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Expression and Purification Analysis of YLip

Bacterial plasmid pET28a vector comprising the complete open reading frame of YLip nucleotide sequence has been transformed into cells of competent *E. coli* BL21 (λDE3). A particular colony of recombinant cells of BL21 (λDE3) that was grown on kanamycin agar plate has been preferred for further overnight growth at 37°C, trailed by inoculation of recombinant cells into a 500 mL kanamycin nutrient production broth. At the mid-exponential period when the $OD_{600\ nm}$ is reached to 0.6–0.8 the temperature of the incubation was reduced to 15°C. Afterward the addition of a molecular reagent such as 0.1 mM concentration of IPTG,

so that the cells of recombinant BL21(λDE3) for an additional 16 h duration were grown in submerged fermentation. Bacterial BL21 (λDE3) cells were collected through centrifugation at 10000 x g for 10 minutes. After centrifugation pellet was re-suspended in binding buffer reaction (Tris-HCl 20 m*M*, NaCl 0.1 *M* and 5% glycerol at pH 8.0), followed with disruption of recombinant cells through sonication method at 4°C.

The supernatant was collected from the cell debris after centrifugation at 15000 g for 15 min and that the concentration of imidazole was corrected to 5 m*M* for purification of the crude enzyme through nickel-chelated affinity chromatography procedure. 1-mL HisTrap column was used to purify YLip. YLip enzyme bound to the column was eluted operating a step-gradient (binding buffer contains 100–500 m*M* imidazole). The dialysis buffer was utilized to remove Imidazole with (pH 10, 25 m*M* KCl, 6% glycerol). AKTA Explorer system utilized the anion-exchange chromatography purification procedure that was equipped with a 1 mL HiTrap Q-Sepharose FF column and equilibration of Q-Sepharose FF column was done with dialysis buffer and a linear gradient of KCl was used up to 25 to 1,000 m*M* concentration. Lipase activity exhibiting fractions were gathered. Purification stages at 4°C were performed. The purified enzymes were kept at -80°C for further analysis.

SDS–PAGE

The SDS–PAGE of purified alkaliphilic cold active lipase was determined through mini-slab gel equipment following the technique of (LaemmLi, 1970) by running 10% polyacrylamide gel for resolving the protein. Gel staining was carried out with Coomassie Brilliant Blue R-250 in methanol/acetic acid/H2O (5:1:5). About 5% of the methanol with 7% acetic acid in H_2O was exploited to decolorize the staining and subsequently the protein with comparative molecular mass was estimated comparing with standard protein markers (Sigma). The concurrent pattern of bands was analyzed using gel documentation technique.

Results

Sampling of Psychrotrophic Bacteria

The soil and water samples were collected from the millions of years old mountain glacier of Rakaposhi (35˙36ºN and 74˙27ºE) located at an altitude of around 3000 meters. The other geophysical parameters included; Temperature (-3 to 18°C); Atmospheric pressure (~790 mb) and pH (5.5 to 10) in the month of July 2017.

Isolation and Screening of Cold Active Alkaliphilic Lipase from Psychrotrophic Bacteria

A total of twenty diverse psychrotrophic alkaliphilic bacterial strains from the soil and water from Rakaposhi glacier were screened for producing organic solvent stable alkaliphilic lipase. Based upon the greater zone of hydrolysis on tributyrin agar (TBA) plate at 20 $\pm$°C at pH 9, five alkaliphilic lipase secreting isolates were subjected for quantitative screening and investigated for maximum enzyme production in lipase stimulating tributyrin broth media utilizing shake flask culture technique. Depending upon maximum lipolytic enzyme production (511 U/mg), one potential psychrotrophic isolate, PAK3 was selected for further studies. The psychrotrophic organism grew healthy in the pH range of 7–11 and temperature range of 10–37°C but did not have the potential to grow beyond 40°C.

Molecular Identification and Relatedness of Strain

NCBI GenBank issued the accession no. MG687270 for our strain which closely resembled to *P. peli* but with less than 79%. Further, it has been observed that *Pseudomonas* spp. (MG687270) had very proximal sequence resemblance with *P. anguilliseptica* (AF439803) and *P. cuatrocienegasensis* (JN64459) with 72% and 78% sequence similarity (e-value 0.0).

Optimization of Cold Active Alkaliphilic Lipase Production

Thermolabile alkaline lipase production by *Pseudomonas* spp. PAK3 instigated subsequently after 144 h and was observed the maximum catalytic activity of 3036 U/mL after 96 h in a logarithmic period at 20°C (Fig. 1A). Though, the maximal cell mass at 600 nm observed was after 120 h of incubation at 20°C in submerged fermentation procedure, which reveals that the enzyme secretion was independent of cell mass (Fig. 1A). The temperature was determined maximum (3643 U/mL) at 25°C after 96 h of incubation in the fermentation culture medium (Fig. 1B). Conversely, there was uninterrupted deterioration in the catalytic activity of the enzyme with a rise in incubation temperature and was nearly inhibited at 50°C. The pH condition of the fermentation culture media intensely affects several enzyme catalytic reactions and carrying of compounds through the membrane of a cell and from it was noticed that lipase hydrolytic activity was maximum at pH 9 with 2976 units (Fig. 1C). The increased catalytic activity of thermolabile alkaline lipase was succeeded with inoculum age and size (2932 unit and 3265 unit) (Fig. 2), tributyrin (3557 units), peptone (3126 units), and Ca^{2+} (3766 units) while lactose, magnesium nitrate and $FeCl_3$ worsened the production (Table 1).

Cloning and Sequence of YLip Gene Analysis

For cloning of the YLip gene encoding solvent resistant alkaline protein, information of nucleotide sequence from *P. mandelii* JR-1 was used including designing of primers. The

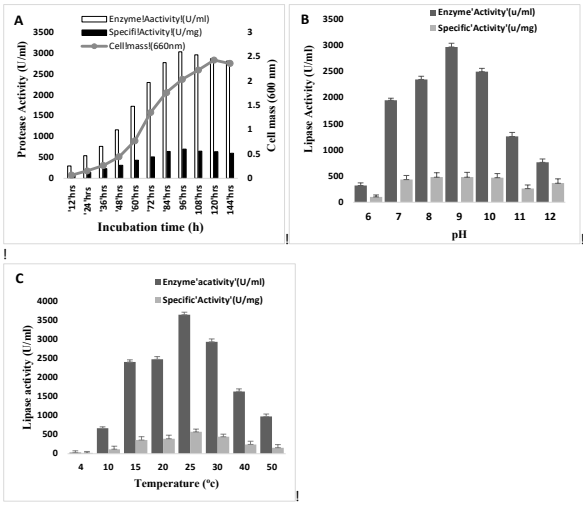

Fig. 1: A: Effect of incubation period on growth and enzyme production by *Pseudomonas* at 20°C at pH 9. **B**: Effect of temperature on lipase enzyme production by PAK03 after incubation at 96 h at pH 9. All values are represented as mean ± SD of three replications. **C**: Effect of pH on lipase enzyme production by PAK03 after incubation at 96 h at 25°C. All values are represented as mean ± SD of three replications

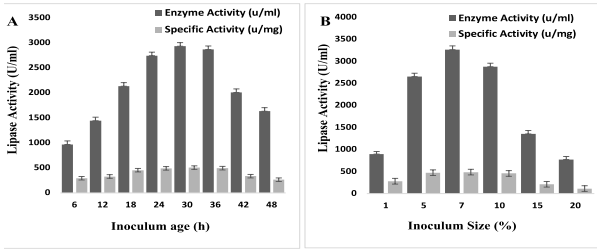

Fig. 2: Effect of inoculum size and age on enzyme production by PAK3 after 96 h incubation at 25°C, pH 9. All values are represented as mean ± SD of three replications

estimated PCR product of YLip gene (891 bp) was amplified and inserted into a pET28 bacterial vector. Protein translated from the YLip gene comprised a Gly-His-Ser-Gln-Gly sequence (Fig. 3), a distinguishing motif of the protein family belonging to serine lipase (Gly-X-Ser-X-Gly) (Cygler *et al*., 1993). The clone yielded a recombinant lipolytic protein of 32 kDa (Fig. 4B). From the multiple sequence alignment, it was characterized that 96% identity of the YLip sequence at the level of the amino acid was observed identical to a protein tolerant toward organic solvents encoding LipS gene, from *P. mandelii* JR-1 (Fig. 3). The Q3KIU1, YLip, rPFL and LipS genes resulted was 296 matching residues of amino acid (Fig. 3). The rPFL gene amplified from *P. fluorescens* exhibited sequence identity of 80% sequence identity but demonstrated a conserved motif of serine lipase family (Fig. 3). Residues of 2 Asp as a calcium binding motif were observed.

Examining the Expression and Purification of the Cloned Lipase Gene YLip

YLip gene complete nucleotide sequence was inserted in a pET28a vector with His_6 residues on N-terminal position. YLip recombinant protein was effectively expressed in *E. coli* BL21 (λDE3) strain as a soluble protein and nickel chelate affinity chromatography (Fig. 4A), was used for purification analysis trailed by another purification step of Q-Sepharose column chromatography procedure (Table 2). Though, YLip large protein fraction did not attach to the resins of nickel and observed 5% of quite a low yield. Profiles of elution from purification steps such as nickel chelate chromatography and Q-Sepharose chromatography procedures held pace through the activity of lipase. YLip protein appeared as a 32 kDa protein band on a Coomassie blue tainted SDS gel analysis (Fig. 4B, lane Q). Sequence (VNLIGHSQGSLTAR) of the peptide was analyzed by the MALDI-TOF MS from the 32-kDa protein band correspondingly matched *P. mandelii* LipS amino acid sequence (underlined in Fig. 3).

Thermal Stability of Purified Lipase Enzyme

Study of thermal stability showed that YLip maintained above 50% of its catalytic activity from 4–50°C (Fig. 5A). The residual activity of 44% lipolysis was observed retained after 1 hour of incubation at 60°C. At low temperatures the activity observed was maintain, when thermolabile enzymes mostly show flexibility in the amino acid structure by the expense of the protein stability through a diminished number of intramolecular non-covalent bonds taking place within the protein molecules such as ionic interactions, hydro-phobic relations and hydrogen bonds, comparative to their counterparts of warmer temperature (Joseph *et al*., 2008). YLip encoding protein was observed distinctive in retaining with the high thermal stability while compared to another lipase enzyme resulting from that of the psychrotrophic bacterium. Comparably, thermolabile esterase enzyme from *psychrobacter cryohalolentis* was observed to sustain nearby 60% of its hydrolytic activity after for 1 hour of incubation at 80°C (Novototskaya *et al*., 2012).

Organic Solvents, Detergents and Metal Ions Effect on Solvent Stable Alkaliphilic Lipase

YLip encoding lipase catalytic activity was estimated in the existence of numerous organic solvents (Fig. 5B). YLip observed was stable in the existence of toward acetone and isopropanol. To our wonder, lipase catalytic activity was observed to be stimulated by ethanol with 96% and DMSO with 92% after incubation for 1 h. Moreover, YLip hydrolytic lipase activity at an elevated level observed for solvents DMSO and ethanol have been retained 83% and 79% of residual catalytic activity after 24 h of incubation of reaction mixture. In contrast, YLip catalytic activity was

Table 1: Effect of carbon, nitrogen and metal ion on solvent stable lipase production by PAK3 at pH 9, 25°C

Additional supplements (1%)	Lipase activity (units)
Control	2325
Carbon source	
Glucose	3477
Tributyrin	3557
Sucrose	2654
Maltose	1532
Lactose	1559
Tween 20	1245
Olive oil	1171
Nitrogen Source	
Yeast extract	2823
Peptone	3125
Ammonium Nitrate	2989
Beef Extract	1076
Sodium citrate	954
Magnesium Nitrate	765
Metal ions	
$FeCl_3$	763
$CaCl_2$	3766
$NaCl_2$	1176
$MgCl_2$	1212
KCl	1654
$ZnSO_4$	669

Table 2: Purification of YLip from BL21 (DE3)

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purity fold
Cell lysate	86.32	7763	89.93	100	1
Nickel-chelate column	2.26	436	192.92	5.62	2.15
Q-Sepharose column	0.86	216	251	2.8	2.8

observed decreased to 50% in aqueous buffer solution. In methanol YLip lipase catalytic activity retained of about 30%, residual activity has experimented as associated to control.

Different detergents effect on the hydrolytic activity of YLip has experimented, as present in (Fig. 5C). The concentration of 0.1% detergents strength was observed overall more effective as compared to a concentration of 1.0%. Detergent concentration at 0.1%, thermolabile alkaliphilic lipolytic activity increased by 62% in the existence of Tween 80 and likewise in the presence of Triton X-100 with 26% of the lipolytic activity has been stimulated as compared to control, but it was constrained 67% of the residual activity by the effect of SDS. In an earlier experiment, some substances of the surface-active surfactants were observed that stabilizes the interfacial region between different molecules, facilitating the approach of the different substrate to the enzyme active site (Karadzic *et al*., 2006). Nucleotide sequence evaluation proposed that two residues of Asp role as the calcium-binding domain of amino acid encoding from YLip gene (Fig. 3). Maximum catalytic activity for YLip was experiential in the attendance of $CaCl_2$ with a 43% stimulation of activity (Fig. 5D). Consequences of KCL and $MgCl_2$ were minimal, but the presence of $CuSO_4$ and EDTA decreased enzymatic activity significantly.

Discussion

Glaciers in Karakorum Range of mountains in Pakistan are archives of psychrotrophic bacteria. Bacterial cold active alkaline protease and lipase are frequently produced throughout starvation and sporulation other than the stress of temperature and pH *etc*. After the quantitative screening, bacteria having the potential to produce maximum production of lipase at low temperature were selected for further studies. The strain PAK3 exhibited highest similarity of 79% with the *Pseudomonas* and validated to be novel specie. The maximum hydrolytic catalysis was observed at a range of 15 to 25°C in the present study. Thermolabile lipases enzymes are frequently extracellular and are greatly affected by temperature, pH, nitrogen source, dissolved oxygen, agitation, inducers, inorganic sources, and carbon source. The most usual technique utilized for the cold active production of lipase is submerged fermentation (Lee *et al*., 2003). Thermolabile lipase yielding bacterium identified as *P. fluorescens* by 16S rRNA technique. *P. fluorescens* showed psychrotrophic characteristics with an optimum temperature growth at 25°C (Gokbulut and Arslanoglu, 2013).

A strong proliferation in the production of lipase enzyme was detected while the pH of the submerged fermentation medium has been transformed from neutral to basic condition. This suggests a robust effect of extracellular pH upon the production of the alkaline tolerant enzyme. The optimum production of lipase was observed at pH 9, demonstrating that *Pseudomonas* spp. PAK3 stand and produce lipase in basic pH circumstances. Thermolabile lipase production in conditions with high pH has a significant role in industrial procedures, such as detergent formulations, sewage treatment, and leather processing. In the production medium lipases are generally secreted from bacteria in the existence of oil or triglycerides fat (Joseph *et al*., 2006). Tributyrin utilized as a variable carbon source in fermentation medium stimulated the maximum production of thermolabile lipase. Glucose and lactose when used as a supplementary carbon source for psychrotrophic bacteria observed to secrete maximum production of lipase related to another source of carbons. Production of lipase from psychrotrophic bacteria alters through species, enzyme specificity, optimum temperature and also optimum pH and extremely effected on the selection of substrate and circumstances (Calik and Zdamar, 2001). Different growth factors and also the well-adjusted nutrients resource has enhanced lipase enzyme production. Consequently, a practicable technique of choosing the strain capable of producing maximum lipase and culture circumstances optimization decrease the expenditure for production and lipase efficiency and stability increases.

At cold temperature psychrotrophic bacteria tend to have respectable growth frequency. Therefore, cold effective lipase enzyme is dependent on temperature and are thermolabile (Rashid *et al*., 2001). Thermolabile enzyme

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Lip9        MKKKSLLPLGLAIGLASLAASPLIQASTYTQTKYPIVLAHGMLGFDNILGVDYWFGIPSA   60
rPFL        ---------------------------MSQELATRYPLVLVPGMLGFVRLLLYPYWYGIIPA   35
Q3KIU1      ---------------------------MSQDSATRYPLVLVPGMLGFIRLVLYPYWYGIIKA   35
Ylip        ---------------------------SQGSATRYPLVLVPGMLGFIRLVLYPYWYGIISA   34
LipS        ---------------------------MSQGSATRYPLVLVPGMLGFIRLVLYPYWYGIISA   35
                                        *    *:**:**. ***** .::   **:**  *

Lip9        LRRDGAQVYVTEVSQLDTSEVRGEQLLQQVEEIVALSGQPKVNLIGHSHGGPTIRYVAAV   120
rPFL        LRRGGAQVIAVQVSPLNSSEVRGEQLLAQIQRIMAETGAARVNLIGHSQGALTARYAAAR   95
Q3KIU1      LRRGGATVIAVQVSPLNSTEVRGEQLLTRIDEILRETGAAKVNLFGHSQGSLTARYAAAK   95
Ylip        LRRGGATVFAVQVSPLNSSEVRGEQLLARIEEILRETGAEKVNLIGHSQGSLTARYAAAK   94
LipS        LRRGGATVFAVQVSPLNSSEVRGEQLLARIEEILRETGAEKVNLIGHSQGSLTARYAAAK   95
            ***.** * ..:** *:::******** :::.*:  :*  :***:***:*. * **.**

Lip9        RPDLIASATSVGAPHKGSDTADFLRQIPP-GSAGEAILSGLVNSLGALISFLSSGSTGTQ   179
rPFL        RPDWVASVTSVAGPNHGSELADYLQRHSPAHSLRGRVLSLLLRGISSLMRLLETGYRGPK   155
Q3KIU1      RPDLVASVTSVAGPNHGSELADYLAKYYPADSAKGRILEALLRSVGWLMALLETGYHGPK   155
Ylip        RPDRVASVTSVAGPNHGSELADYLEKHYPADTFKGRVLSVLLRWIGALMSLLETGYRGPK   154
LipS        RPDRVASVTSVAGPNHGSELADYLEKHYPADTFKGRVLSVLLRWIGALMSLLETGYRGPK   155
            *** :**.***..*:**: **:* :  *  :    :*. *:. :. *: :*.:*  * :

Lip9        NSL---GSLESLNSEGAARFNAKYPQGIPTSACGEGAYKVNGVSYYSWSGSSPLTNFLDP   236
rPFL        QPVDIHASHQSLTTEGVALFNRQYPQGLPQEWGGQGPAQVDGVHYYSWSGILQPGKTNRG   215
Q3KIU1      GNLFDGTNRSCRLFARTFVREPGQCDGMVGRYSSHLGQVIGDDYPLDHFDIVNQSLGLVG   275
Ylip        LPVDIPASHHSLTTQGVALFNQRYPQGLPETWGGHGPEEVNGVRYYSWSGTLQPGNTDRG   214
LipS        LPVDIPASHHSLTTQGVALFNQRYPQGLPETWGGHGPEEVNGVRYYSWSGTLQPGNTDRG   215
              :   .* .**.::*.* **  ****:*    *.*  :*:** ******     :

Lip9        SDAFLGASS-------LTFKNGTANDGLVGTCSSHLGMVIRDNYRMNHLDEVNQVFGLTS   289
rPFL        RNLFDGTNRSCRLFARTFVREAGQCDGMVGRYSSHLGQVIGDDYPLDHFDIVNQSLGLVG   275
Q3KIU1      GNLFDGTNRSCRLFARTFVREPGQCDGMVGRYSSHLGTVIGDDYPMDHFDIVNQSLGLVG   275
Ylip        GNRFDGTNRSCRLFARTFVRETGHCDGMVGRYSSHLGTVIGDEYPMDHFDIVNQSLGLVG   274
LipS        GNRFDGTNRSCRLFARTFVRETGHCDGMVGRYSSHLGTVIGDEYPMDHFDIVNQSLGLVG   275
             : * *:.          .::    **:**  ***** ** *:* ::*:* *** :**..

Lip9        LFETSPVSVYRQHANRLKNASL      311
rPFL        K-GAEPIRLFVEHARRLKAAGL      296
Q3KIU1      K-GADPVRLFVEHAARLKAAGV      296
Ylip        K-GADPVRLFVEHAARLKGAGV      295
LipS        K-GADPVRLFVEHAARLKGAGV      296
              :.*: :: :** *** *.:
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Fig. 3: Multiple Sequence Alignment for YLip (*Pseudomonas* spp. PAK3), LipS (*P. mandelii* JR-1), Q3KIU1 (*P. fluorescens* Pf0-1), rPFL (*P. fluorescens* JCM5963), and Lip9 (*P. aeruginosa* LST-03). GXSXG motif (yellow). LipS possesses a catalytic triad consisting of Ser[83] (yellow), Asp[241] (yellow), and His[263] (yellow) residues. Calcium binding motif (_)

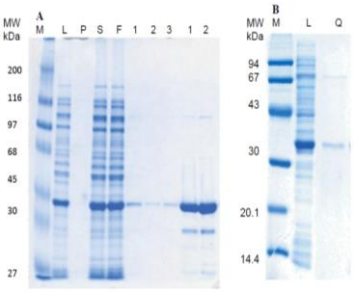

Fig. 4: Expression and Purification of yLip. **A**: SDS gel after nickel chelate affinity chromatography. The fusion protein was expressed in *E. coli* strain BL21. A lysate (L, lane 2) was made in Cell Lytic B and separated by centrifugation into a pellet (P, lane 3) and a supernatant (S, lane 4) fraction. The supernatant was applied Affinity Gel column and the flow through (F, lane 5), wash (1-3, lanes 6–8) and elution (1–4, lanes 9-10) fractions were collected. **B**: SDS gel after anion-exchange column chromatography. M, molecular weight marker. L, cell lysate. Q, eluted fraction from Q- Sepharose column (13.5 mL). The molecular weight of LipS was calculated to be 32 kDa

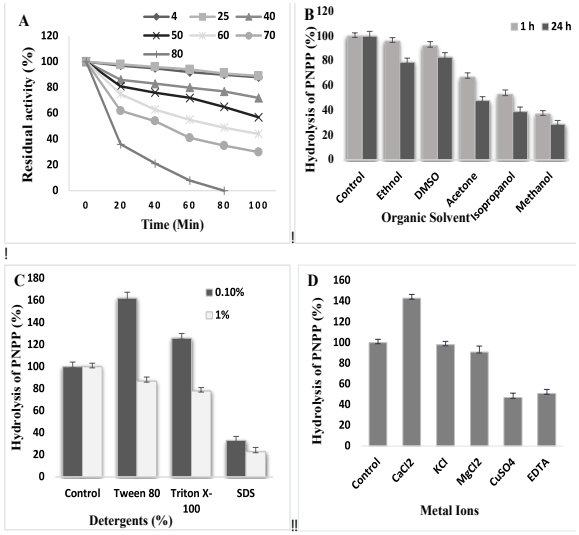

Fig. 5: A: Thermal stability of Ylip. Aliquots of YLip were pre-incubated in reaction buffer (100 m*M* Tris-Cl, 100 m*M* NaCl, 0.5% Triton X-100, pH 8 pH 9) at 4, 25, 40, 50, 60, 70 and 80°C for the indicated durations. Lipase activity was measured in a reaction buffer at 25°C for 10 min with 0.03 g PNP. Prior to incubation, the activity of YLip at 25°C was 100%. Data correspond to the mean for three experiment. **B**: The effects of organic solvents was measured after incubation of Ylip for 1 h and 24 h in a reaction buffer (100 m*M* Tris HCl, 100 m*M* NaCl, 0.5% Triton X-100, pH 9) containing 30% (w/v) of each organic solvent at 25°C for 10 min with 0.03 g PNP. **C**: The effect of detergents was measured in the same reaction buffer containing 0.1% (w/v) or 1.0% (w/v) of detergent at 25°C for 10 min with 0.03 g PNP. **D**: The effects of metal ions were measured in the same reaction buffer containing 5 m*M* of each metal ion at 25°C for 10 min with 0.03 g PNP. Data correspond to mean ± SD from three experiments

decreased thermostability and acquired enhanced hydrolytic catalysis at low temperatures. Joseph *et al.* (2011) experimented semisolid fermentation process using *Micrococcus roseus* for production of thermolabile lipase enzyme isolated from the Himalayas. Maximum production of lipase was observed by the addition of groundnut oil cake at 15°C for 2 days. Glucose at 1% as carbon and ammonium nitrate 2% as supplementary nitrogen basis increased the production of lipase. Maximum hydrolysis was observed at 10 to 15°C and at pH 8. *Pseudomonas* spp. used tryptone, yeast extract as nitrogen and carbon sources for optimum growth and thermolabile alkaliphilic lipase production. Tributyrin and Tween 80 was observed to stimulated cold adapted alkaliphilic lipases secretion at 4°C with 7.6 pH optimum (Choo *et al*., 1998). Psychrotrophic *Pseudomonas* is proficient of cultivation on lipid as the only carbon nutrient. Different psychrotrophic microorganisms from a soil sample of the Arctic territory was isolated by (Dey *et al*., 2014). Lipase enzyme was secreted in the extracellular minimal media with only olive oil at a concentration of 1%. Soybean oil was observed to stimulate the maximum production of thermolabile lipases within 4 days from *Acinetobacter* spp. at 4°C (Suzuki *et al*., 2001). Maximum production of lipase in fermentation medium was experimented from *Aeromonas* spp. at 10°C in 8 days by using carbon and nitrogen source such as tryptone and yeast extract and tributyrin utilized as an inducer of the enzyme (Lee *et al*., 2003). *Serratia marcescens* was observed to secrete thermolabile lipase after 1 week of incubation at 6°C in existence of skim milk. It was observed that Tween 80 and 20 were found the best stimulator for thermolabile lipase for *Psychrobacter* spp. secretion along with nitrogen source such as yeast extract in 2 weeks at 25°C.

Pseudoalteromonas spp. secreted lipases at 25°C in almost 2 weeks along with nitrogen source and Tween 80 and olive oil as stimulator (Zeng *et al*., 2004). *Pseudomonas* spp. strain KB700A was isolated and studied the optimization production factors such as varying carbon and nitrogen source for lipase enzyme and also the usage of tryptone and tributyrin in fermentation medium for best growth and maximum production of thermolabile lipases (Rashid *et al*., 2001). Similarly, another researcher reported on *Bacillus sphaericus* MTCC 7526 isolation that was observed to secreted thermolabile alkaline lipase enzyme by using Lactose/Tributyrin and peptone as the best carbon and organic nitrogen source at pH 8 and 15°C after 48 h of incubation (Joseph *et al*., 2006). Lactose/Tributyrin and peptone encouraged secretion of thermolabile lipases by *Microbacterium phyllosphaerae* MTCC 7530 with optimum 8 pH after incubation of 36 h at 4°C (Joseph *et al*., 2006).

A promising zone of exploration in enzymology area is to acquire radically diverse and novel hydrolytic enzyme through different molecular methodologies such as protein engineering, DNA recombinant technology, metagenomic approach and directed evolution. Recombinant protein expression, purification and molecular analyses of thermolabile alkaliphilic lipase gene cloned from *P. fragi* strain and the recombinant cold adapted alkaliphilic lipase gene after expression kept considerable hydrolysis at a cold temperature (Alquati *et al*., 2002). Separation of lipase enzyme gene straightforwardly from ecological DNA, the PCR technique was utilizing for amplification based on primers designed on alkaliphilic lipase consensus (Bell *et al*., 2002). Gene cloning, expression purification and enzyme biochemical characterization were experimented out in *Psychrobacter* spp. (Kulakova *et al*., 2004). Gene lipP of 837 bp in length encoding for a cold-active lipase secreted from *Moritella* spp. a psychrophilic bacterium was separated from samples collected from Antarctic region has been cloned and sequenced. The determined amino acid sequence disclosed a protein sequence of 278 long chains of amino acid residues with a molecular weight of 30 kDa.

Nucleotide sequence exhibited sequences of pentapeptide comprising the hydrolytic active serine motif (Gly-Trp-Ser-Leu-Gly) and also preserved dipeptide His-Gly in the N-terminal side of this hydrolytic lipase. Cloned lipase gene encoding lipase protein has been cloned into pET28a bacterial plasmid vector to create the protein with recombinant solvent stable lipase enzyme and expressed in *E. coli* BL21 strain after induction with 1 m*M* IPTG (Yang *et al*., 2008). The modern development in research of lipase is the innovative and also advanced catalytic lipase development through the molecular methodologies for example on the metagenomic method directed evolution and discovering natural populations (Gupta *et al*., 2004). Molecular biotechnology signifies a very striking technique that could be utilized to enhance lipase expression generally in the situation of isoenzymes because of the very low yield after the purification procedure. Lipolase was the earliest lipase resulted by genetic engineering was announced as Novozymes in 1988 in the market. An increasing number of lipases through recombinant technique is endorsed to the advancement in the modern molecular techniques (Kademi *et al*., 2005).

Currently a novel lipase has been separated from that of the metagenomic archive of bacteria from Baltic sea deposit (Hardeman and Sjoling, 2007). Microorganisms DNA has been isolated and replicated into a duplicate regulator fosmid bacterial vector engendering a collection of 47,000 duplicates through 24 to 39 kb of inserts. Nucleotide from lipase catalytic reaction from the sub clones was sequenced and of 978 bp of gene translating a 35± kDa lipase Lip1 with that of 54% genome resemblance to a *P. putida* amino acid. Active site of catalytic triad conserved regions such as Glu242 Ser148 and His272. Lipase Lip1 was overexpressed, purified and exhibited to fatty acids of hydrolysing p-nitrophenyl esters with C14 sequence size. Novel lipo1 a psychrophilic producing esterase achieved straight from the DNA recombinant technique was extricated from the stimulated slurry (Roh and Villatte, 2008). Psychrotrophic *P. mandelii* lips gene encrypting a unique alkaline organic solvent resistant lipase was extracted, cloned and expressed in *E. coli*. The expressed protein was purified by standard practices and molecular mass of 32 kDa has resulted from SDS-page analysis. Lipolytic activity of 80 percent of the residual activity was observed at basic pH conditions and at 40 to 50^{o}C the optimum temperature was observed for hydrolytic activity has experimented. It preserved its hydrolytic catalytic reaction in the existence of the organic solvents and also observed that the catalysis activity was augmented in the existence of DMSO and ethanol (Kim *et al*., 2013). The studied enzyme was active in the presence of numerous organic solvents. With this concern, YLip was alike to the organic solvent resistant thermolabile lipase from *P. mandelii* in relations of relative stability and stimulated catalytic hydrolysis in the existence of different organic solvents. Highest lipolytic activity for Ylip was experimented in the existence of $CaCl_2$, whereas K^+, Mg^{2+}effects were minimum. The presence of $Cu^{2+}$and EDTA declined lipolytic activity significantly (Kim *et al.*, 2013).

Conclusion

As per guidelines and minimal standards of International Journal of Systematic and Evolutionary Microbiology (IJSEM), a strain exhibiting less than 79% genetic similarity can be considered as novel species. We presume, our isolate PAK3 to be a *Pseudomonas* spp. MG 687270 as novel species. Being isolated from a unique but physiologically challenged geo-environment, the lipolypitc activity Ylip seems far more specific and efficient than previously reported counterparts. This low temperature active and broad pH stable hydrolase can prove to be a competitive addition in commodities of industrial biotechnology.

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[Received 28 Jan 2019; Accepted 29 May 2019; Published (online) 10 Nov 2019]