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Isolation, Identification and Characterization of a Lipase Producing *Pseudomonas*

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Abstract: Lipases are hydrolytic enzymes and catalyze the hydrolysis of long-chain triacylglycerols into glycerol and fatty acid. Lipases are produced by plants, animals and microorganisms including bacteria and fungi. However microbial lipases, especially from bacteria, are more useful than their plant and animal derivatives because of several important properties. The primary goals of this research work is to isolate and identify a lipase producing *Pseudomonas* species from waste water samples collected from Dir lower, Peshawar and Kohat Khyber pakhtunkhwa Pakistan. The isolated bacteria were identified as *Pseudomonas* biochemically. The other purposes of this study are production, partial purification, characterization of lipase activity at different pH and incubation time, production and determination of molecular weight analysis. The lipase was partially purified up to 30% saturation using ammonium sulphate precipitation. Purity of lipases was cheeked by SDS-PAGE, showing the same banding pattern of all the lipases and the molecular weight were determined approximately 50kDA by comparing with protein marker bands. Spectrophotometric lipase assay was used for enzyme characterization. All the 7 isolates shows maximum activity at pH 7 after 48 hours of incubation and 37°C. Among all the isolates, isolate HSWPC shows highest activity of 110.11U /ml at pH 7 after 48 hours of incubation and 37°C. *Pseudomonas* lipases are widely used in food industry, detergent, paper, textile, leather and pharmaceutical industries because of their stability, selectivity and broad substrate specificity.

Keywords: Lipases, *Pseudomonas*, Production, Partial Purification, Molecular Characterization, Lipase Assay

1. Introduction

Lipases are ubiquitous enzymes widely present in many species of plants, animals, fungi, yeast and bacteria [1]. They hydrolyze long chain triacylglycerol into monoacylglycerol, diacylglycerol, glycerol and fatty acids [2]. Lipase producing microorganisms including bacteria, yeast and fungi are found in various habitats for example coal tips, compost heaps, decaying food, dairies, industrial wastes, oil-processing factories, oil seeds, soil contaminated with oil and waste water [3]. Among all these microorganisms several species of bacteria including mainly *Achromobacter, Alcaligenes, Arthrobacter, Pseudomonas*, *Staphylococcus* and *Chromobacterium species* are very efficient in producing extracellular lipases [4] while *Bacillus* and *Pseudomonas,* spp. are the most efficient [5].

Long chain triacylglycerols are the normal substrate for lipases. Lipases hydrolyzed carboxyl ester bonds in aqueous conditions. Moreover, lipases also catalyze the reverse reaction i.e acidolysis, alcoholysis and esterification [6]. The catalytic ability of lipases can be further improved by molecular imprinting, solvent engineering as well as by molecular techniques for example directed evolution and protein engineering [7].

Because of their higher activities at neutral and alkaline pH, bacterial enzymes are preferred than fungal enzymes [8]. Most of the well-studied microbial lipases are inducible extracellular enzymes which upon their synthesis, are secreted to the extracellular environment [9]. Additionally, it has been shown that lipases are produced in the presence of inducers like tributyrene, triacylglycerols, fatty acids, esters, Tween 20 and 80, bile salts, glycerols and oil [10]. Moreover, many, if not all, commercially useful extracellular lipases are isolated from different bacterial species, including *Bacillus* and *Pseudomonas* [3, 11]. Among the *Pseudomonas* species, *Pseudomonas* aeruginosa, *Pseudomonas* cepacia and *Pseudomonas*

fluorescence, are the major producers of lipases [12]. Additionally, lipases produced by these bacteria are important in the sense that they are temperature resistant at higher pH, unlike their other counterparts isolated from alternative resources [13].

Mostly commercial enzymes are hydrolases including carbohydrases, proteases, pectinases and lipases. These enzymes catalyze the hydrolysis of natural organic compounds into glucose, peptides, glycerol and fatty acids respectively [14]. Lipases have broad applications in food industry, detergent industry, paper and pulp industry, leather and pharmaceutical industry. The main purpose of this study is to isolate lipase producing pseudomonas bacteria from service dig waste water of dir lower, peshawar and kohat districts, khyber pakhtankhwa Pakistan. Different incubation period and pH were optimized at 37°C for enhanced lipase production.

2. Materials and methods

2.1. Collection of Waste Water Samples

Waste water samples were collected from Mayar Dir lower, Timergara Dir Lower, Kohat university, Kohat city, ring road Pesharwer, Gulbahar Peshawer and Hayatabad Peshawer service stations in properly labeled pre-sterilized bottles. All these samples were immediately transferred to laboratory and stored at 4°C till analysis for lipase producing *Pseudomonas* bacteria.

2.2. Isolation of Pure Bacteria

The water samples were serially diluted with sterile distilled water 10^{-1} up to 10^{-5} each. The diluted samples were cultured in nutrient broth for 24 hours and subsequently sub-cultured on nutrient agar plates for 24 hours to obtain isolated colonies. A total of 27 different bacterial isolates were obtained from the nutrient agar cultures.

2.3. Screening of Lipase Producing Bacterial Species

The pure isolates were screened for lipase production on tributyrene agar plates. The bacterial isolates were streaked, on tributyrin agar plates and incubated for 48 hour at 25°C, 30°C and 37°C. Formation of clear zones around colonies at 37°C indicates lipase production [15].

2.4. Characterization

The formation of clear zones around colonies on tributyrene agar plates were consider as lipase positive bacterial isolates. The lipase producing bacterial isolates were characterized and identified as pseudomonas species on the basis of biochemical and morphological characters according to Bergey's manual of determinative bacteriology.

2.5. Enzyme Production

The composition of medium for the production of lipase was peptone 0.5%, yeast extract 0.3%, NaCl 0.625%, $MgSO_4 \cdot 7H_2O$ 0.01%, $CaCl_2.2H_2O$ 0.01% and olive oil 1% at pH 5, 7 and 9. 5ml of overnight culture was incubated in 250 ml Erlenmeyer flask, containing 50 ml nutrient broth on a rotary shaker at 200 rpm and incubated at 37°C 3 [1]. After 24, 48 and 72 hours of incubation the culture was centrifuged at 10,000 rpm for 20 min at 4°C and the cell free culture supernatant fluid was used as the source of extracellular lipase enzyme [16].

2.6. Partial Purification

The extracellular lipase was partially purified using ammonium sulphate fractionation, which involves the gradual addition of solid ammonium sulphate with constant stirring at 4°C to obtain 0-30% fraction. The pellet was collected and re-suspended in 5ml of 0.05M phosphate buffer at pH 7 and tested for enzyme activity 8.

2.7. Molecular Characterization

2.7.1. SDS PAGE

Molecular mass of partially purified lipases were determined by using SDS-PAGE according to Laemmli (1970) protocol [17]. 12% polyacrylamide resolving gel was used along with 4% stacking gel. Partially purified lipase and protein marker were loaded into SDS gel. The electrophoresis was carried out at 100 volts for 2 hours. Gel was stained with Bromophenol Blue for 35 minutes. The gel was placed in destaining solution for 5 hours become clear and photograph taken through digital camera. Protein marker (Fermentas) was used for the determination of molecular mass of lipases.

2.7.2. Lipase Assay

The activity of lipase was determined spectrophotometrically using p-nitrophenyl palmitate (p-NPP) as substrate as was earlier described by Krieger et al (1999) [18] with slight modifications. The reaction mixture contains one part of solution A and nine parts of solution B. Solution A contains 3 mM Para nitro phenyl palmitate while solution B consist of 100 mM potassium phosphate buffer pH 7, 0.4% triton X100 and 0.1 gum Arabic. 50 µl of lipase containing diluted solution [19] was added to 950 µl of substrate solution and incubated at 37°C for 15 min. Subsequently the reaction was stopped by boiling for 10 minutes. Finally the reaction mixture was centrifuged at 10,000 rpm for 10 minutes. The amount of P. nitrophenol palmitate thus released was measured spectrophotometrically at 410 nm against a blank containing only buffer. One International Unit (IU) of lipase activity was defined as the amount of enzyme that catalyze the release of 1µmol p-nitrophenol per minute from P. nitrophenyl palmitate under assay conditions 8. The enzyme activity was calculated by using following formula.

$$OD_{410} = 5.43020 \times \text{Conc. (mM)} + 0.00303 \quad (1)$$

$$\text{Conc (mM)of p} - \text{nitrophenol released} = \frac{\text{OD}_{410} - 0.00303}{5.43020} = A \quad (2)$$

$$\text{Enzyme (U mL-1)} = \text{A x 20 (dilution factor)} \quad (3)$$

2.7.3. Optimization of Incubation Period for Lipase Activity

The effect of incubation period on lipase activity was studied by incubating bacterial culture for 72 hours at 37°C in shaking incubator. The samples were removed after every 24 hours for determination of lipase activity.

2.7.4. Optimization of pH for Lipase Activity

The effect of pH on lipase activity was determined at different pH ranging from 5 to 9 of the fermentation medium.

3. Results

3.1. Isolation of Bacterial Isolates from Waste Water Samples

In the present study seven waste water samples of service digs were collected from different sites of Lower Dir, Peshawar and Kohat districts of Khyber Pakhtunkhwa, Pakistan. The choice of area was based on: place with higher probability of lipase producing bacteria, ease of access of that area and area not widely explored for the same purpose (table 1). In total seven areas were selected; two each from district Lower Dir and Kohat while three areas were explored in district Peshawar.

Table 1. *Collection of waste water samples from district Lower Dir, Kohat and Peshawar. Collections of samples were made according to established protocols already mentioned in materials and methods section.*

S.NO	Waste water sample	Location	District
01	Service dig	Mayar	Lower Dir
02	Service dig	Timergara	Lower Dir
03	Service dig	Kohat university	Kohat
04	Service dig	Kohat city	Kohat
05	Service dig	Ring road	Peshawar
06	Service dig	Hayatabad	Peshawar
07	Service dig	Gulbahar	Peshawar

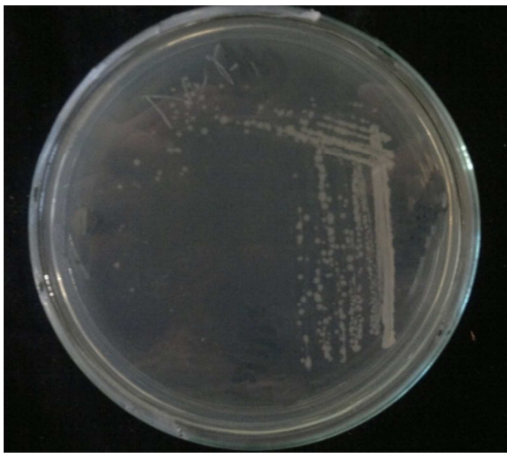

Figure 1. *Growth of bacteria on nutrient agar: Bacterial samples were subcultured on nutrient agar plates using streaking method. Individual colonies were used for further analysis.*

The collected waste water samples were processed for the isolation of various bacterial species. For the purpose, waste water samples were initially cultured in nutrient broth followed by sub-culturing on nutrient agar plates while using streaking method (figure 1). A total of 27 different bacterial isolates were obtained from the nutrient agar cultures. Size, color and morphology of the colony were some of the parameters that were kept in mind while picking certain colony. The obtained bacterial isolates were subsequently tested for their abilities to produce lipases.

3.2. Screening of Lipase Producing Bacteria

The isolated bacteria were subsequently screened on tributyrene agar for lipase production (figure 2). Bacterial isolates were streaked on tributyrin agar plates and incubated for 48 hours at different temperature. Formation of clear zones around colonies indicated lipase production.

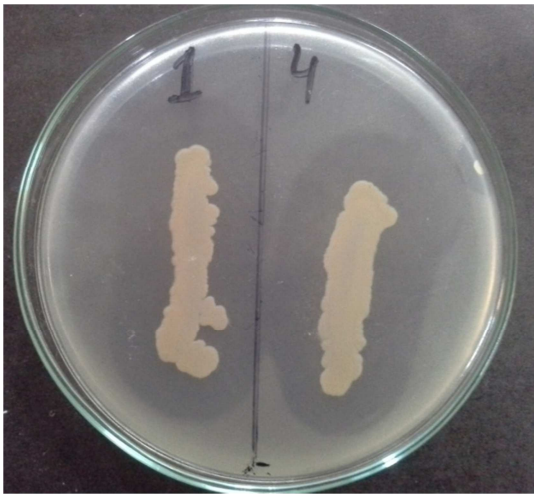

Figure 2. *Screening of lipase producing bacteria on tributyrene agar plates. Formation of clear zones around bacteria is direct evidence showing that bacteria have lipase activity.*

Table 2. *Shows total number of isolates and lipase producing isolates. Among 27 total isolates, 13 showed lipase activity.*

S.NO	Sample	Total isolates	Lipase producing isolates
01	MSWD	3	2
02	TSWD	4	2
03	KUSW	5	2
04	KCSW	3	1
05	RSWP	5	3
06	HSWP	4	1
07	PCGSW	3	2
Total		27	13

Following the above mentioned protocol, all the 27 bacterial isolates were screened for lipase production. Among all, only 13 isolates showed lipase activity while the remainder bacteria showed either no or very less activity to be examined by the aforementioned protocol (table 2). It is obvious from the table that lipase producing bacteria are

present everywhere irrespective of habitats and localities. Although, some places can be richer from lipase producing bacteria than others.

3.3. Identification of Lipase Producing Pseudomonas

The goal was to isolate the lipase producing *pseudomonas* species, therefore, in order to find lipase producing *pseudomonas* species among these 13 isolates, bacteria were grown on cetrimide agar medium. Cetrimide agar is particularly used for the isolation of *pseudomonas Species* among alternative microbial flora. It was found that among these 13 isolates, 7 isolates were *pseudomonas* positive while remaining 6 isolates were *pseudomonas* negative (figure 3).

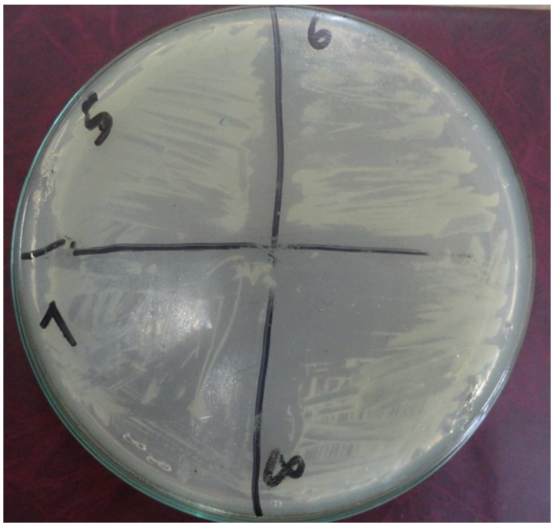

Figure 3. *Growth of lipase producing bacteria pseudomonas on cetrimide agar. Characteristic yellow-green color is the indicative of production of pseudomonas pigments in the presence of cetrimide.*

3.4. Biochemical Characterization of Lipase Producing Pseudomonas

It was found that bacterial isolates B, D, A, A, D, C and A from the respective areas are lipase producing pseudomonas positive (table 3). As it is clear from the table 3, lipase producing pseudomonas species are found in all the selected areas.

Furthermore, gram staining showed that all of the 7 pseudomonas were gram negative. While microscope observation revealed that all of them were rod shaped bacteria (Table 3).

Table 3. *Cell morphology and gram reactions of lipase producing pseudomonas. Lipase producing pseudomonas bacteria were found in all areas analyzed. The pseudomonas species we analyzed were all gram negative and rod-shaped.*

S.NO	Sample	Isolates	Cell Morphology	
			Gram staining	Shape
01	MSWD	B	Negative	Rod
02	KUSW	D	Negative	Rod
03	KCSW	A	Negative	Rod
04	RSWP	A	Negative	Rod
05	RSWP	D	Negative	Rod
06	HSWP	C	Negative	Rod
07	PCGSW	A	Negative	Rod

Different biochemical tests were performed in order to further analyze lipase producing pseudomonas bacteria. All the tests were performed according to established protocols also mentioned in the materials and methods section. It showed that all the isolated *pseudomonas* species showed catalase and oxidase activities (Table 4). Moreover, Simmon citrate assay was performed to determine the abilities of bacteria to use citrate as an energy source. It was found that after 24 hours, all of the bacteria inoculated deep into the Simmons citrate medium gave blue color showing that all of them are capable of using citrate as energy source. Similarly, motility assay showed that all of the bacteria under investigation are motile in nature. Additionally they also showed Urease production. However, they were indole and TSI (triple sugar iron) negative in nature.

Table 4. *Biochemical characteristics of pseudomonas that were analyzed: All of the lipase producing pseudomonas isolates are catalase, oxidase, Simmon citrate, motility and Urease positive, while indole, TSI and gram negative.*

S. No	Sample	Isolates	Catalase	Oxidase	Simmoncitrate	Motality	Indole	Urease	TSI	Gram staining
01	MSWD	B	+	+	+	+	-	+	-	-
02	KUSW	D	+	+	+	+	-	+	-	-
03	KCSW	A	+	+	+	+	-	+	-	-
04	RSWP	A	+	+	+	+	-	+	-	-
05	RSWP	D	+	+	+	+	-	+	-	-
06	HSWP	C	+	+	+	+	-	+	-	-
07	PCGSW	A	+	+	+	+	-	+	-	-

3.5. Molecular Characterization of Lipases Produced by Various Pseudomonas Isolates

Although different bacterial isolates were obtained showed lipase activity, however, it was not sure if the enzymes they produced are identical to each other or they belong to different groups. For this purpose, lipases were partially purified from different isolates of *pseudomonas* and subsequently subjected to SDS-PAGE to separate different lipases, if any, based on their molecular weights. We found that all lipases isolated from various bacterial isolates were identical to each other based on their molecular weight. All the lipases had a molecular weight of approximately 50kDa, when compared to the protein maker bands (Figure 4).

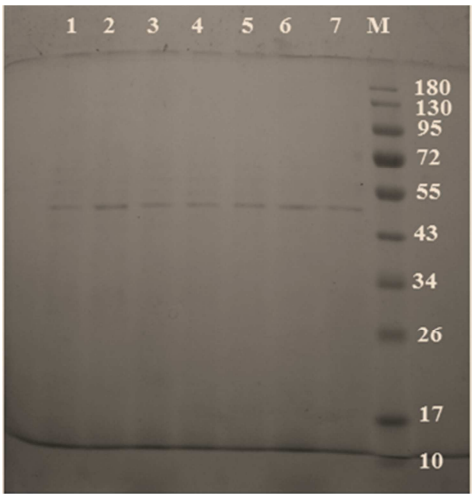

Figure 4. *SDS-PAGE of partially purified lipase enzymes from pseudomonas.*

Lane denoted with M shows protein marker bands (Fermentas) (180,130, 95, 72, 55, 43,34, 26, 17, 10kDa). Bands at approximately 50kDa shows that all the lipases isolated from different bacteria are identical to each other.

3.5.1. Assay of Lipase Activity at different pH

Lipases obtained from bacteria should work at various harsh conditions that will subsequently help bacteria to survive in various environmental conditions. However, there must be optimum conditions at which they should have maximum activity. Therefore, in order to find the optimum conditions for the lipases at which they show maximum activity, p-nitrophenyl palmitate (p-NPP) which act as a substrate for lipases were incubated with lipases at various pH and incubation times. It was found that most of lipases isolated from various bacteria, showed activities at various pH, however, lipases activities were maximum at pH 7 (Figure 5).

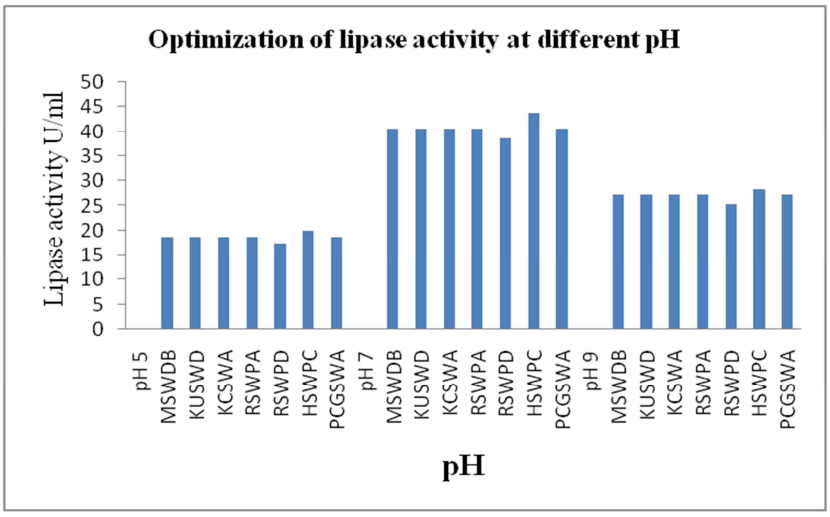

Figure 5. *Effect of pH on lipase activity.*

3.5.2. Assay of Lipase Activity at Different Time Period

Similarly, p-NPP was incubated with lipases at 37°C at various incubation times i.e. 24, 48 and 72 hours. It was found that lipases showed maximum activity after 48 hours of incubation (Figure 6).

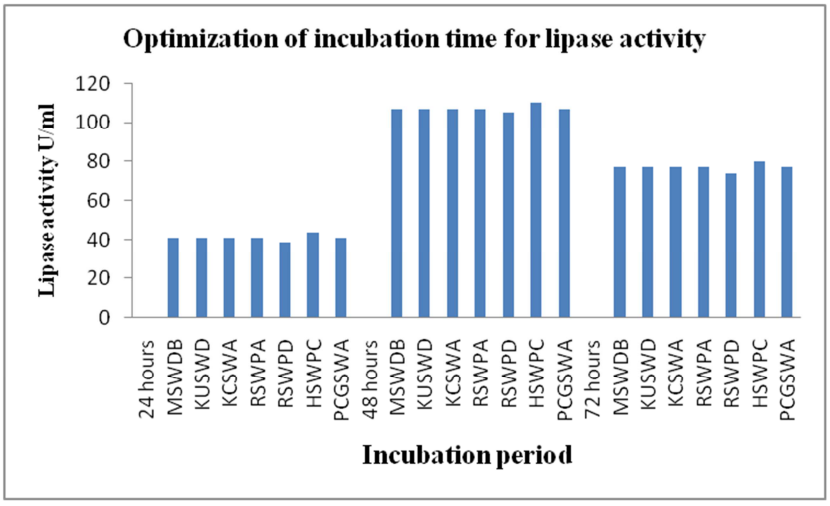

Figure 6. *Effect of incubation period on lipase activity.*

4. Discussion

Lipases are an important group of hydrolases catalyzing the hydrolysis of triacylglycerol there by having various industrial applications in food, dairy, cosmetic, detergent oleo chemical and pharmaceutical industries [20, 21]. These enzymes are widely present in many organisms including microorganisms 1. Among these microorganisms, several species of bacteria including mainly *Achromobacter, Alcaligenes, Arthrobacter, Pseudomonas*, *Staphylococcus* and *Chromobacterium species* are very efficient in producing extracellular lipases 4. *Pseudomonas* lipases are especially interesting due to their high activity at both neutral and alkaline pH [22].

In present study 7 waste water samples of service digs were collected from different sites of lower Dir, Kohat and Peshawar districts of Khyber Pakhtunkhwa. Areas, those were not previously explored for the same purpose and those containing potential lipase producing bacteria were selected for the purpose. These samples were processed for the isolation and screening of lipase producing pseudomonas species as discussed in material and methods (figure 1, 2). A total of 27 bacterial isolates were isolated on nutrient agar and screened for lipase production on tributyrene agar. Only 13 isolates out of 27 produced clear zone around colonies showing the lipase activity (figure 2) [23]. The lipase producing bacterial isolates were separated into *pseudomonas* positive and *pseudomonas* negative on the basis of growth on pseudomonas cetrimide agar medium. Among 13 *pseudomonas* isolates seven isolates are *pseudomonas* positive and six were *pseudomonas* negative (Figure 3) [24]. The results are similar to that of Prasad et al for the isolation and screening of a lipase producing *pseudomonas* from different industrial effluents [25].

The lipase producing bacterial isolates were studied for morphological characteristics according to standard microbiological protocols on the basis of culture, size, color and shape of the colony. In this work the seven lipase producing bacterial isolates selected from service digs waste water samples were identified as *pseudomonas* species on the basis of morphological and biochemical tests according to Bergey's manual of determinative bacteriology (table 3, 4) [26].

The cells free crude lipases produced by seven lipase producing *pseudomonas* isolates were partially purified up to 30% saturation using ammonium sulphate. Purity of lipases was cheeked by SDS-PAGE, showing the same banding pattern of all the lipases and the molecular weight were determined approximately 50kDA by comparing with protein marker bands (figure 4). The results are in agreement with Naeem et al (2001) who showed similar results for psychrotrophic *pseudomonas* species strain KB700A [27]. However the molecular weight of extracellular lipases obtained from *pseudomonas* range from 29 to 65kDA [28, 29].

Additionally, the lipase activity was tested at different pH. Lipolytic activities of all the seven isolates were maximum at pH 7. The isolate HSWPC shows maximum activity of 43.8U/ml followed by MSWDB, KUSWD, KCSWA, RSWPA, PCGSWA 40.50U/ml while RSWPD showed maximum activity of 38.66U/ml at pH 7 (figure 5). However it was observed that lipase activity decreased directly with increase or decrease in pH of the medium. The results are in accordance with M. P. Prasad and K. Manjunath (2012) who found similar results for the *pseudomonas* species at pH 7 25.

Moreover, the incubation period was also optimized for lipase activity. The results showed that lipase activity increases after 24 hours of incubation. The enzymatic activity of lipase was at maximum after 48 hours of incubation period. HSWPC shows maximum activity of 110.11U/ml followed by MSWDB, KUSWD, KCSWA, RSWPA, PCGSWA106.79U/ml while RSWPD showed 104.95U/ml activity after 48 at pH 7 (figure 6) 12. The results were similar to M. Veerapagu et al (2013) results for *Pseduomonas* gessardii after 48 hours of incubation.

5. Conclusion

As a conclusion, a lipase producing *Pseudomonas* species was isolated from service digs waste water collected from Dir lower, Peshawar and Kohat districts of KPK by using tributyrene agar plate assay and identified as Pseudomonas species biochemically. The partially purified lipases were characterized for lipolytic activity at different pH and incubation time. The molecular mass of purified lipase was estimated to be approximately 50 kDa by SDS-PAGE. All the 7 isolates shows maximum lipolytic activity at pH 7 after 48 hours of incubation and 37°C. Among all the isolates, isolate HSWPC shows highest activity of 110.11U /ml at pH 7 after 48 hours of incubation and 37°C. Due to their large scale application in industrial and health sectors, attention is given to isolate *Pseudomonas* lipases.

Recommendation

Molecular analysis of these isolates is recommended for species identification. Further research is needed for cloning and expression of lipase gene.

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