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Research Article

Optimization Study of Lipolytic Enzyme from *Bacillus cereus*, PCSIR NL-37

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HM performed the experiments. NA, TZ, ZH and SSA designed the experiments and drafted the manuscripts. SSA finalized the draft.

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Abstract | In this study, the optimum growth conditions and lipolytic enzyme activity shown by the bacterial isolate (PCSIR NL-37) from the soil of Lahore (Punjab, Pakistan) are being reported. Lipolytic bacterial strains (100) isolated from soil and water samples from oil industry, mobile oil filling areas and kitchen wastages were screened on tributyrin agar and rhodamine agar plates. The strain PCSIR NL-37 was identified as *Bacillus cereus* following morphological, biochemical and molecular characterization. Best medium for lipase production was olive oil and mustard oil cake and the best carbon sources were fructose and maltose whereas yeast extract was found to be the best nitrogen source, showing 55U/mL enzyme activity. The best inoculum size for the growth of this strain was 4-5%. Optimum pH was 8 and best temperature was 40°C for the reported strain. Addition of divalent Mg^{2+} and Ca^{2+} ions resulted in 2-fold increase in enzyme activity while SDS completely inhibit the lipase activity to zero.

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Introduction

Lipolytic enzymes hydrolyze triacylglycerol into fatty acid and glycerol. These enzymes are water soluble and act upon oil water interface (Reis *et al.,* 2008). They play an efficient role in reaction like trans-esterification, esterification, hydrolysis, aminolysis and acidolysis. Lipolytic enzymes are widely distributed in plants, animals and microorganisms. The most important and cost-effective source of extracellular lipases are the microorganisms. As mentioned by Vakhlu and Kour (2006), more stable form of lipases are the microbial lipases. Bacteria like *Pseudomonas* spp. *Staphylococcus* spp., *Chromobacterium* spp., *Alcaligenes* spp., *Achromobacter* spp. and various *Bacillus* spp. are commonly used for lipase production at commercial scale (Chahinian *et al.,* 2000). Lipolytic enzymes are mostly glycoproteins while other extracellular bacterial lipolytic enzymes exhibit lipoproteins model.

Microbial lipolytic enzymes have proved their role in various fields, such as food, feed stock, detergent, textile, biodiesel, oil processing industries, pharmaceuticals, cosmetics, agrochemicals as well as in waste water treatment. Because of immense applications of the lipolytic enzymes and the bacterial strains producing them, it is very important to search for the high enzyme producing bacteria with

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improved properties.

The optimum production of these extracellular enzymes can be achieved by monitoring nitrogen and carbon sources, presence of lipids, temperature, inoculums size, pH, and agitation *etc.,* in fermentation medium. During this study, the soil samples were collected from various places. The lipase producing strains were isolated, identified and characterize to optimize their growth conditions and enzyme activity.

Materials and Methods

Sample collection

Soil samples for bacterial strain isolation were collected from various places like industrial, automobile and kitchen wastages. The samples were properly labeled and preserved.

Screening of lipolytic bacteria

Each soil sample (1g) were added to 9 ml of sterilized water, mixing them thoroughly. These solutions were serially diluted to isolate lipolytic enzyme producing organisms. Further screening was carried out using tributyrin-agar base medium plates. The appearance of clear zone on the plates was observed after 24 hours and measurement of clear zones was recorded in order to monitor the lipolytic activity (Akano andAtanda 1990; Ertugrul *et al.,* 2007). These isolates were coded, preserved and stored by the routine microbiological techniques for further studies.

Rhodamine-olive plate agar plate assay

For confirmation of lipase production from bacterial isolate, a very specific and sensitive plate assay (Rhodamine-olive agar plate) was used (Bornscheuer *et al.,* 2002).

Preparation of inoculum

For this study, the high lipase producing isolate PCSIR NL-37 was used. Pure freshly grown culture from preserved slant was used to inoculate 50ml of nutrient broth in a 250mL conical flasks which were incubated at 37°C for 18 hours in shaking water bath.

Media for lipase production

Composition of the medium for lipolytic enzyme production was; glucose 0.5%, peptone 0.5%, yeast extract 0.5%, olive oil 5%, $MgSO_4 7H_2O$ 0.05% and NaCl 0.3% in distilled water which was autoclaved at 120°C for 15 minutes. The medium was cooled before pouring 2% inoculum from overnight grown culture. Lipase production was carried out at 37°C in shaking water bath.

Qualitative assay of crude lipase activity

Two different methods *viz.* phenol red-agar well assay and tributyrin-agar well diffusion assay were used to study the crude lipase activity.

Phenol red-agar well plate assay

To prepare the plates for this assay, phenol red 0.1g/L, olive oil (10ml/L), $CaCl_2$1g/L and agar 2g/L were used. Well were prepared in phenol red agar and 10-50µL lipase enzyme broth was poured into the well. The change in color around the well was noted after 48 hours (Singh *et al.,* 2006).

Tributyrin-agar well-diffusion assay

In this method, 1% tributyrin (v/v) was used as substrate for enzyme. Homogenizer was used to emulsify the Tributyrin-agar. Freshly extracted crude lipase enzyme (10-50µL) was added into wells prepared in agar plates. Plates were incubated at 37°C in incubator. The developed clear zones around the wells were measured (mm) after 48 hours and the data was used for further analysis along with titrimetric assay (Kim *et al.,* 2001).

Physical and biochemical characterization of isolates

Various physical and biochemical tests were performed for identification of the bacterial isolates. These include colony morphology, Gram's staining, oxidase test, catalase test, coagulase test, urease test, nitrate reduction, citrate utilization, tyrosine decomposition, starch degradation, methyl redtest and motility test (Selva *et al.,* 2008; Mazhar *et al.,* 2016).

Molecular Characterization

Genomic DNA from PCSIR NL-37 was prepared using Pure Genome DNA Extraction kit according to the prescribed protocol. Pure extracted DNA was used for amplification of 16S rDNA region using standard ribotyping primers (Edwars *et al.,* 1989). Amplified DNA product was sequenced and analyzed for the species identification using NCBI BLAST facility. New sequence was submitted to GenBank Database.

Production medium

For solid state fermentation (SSF), oils waste, agro-industrial and fruit waste were used as substrate. Substrate (10g) was mixed with 50ml of minimal medium, composed of diammonium hydrogen phosphate 1g/L, magnesium sulphate heptahydrate 0.40g/L, peptone 2g/L, sodium chloride 2.5g/L, calcium chloride 0.4g/L, Tween 80,1–2 drops, pH 7.0 in 250mL conical flasks. Production medium in flasks was inoculated overnight at 37°C for 48 hours in static conditions.

Optimization studies

The various parameters selected for extracellular production of lipolytic enzyme from PCSIR NL-37 were according to Pau and Omar (2004).

Effect of pH

To set different pH from 4.0-9.0 in the medium, HCl and NaOH was used all other parameters were left unchanged.

Effect of temperature

Production medium in Erlenmeyer flasks (250mL) as prepared above was incubated at different incubation temperature from 20-60°C at the intervals of 5°C.

Effect of inoculum size

Varying inoculum sizes from 4-8% (v/v) were tested and the harvested cell free broth after centrifugation for 10 minutes at 10,000rpm and 4°C, was assayed for lipolytic activity.

Effect of carbon source

Various carbohydrates were added to culture media for use as carbon source to find their role in achieving optimum lipase activity. These were glucosefructose, lactose, mannitol, maltose, sucrose, and starch.

Effect of nitrogen source

Effect of both organic and inorganic nitrogen sources was studied in single and indifferent combination for the production of lipolytic enzymes. Peptone, tryptone, beef extract, yeast extract, ammonium sulphate, ammonium chloride and ammonium nitrate were used.

Effect of detergents

Effect of different detergents in the culture medium on bacterial growth and enzyme activity was evaluated by adding (1%) SDS, Tween-20,Tween-80 and Triton-100 (v/v).

Effect of metal ions

Effect of different metal ions in the medium on lipolytic activity was determined by supplementing 1mM of calcium, magnesium sodium zinc, iron potassium and copper in the production medium.

Results

Screening and isolation of Lipolytic bacterial strains

Hundred bacteria were isolated from different environmental sources as shown in Table 1 and screened on tributyrin agar plates and rhodamine-olive agar plates. Twenty-six colonies were screened on the basis of appearance of transparent zones of lipolytic activity.

Figure 1 demonstrates the zone of hydrolysis on tributyrin-agar after 48 hours of incubation at 37°C. By the results of different qualitative and quantitative assay 6 isolates showed considerable lipase activity among them, PCSIR NL-37 exhibited lipase production at maximum (Table 2).

Table 1: Bacterial isolates from various environmental sources

S. #	Collection area	Number of bacterial isolates	Lipase producing isolates
1	PCSIR	30	12
2	Industrial wastes	20	06
3	Kitchen wastes	20	02
4	Automobile wastes	30	05

Table 2: Clear zones on tributyrin-agar plate

S. #	Isolates #	Zone diameter (mm)
1	PCSIR NL 29	1.00±0.00
2	PCSIR NL 30	0.70 ±0.203
3	PCSIR NL 31	0.97 ±0.06
4	PCSIR NL 32	0.53 ±0.06
5	PCSIR NL37	1.97 ±0.06
6	PCSIR NL 38	1.53 ±0.23

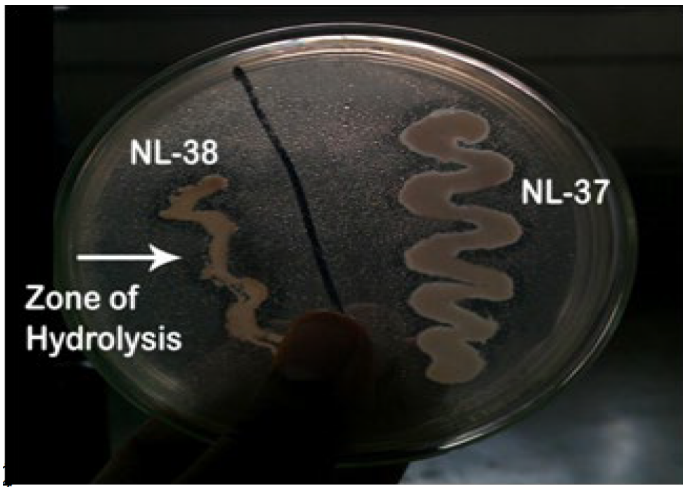

Figure 1: Clear zones shown by two lipolytic bacterial isolates on tributyrin-agar plates

Screening using Tween 80-agar plates with indicator

On phenol red well agar assay the clear yellow zones around the wells showed lipase activity. Change in coloration of the media from reddish pink to lime yellow was observed because of pH change from basic (from pH 7.2 to below 6.5). The bacterial isolate NL-37 showed maximum clear yellow zone around well for 24 hours incubation at 37°C. Screening using Tween 80 plates showed precipitates around the lipase producing bacterial isolates (Gopinath *et al.*, 2005). Liberated fatty acids binds with calcium forming a complex visible as insoluble crystals around bacterial colonies of PCSIR NL-37.

These isolates were also screened quantitatively through submerged fermentation media containing olive oil at 37°C for 48 hours. The best lipase producers were NL-37 showed the lipase activity of 26.30U/mL. It was found that PCSIR NL-37 showed remarkable lipase production as compared to other isolates, so this isolate was

selected for further characterization.

Morphological and Biochemical Characterization of Isolate

PCSIR NL-37 was found to be Gram positive rod, spore former, had flagella. Colonies of NL-37 were characteristically very large, 5mm in diameter white pigmented irregular and undulate edges were observed (Table 3).

Table 3: Summary of morphological and biochemical characterization

S. #	Characteristics	Strain NL-37
Morphological		
1	Shape	Rods (chain form)
2	Gram staining	+
3	Motility test	+
4	Spore staining	+
Growth		
1	Temperature	15-45°C
2	pH	5-12
Biochemical tests		
1	Oxidase test	-
2	Catalase test	+
3	Voges-Proskauer	+
4	Indole test	-
5	Methyl red test	+
6	Nitrate reduction test	+
Hydrolysis		
1	Casein	+
2	Starch	+
3	Gelatin	+
Growth in carbohydrates		
1	Glucose	+
2	Maltose	-
3	Sucrose	+
4	Fructose	-
	Mannitol	-

Genotypic characterization

16S rRNA region was amplified to yield DNA fragment of 500 bp in length. It was sequenced using sequencing facility at CAMB, PU, Lahore. PCSIR NL-37, 16SrRNA sequence was blasted using the NCBI facility BLASTN. It showed maximum homology (98%) with *B. cereus*. New sequence was published in GenBank database with the accession number KT374118.

Fermentation media

The submerged fermentation media contain tributyrin, olive oil and mustard oil whereas solid state fermentation preferred agro industrial wastes. The maximum enzyme activity was shown with olive oil (59U/mL) with decreasing trend with tributyrin and mustard (Figure 2).

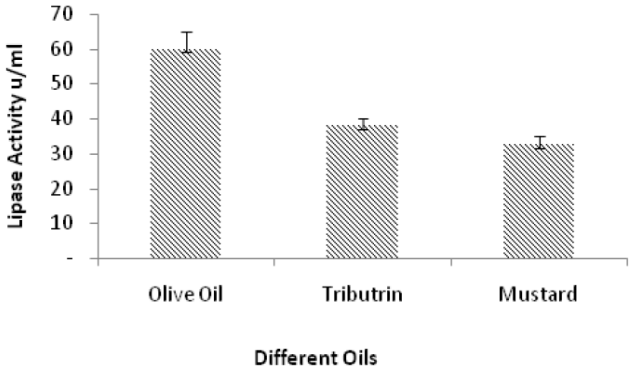

Figure 2: Lipase production by *Bacillus cereus* PCSIR NL-37 during submerged fermentation

Effect of Temperature

The optimum temperature for NL-37 was 40°C, where it showed maximum lipase production of 30.37U/mL. However, lipase production was decreased from 45-55°C. The NL-37 showed minimum lipase production at 55°C (Figure 3)

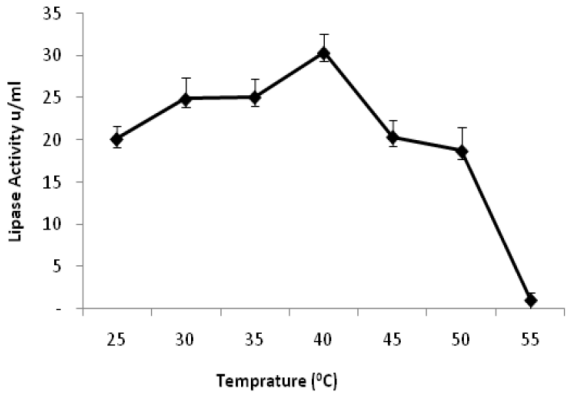

Figure 3: Effect of temperature on lipase activity of *Bacillus cereus* PCSIR NL-37

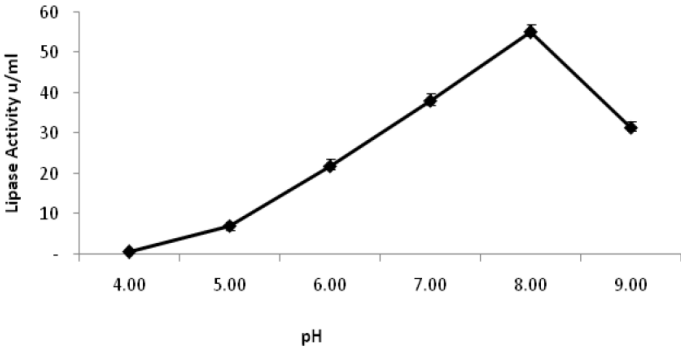

Figure 4: Effect of pH on lipase activity shown by *Bacillus cereus* PCSIR NL-37

Effect of pH

Figure 5 showed the highest lipolytic activity (55.17U/mL) by PCSIR NL -37 at pH-8, however, from

pH 4 to pH 8, the activity showed gradual rise upto pH 8, after which the decline was noticed (Figure 4).

Effect of Inoculum size

NL-37 showed best lipase production (58.13U/mL and 58.20U/mL) at 4% and 5% inoculums size, respectively, whereas below 4% and above 5% of inoculum size, the lipase production was decreased (Figure 5).

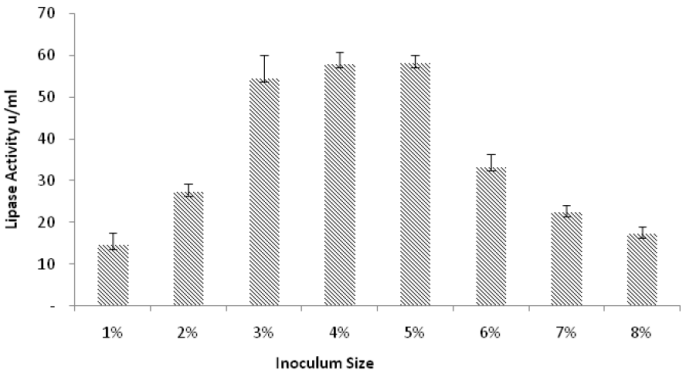

Figure 5: Effect of different inoculum sizes on lipase activity of *Bacillus cereus* PCSIR NL-37

Effect of carbon source

To study the efficiency of various carbon sources for lipase production some carbohydrates were added to the culture medium. Fructose and maltose enhanced the lipase activity from PCSIR NL-37, expressed, 28.53U/mL and 30.17U/mL, respectively (Figure 6).

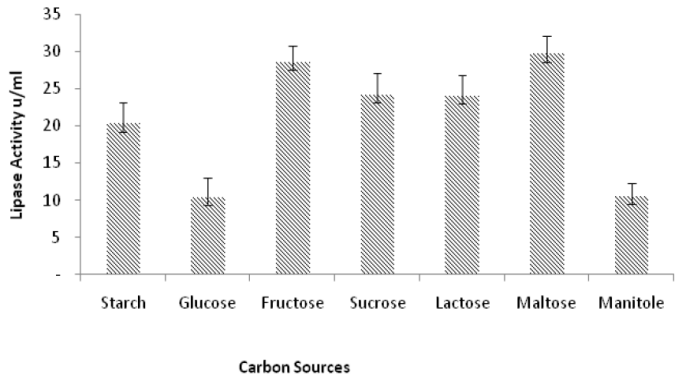

Figure 6: Effect of different carbon sources on lipolytic activity shown by *Bacillus cereus* PCSIR NL-37

Effect of Nitrogen source

As shown in Figure 7, yeast extract supplemented in the media was found best source of nitrogen for maximum production of lipolytic enzyme in the current study. PCSIR NL-37 strain expressed 50.83U/mL enzyme activity. Ammonium chloride and peptone when supplemented also have significant effect on enzyme production (Figure 7).

Effect of incubation time for incubation

Lipase production was tested by incubating fermentation media for different time periods from 24 to 120 hours. Figure 8 shows that optimum time of incubation for lipolytic enzyme production in our study was 72 hours.

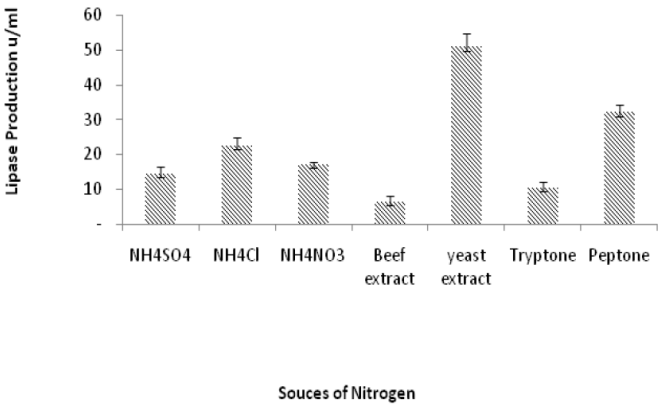

Figure 7: Effect of nitrogen source on lipase activity shown by *Bacillus cereus* PCSIR NL-37

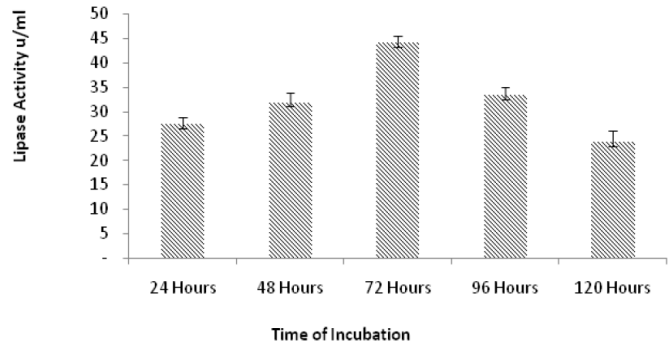

Figure 8: Effect of incubation time on lipolytic enzyme activity shown by *Bacillus cereus* PCSIR NL-37

Effect of metal ions

Various metal ions effect the lipase production to various extents. Figure 9 shows that the lipase production by PCSIR NL-37 was found to be maximum (40U/mL) with Mg^{+2} and Ca^{+2} ions added to the bacterial culture media.

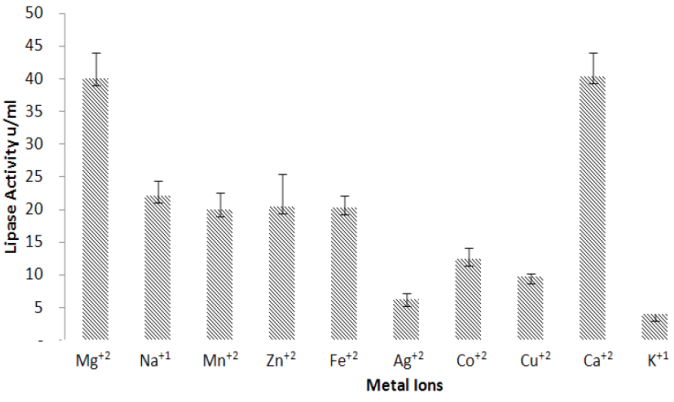

Figure 9: Effect of various metal ions on lipase activity of *Bacillus cereus* PCSIR NL-37

Effect of Detergents

The role of different detergents like, Tween-20, Tween 80, SDS and TritonX100 were studied for enzyme production by NL-37 *Bacillus* strain (Figure 10).

SDS showed complete inhibition of enzyme activity while Tween and Triton gave moderate effect. The maximum enzyme activity (23U/mL) was noticed in case of Triton X100.

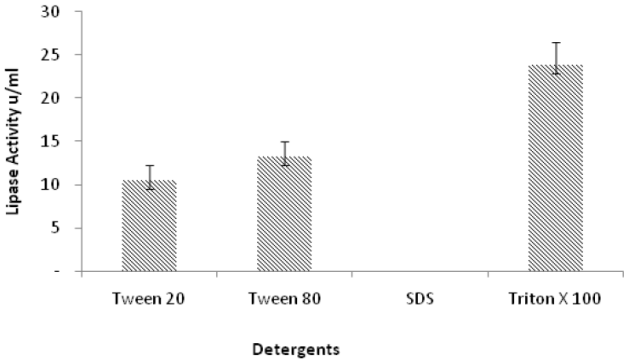

Figure 10: Effect of different detergents on lipase activity of *Bacillus cereus* PCSIR NL-37

Discussion

In this study, an effort has been made to isolate and characterize the local bacterial strains from Lahore which show considerable lipolytic activity. As discussed earlier, commercially these bacteria are of high significance due to their industrial application. They also play an important role in biodegradation of oily and fatty domestic and industrial waste.

Three qualitative methods were performed for screening lipase producing isolates. Clear zone around the colony on tributyrin plates was an indication of lipase producer. Tween-80 plates and Phenol red plates were also found very effective in the screening of lipolytic enzymes from *Bacillus* spp. In the present study many bacterial strains with lipolytic activity have been isolated but only one (PCSIR NL-37) with highest lipid degrading activity was selected for further characterization. Partial 16S rRNA gene was sequenced and analyzed using NCBI database. This sequence data was published in GenBank database accession # KT374118. After morphological, biochemical and molecular characterizations, the selected strain was identified as *Bacillus cereus.*

The selected isolate was tested for optimized growth condition and lipolytic enzyme activity. Incubation time for growth of bacteria and enzyme production play a pivotal role in selection of organism for commercial applications. The isolate was cultured for various time intervals. The maximum lipolytic activity was achieved after 72 hours incubation at other optimum conditions. Pogaku *et al.* (2010) on the other hand reported two days as the best time for the enzyme production for *Staphylococcus* spp. The best temperature for the growth and lipase production from PCSIR NL-37 was 37°C. RSJ1 strain of *Bacillus* sp. reported by Sharma *et al.* (2001) can grow at higher temperature like 50°C. *B. subtilis* strain IFFI 10210 reported by Ma *et al.* (2006) and *B. subtilis* PCSIR NL-39 showed 45°C as the optimum temperature for bacterial growth and enzyme production (Mazhar *et al.*, 2016; 2017).

In case of optimum pH, the maximum enzyme activity (55.17U/mL) observed in this study by *B. cereus* was at pH 8. Similarly, the best pH for maximum enzyme production was pH 8.0–8.5 shown by *Citrobacter freundii* Vijay *et al.* (2006) and by *Pseudomonas* sp. (Kanwar *et al.*, 2002).

For bacterial growth, addition of suitable carbon source in culture media is of fundamental importance because bacteria can biosynthesize any new product in high quantity only in the presence of sufficient raw material including carbon. Lotti *et al.* (1998), emphasized source of carbon as an essential component in the production of lipolytic enzymes. Effect of different lipid sources were studied (Figure 2) and olive oil was found best inducer. Effect of carbon source other than lipids was also investigated on lipase enzyme production by *B. cereus.* A range of carbon sources were tried. Maltose and fructose were the ideal carbon source amongst carbohydrates. However, lipase production was much lower with other tested carbon sources. In contrary Kamini *et al.* (2001) reported, lactose to enhance lipase production from *Cryptococcus* sp. Similarly, the type of nitrogen source used in the medium has its effect. In this work yeast extract was found to be the most suitable nitrogen source with 52U/mL enzyme activity followed by peptone with 36U/mL activity.

Metal in 1mM concentrations in the current study showed similar effect as reported by Nawani *et al.* (2006). They also reported Mg^{2+} to enhance the enzyme production. Lipase from *B. cereus* PCSIR NL-37 was activated by Ca^{2+} and Mg^{2+} whereas Ag^{+2}, K^{+} and Cu^{2+} ions inhibit its activity (Figure 9).

The influence of various surfactants on lipase activity was also investigated and has been shown in Figure 10. SDS at 1.0% concentration completely inhibit the lipase activity in *B. cereus* during this study. Tween-20 produced maximum decrease in with 10U/mL lipase activity while Tween-80 with small rise showed 13.5U/mL activity. The maximum lipase activity (23U/mL) was found in case of Triton-100. Similar results were reported by Matsumae *et al.* (1994) with lipase producing strain of *Serratia marcescens* SR41 8000. In contrast to these studies, lipolytic activity has been shown totally lost with the Tween-20 and Tween-80, but when incubated with Triton X100, the effect was neutral using lipase producing *Bacillus thermocatenulatus* (Schmidt-Dannert *et al.*, 1994).

Conclusions and Recommendations

The isolated *Bacillus cereus* strain PCSIR NL-37 showed significant potential for extracellular lipase production. Among different lipid materials, olive oil and mustard oil cake showed best medium for lipase production. Among carbohydrates maltose and fructose produce high enzyme activity. The isolated strain produced maximum enzyme activities with 4-5% inoculum size, at 40°C and pH 8.0 after 72-hour incubation period. Addition of 1% of yeast extract and peptone showed remarkable increase in lipase activity.

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