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Purification and Cloning of Lipase from Micro-Organism

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ABSTRACT

Lipases are a class of serine hydrolase, which belongs to the α/β hydrolases super family. Triacylglycerol acyl esters hydrolase are carboxyl esterase that catalyses both hydrolysis and synthesis of ester formed from glycerol. Lipase enzyme that catalyzes the hydrolysis of stored triacylglycerol releasing di and monoglycerol. The primary goals of this study were to isolate and identify a lipase producing bacterium from soil sample from Sadat by 16SrRNA sequence analysis, find the sequence of lipase gene by degenerate PCR and inverse PCR and analysis of lipase gene for some important feature like active site residues. On this study anew lipolytic strain was isolated, on measurement of biochemical test information, the strain was identified as Bacillus sp BM1. The maximum zone of hydrolysis (9.8mn) and specific lipase activity (86.15U/mg) was noticed for crude lipase production by the strain. The optimum temperature and pH for the lipase were found to 45 C and pH 7, respectively.

Keywords: *Lipase, Purification, cloning, micro-organisms.*

INTRODUCTION

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are part of the family of hydrolases that act on carboxylic ester bonds. They are produced by the pancreas, liver, intestine, tongue, stomach, and many other cells (Gomella, 2007). The physiologic role of lipases is to hydrolyse triglycerides into diglycerides, monoglycerides, fatty acids, and glycerol. These enzymes are widely found throughout the animal and plant kingdoms, as well as in moulds and bacteria (Houde*et al.*, 2014).

Lipases catalyze a broad range of reactions known as bioconversion reactions. Esterification, acidolysis, interesterification and amino lysis come under bioconversion reactions. Lipases can act on esters of fatty acids, synthetic triglycerides, and natural oils and many more substrates (Buchon *et al.*, 2000).

The production of microbial lipases highly depends upon the composition of medium and carbon sources besides physiochemical factors (pH, temperature). The production of lipases generally carried out in the presence of lipid (oil or any inducer i.e: glycerols, triglycerols, bile salts, fatty acids, tweens and hydrolysable esters) (Gupta *et al.*, 2004; Sharma *et al.*, 2001).

Lipases are involved in diverse biological processes which range from routine metabolism of dietary triglycerides to cell signaling (Spiegel *et al.*, 1996) and inflammation (Tjoelker *et al.*, 1995). Thus, some lipase activities are confined to specific compartments within cells while others work in extracellular spaces.

Lipases serve important roles in human practices as ancient as yogurt and cheese fermentation. However, lipases are also being exploited as cheap and versatile catalysts to degrade lipids in more modern applications. For instance, a biotechnology company has brought recombinant lipase enzymes to market for use in applications such as baking, laundry detergents and even as biocatalysts (Guo and Xu, 2005). in alternative energy strategies to convert vegetable oil into fuel (Gupta *et al.*,

2004). High enzyme activity lipase can replace traditional catalyst in processing biodiesel, as this enzyme replaces chemicals in a process which is otherwise highly energy intensive (Harding *et al.*, 2008).

Industrial application of lipases requires process intensification for continuous processing using tools like continuous flow microreactors at small scale. Lipases are generally animal sourced, but can also be sourced microbially (Bhangale, 2012).

MATERIALS AND METHODS

Sample collection, preparation and preservation

Soil samples were collected from Sadat City, Egypt in sterile plastic bags and diluted before culturing on nutrient agar. Bacterial colonies were isolated and further purified according to standard protocols (add ref. here). Isolated colonies were preserved in LB broth as previously described and were stored in -80 °C

Phenotypic characterization and Morphological examination

Bacterial isolates were initially screened for the highest lipase productivity. The isolates with maximum productivity were further characterized using gram staining technique (Ref). Isolates were further tested for different enzymatic activities using standard biochemical assays,namely, oxidase test (Ref), gelatin hydrolysis test (Ref), starch hydrolysis test (Ref), catalase test (Ref) and Nitrate reduction test(Ref). Additionally, Microscopy with high magnification (1000 X) was used to study colony texture and color, cell shape, motility and spore formation

Lipolytic (Hana) gene detection by PCR

Lipolytic (Hana) 16S rRNA geneswere amplified from two bacterial isolates using PCR method. Briefly, genomic DNA was isolated using Gene JET Genomic DNA Purification Kit (Thermo Scientific) according to manufacturer's instructions, and DNA yield was confirmed by visualizing DNA bands on 1% agarose gel. PCR was performed separately for lipolytic (Hana) gene and 16S rRNA. PCR was prepared in a final volume of 50 ul, containing10 mM d NTPs mix, 10X PCR buffer, 20 mM MgCl2 and 1.25 U of Taq DNA polymerase (Thermo Scintific), specific primers for 16S rRNA or Hana gene (Table 1) in a final conc. of 0.5 µM each. Genomic DNA was added in a final amount of 0.1 to 10 ng. PCR amplification was performed on ledom Touch Screen Thermal Cycler model A100/A200 (Hangzhou Long Gene Scientific Instruments Co., Ltd) using the following cycling conditions: initial denaturation at 94°C for 5 min, followed by 35 cycles (94°C denaturation for 30 sec, 55°C annealing for 30 sec, 72°C extension for 1min) and final elongation at 72°C for 5 min.

Gene cloning and transfection

PCR products were analyzed in a 1.0% agarose gel, excised from the gel. DNA fragments of interest were then excised from the gel and purified using Gene JET gel extraction kit (Thermo scientific) as indicated by manufacturer. The purified products were cloned into Escherichia coli strain DH5α using pGEM-T Easy vector (Promega Co., Madison, USA). Briefly, purified PCR fragment was ligated into pGEM®-T Easy Vector in a total volume of 20µl, comprising 2µl pGEM®-T Easy Vector, 10µl 2 x ligation buffers, 1µl T4 DNA ligase, 4µl PCR product, and 3µl nuclease - free Water. The ligation reaction mixture was incubated over night at 4°C. For cell transfection, 100µl of competent cell suspension was taken in a sterile eppendorf tube and chilled on ice, 4 µl of ligated mix was added and left on ice for 30 min, followed by heat shock at 42oC for 2min and were again left on ice for 3 min. 0.9ml of freshly prepared LB was added and incubated at 37oC with gentle shaking. Aliquots of the expressed culture were plated on a selective medium containing ampicillin (100 µg/ml), IPTG (0.1mM/ml), X-Gal (20 mg/ml), 0.6% CMC and incubated at 37oC incubator for 16 hours and the transformants were scored (Sambrook and Russel, 2001). The positive white recombinant colonies were subcultured on ampicillin plates for further screening and the positive clone which harboring the target gene was chosen to extract plasmid pcel12A extraction for further studying.

Subcloning

Plasmid pcel12A from positive clone was digested by BamHI and SalI. Subsequently, the resulting DNA fragments were separated by agarose gel electrophoresis, excised and purified. The resulting DNA fragment was ligated into pET22b+, and used to transform E. coli BL21 as recommended by the manufacturer (Invitrogen). The resulting recombinant E. coli strains were screened on the corresponding CMC agar for the presence of gene conferring lipase activity. The

recombinant plasmid derived from positive clones named pHana1.b was assayed for CMCase

DNA Sequencing

Sequencing was carried out on applied Biosystems model 373 A (Lincoln, Nebr) model 4000L automated DNA sequencer with appropriate dye primers (Macrogen Inc., Souel, Korea). The nucleotide sequence data was analyzed with GENETYX computer software (Software Development Co. ltd., Tokyo, Japan). Homology searches in Gene Bank were carried out with a Blast Program (Kenji *et al.,* 1996). All coding sequences were examined for similarities to protein families and domains using searches against the databases at the Gen Bank. Multiple alignments of deduced protein sequence were performed with ClustalW2, version 2.0.12 (http://www.ebi.ac.uk/Tools/clustalw2/index. html), (Thompson *et al.*, 1994) and examined with the Bio edit program (Hall, 1999). Sequence data are deposited with the Gene Bank Data Library under accession number.

Production and Purification of lipolytic (Hana) protein

The selected bacterium was initially enriched and Lipase crude enzyme was produced by fermentation as described previously (ref). Next, fermented containing lipase were filtered through Whitman No. 1 filter paper and preserved in the refrigerator at 4 ºC as a crude lipase filtrate according to (Ammar *et al.*, 1975). The crude enzyme was precipitated by adding ammonium sulphate to 60% saturation. The mixture was centrifuged at 10,000 rpm for 15 min at 4°C. The precipitates were then re-suspended in 50 mMTris-HCl, pH 8.0 and dialyzed against the same buffer overnight at 4°C with three buffer changes. Dialyzed enzyme solution was concentrated with 4M sucrose solution to get concentrated enzyme free from salt and metal ions (Lowry *et al.,* 1951)

Estimation of Enzyme activity and protein concentration

The lipase enzyme activity was assessed using gelatin clearing zone (GCZ) as previously mentioned (El-Safey and Ammar, 2002) and dinitrosalicylic acid (DNS) method (Mamo and Gessesse, 1997). For protein quantification, Bovine serum albumin (BSA) was used as standard protein and the concentration was measured by dye binding procedure (Bradford, 1976).

Enzyme (Hana1) characterization

Optimum temperature and PH of the enzyme was determined by carrying out the assay at various temperatures (5,10, 20, 30, 37, 40, 50, 60, 70 and 80°C) and at different buffers with different PH values as described before (Miller, 1959).

Optimization of culture conditions for the bacterial growth and lipase production by isolated strain

To select the optimal PH, temperature, aeration and agitation, enzyme production was investigated at different pH environments (pH 5.0-12.0), at different temperatures (25^{o}C-60^{o}C), aeration conditions with respect to volume of media in 250 ml conical flasks (25ml-150ml) and speed of agitation from static to 150 rpm, respectively in separate flasks. The samples were collected every 24 h for 72 h to measure the enzyme activity.

Effect of carbon sources on enzyme production

To find the optimum carbon source for enzyme production, five carbon sources (1%) (starch, glucose, sucrose, lactose and xylose) were selected and added to nutrient broth. The organism was inoculated and incubated for 48 hrs at 37°C and the enzyme activity was assayed in the culture supernatant (Lesuisse*et al.,* 1993). To optimize the nitrogen source for enzyme production, five different nitrogen sources (1%) (yeast extract, gelatin, casein, urea and ammonium chloride) were added to nutrient broth and the organism was inoculated and incubated for 48hrs at 37˚C The enzyme activity was assayed in the culture supernatant (Lesuisse *et al.,* 1993).

RESULTS

Isolation of lipolytic bacteria

A total of thirty bacterial colonies were successfully isolated and purified from soil samples collected from Sadat City, Egypt. all isolates subjected to purification and tested for the ability production of lipase enzyme (Figure 1).

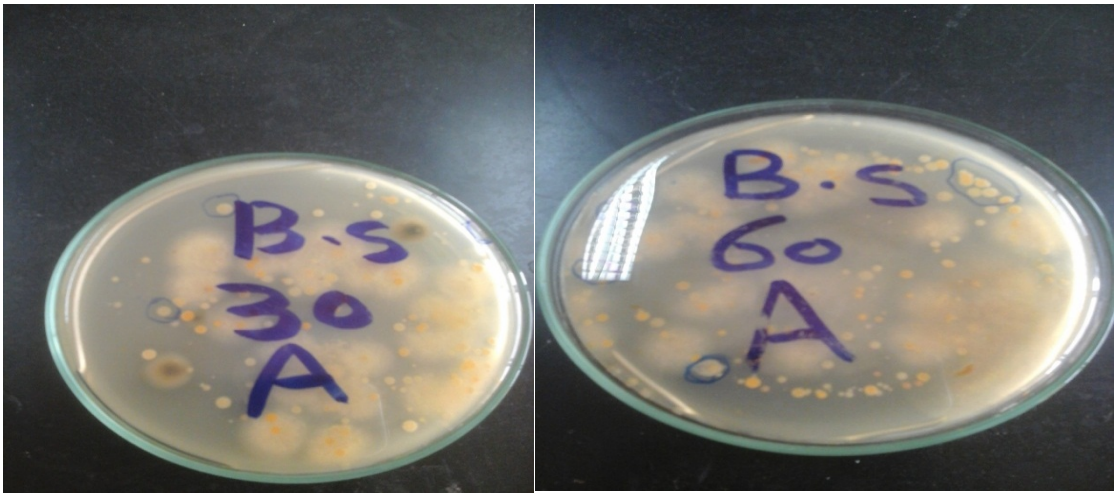


Figure 1. Two bacterial isolates (Hana 1, left side and Hana 2, right side) with highest lipase productivity.

Biochemical and morphological characterization of colonies

To further characterize isolated bacteria, several biochemical and morphological assays were performed. The results revealed that isolated bacteria are gram negative bacilli. Furthermore, they were positive for casein hydrolysis. Additionally, the isolated bacteria showed a rapid and sustained gas production indicating the ability to produce catalase. Also, they showed positive Oxidase and indole production test (Table1).

Table1. Biochemical and morphological characterization of colonies

Characterization of selected isolates	Isolate Hana1	Isolate Hana2
Gram stain	+	+
Cell shape	Long- rod shape	Long- rod shape
Motility	+	+
Oxidase	+	+
Indol production	-	-
Methyl Red Voges-Proskaeur	+	+
Catalase	+	+
Urea	-	-
Citrate utilization	-	+

Scanning electron microscope analysis (SEM):

To gain more insight into the morphology and structure of isolated bacteria, SEM was used.

PCR amplification, sequencing and phylogenetic analysis of 16S rRNA

The two isolates Hana1 and Hana2 were selected to extract their genomic DNA. The PCR product was about 1.5kbp as shown (Figure2). Two bands of the PCR product were excised from the agarose gel electrophoresis, purified and shipped to Macrogen for sequencing Multiple alignment of 16S rRNA genes of *Bacillus Cereus* strain Hana1 and *Bacillus Cereus* strain Hana 2 with the closest bacterial strains 16S rRNA genes of the closet bacterial strains 16s rRNA genes. KC683828.1: *Bacillus cereus* strain MFS16, KX418567.1: *Bacillus anthracis* strain 58283. MF360074.1: *Bacillus cereus* strain MI45, *Bacillus Cereus* strain Hana1 and alignment of 16s rRNA of *Bacillus cereus* strain with the closet bacterial strains 16s rRNA genes. KM025354.1: *Bacillus subtilis* strain TAD21, KY515421.1: *Bacillus cereus* strain R241, *Bacillus cereus* strain Hana-2 using the CLUSTAL W 2.1 multiple sequence alignment software. Phylogenetic tree based

on comparison of the 16S rRNA sequences of lipase producing bacterial isolates, *Bacillus cereus* strain Hana1 and *Bacillus Subtilis* strain Hana2 and some of the closest phylogenetic relatives using the CLUASAL W 2.1 multiple sequence alignment software. The phylogenetic tree was constructed from evolutionary distances using the neighbor-joining method of Mega 4 program package (Kumar *et al.*, 2004).

Detection of lipase gene by PCR

PCR amplification for bacterial strains named *Bacillus Cereus* Hana1 showed a clear band at 1500bp on agarose gel (Figure4). The target band was excised, eluted and purified.

Characteristics of purified Lipase enzyme

We sought to determine the optimal pH for enzymatic activity, there was a remarkable lipase production (140.29 U/ml) at pH 7 (Table 2) (Figure5). To address the effect of temperature on lipase production, we tested enzyme production at different temperature conditions, results indicated that the maximum productivity was reached at temperature 45°C (Figure6). Furthermore, degree of salinity influenced enzyme production as we showed that production was higher at 4%NaCl (Figure7).

Finally, we identified that enzyme production is influenced by carbon and nitrogen sources, xylose and yeast extract were associated with higher lipase production (figure 8).

Table (2): Production of lipase at different pH values.

Growth at PH	Results
4.0	+
5.0	+
6.0	+
7.0	+++
8.0	+
9.0	-

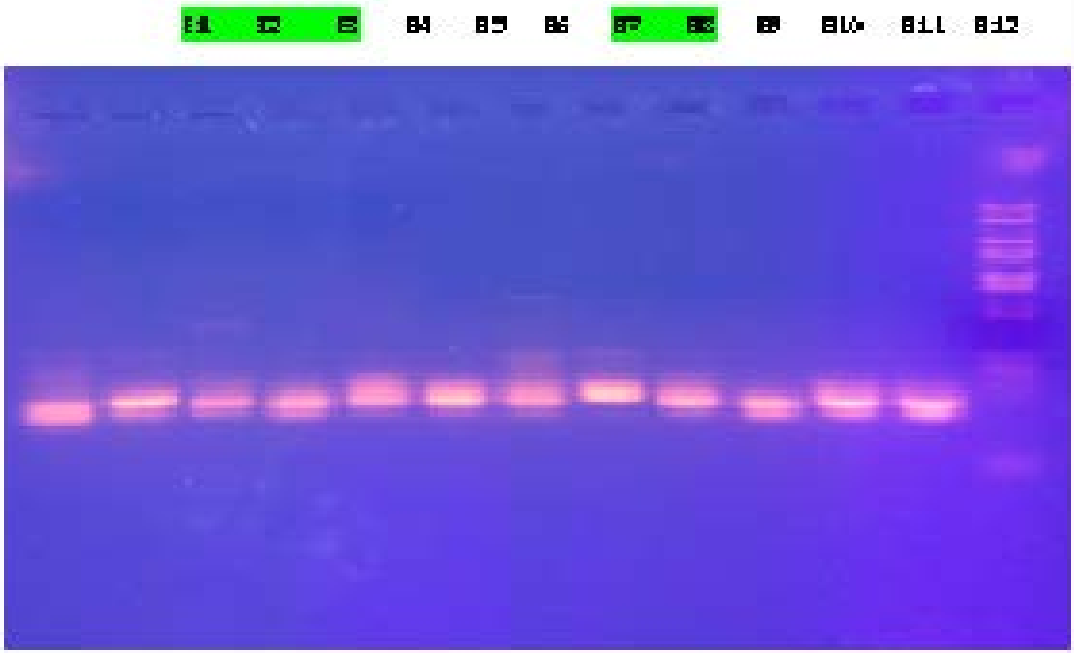


Figure (2): Agarose gel electrophoresis shows the PCR products of 16SrRNA of *Bcilllus cereus* Hana1 and *Bacillus subtilis* Hana 2.

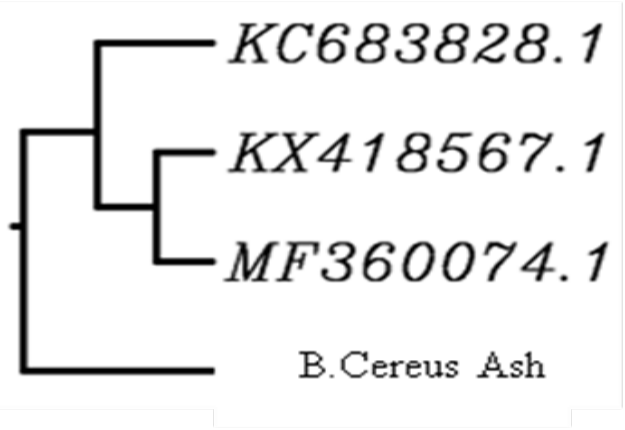


Figure (3): phylogenetic tree of 16srRNA of *Bacillus Cereus* strain and some of their closest phylogenetic relatives

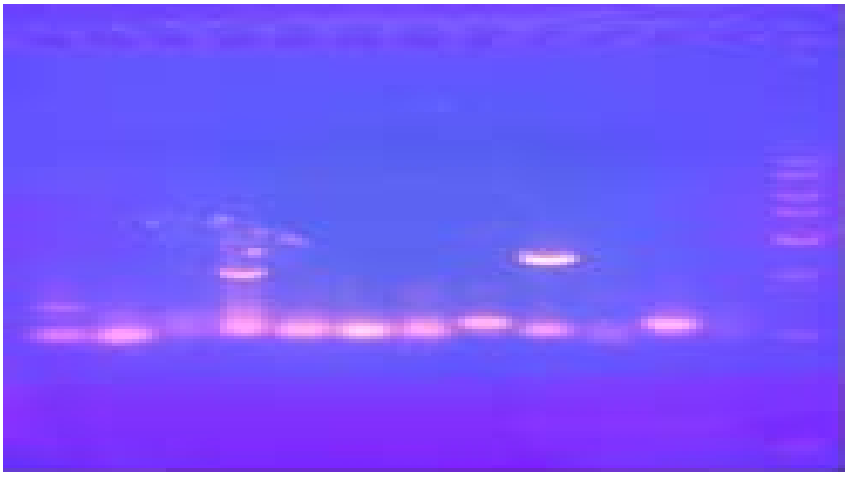
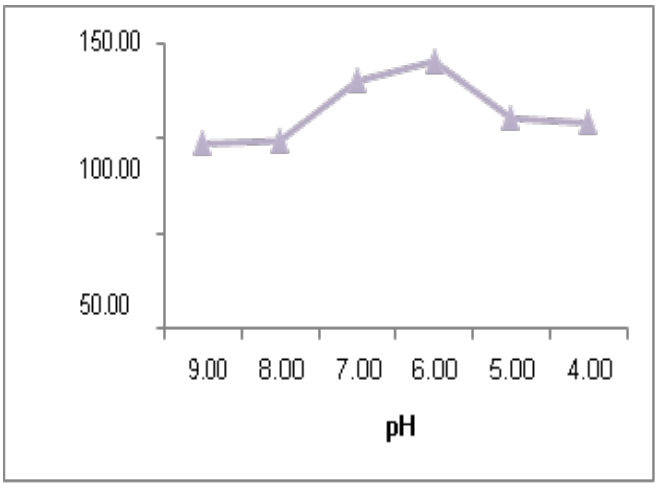


Figure (4):PCR product from B. *cereus* strain using primers Hana-1 gDNA with primers ashbc1



Figure(5): Production of lipase at different pH.

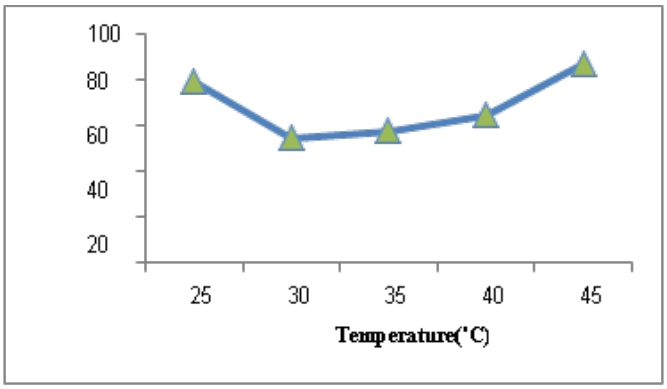


Figure (6): Production of lipase at different temperature.

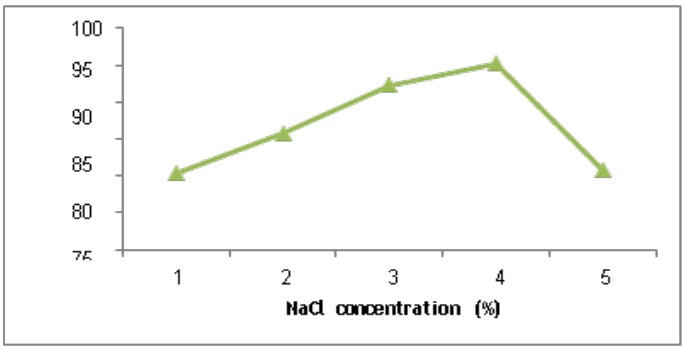
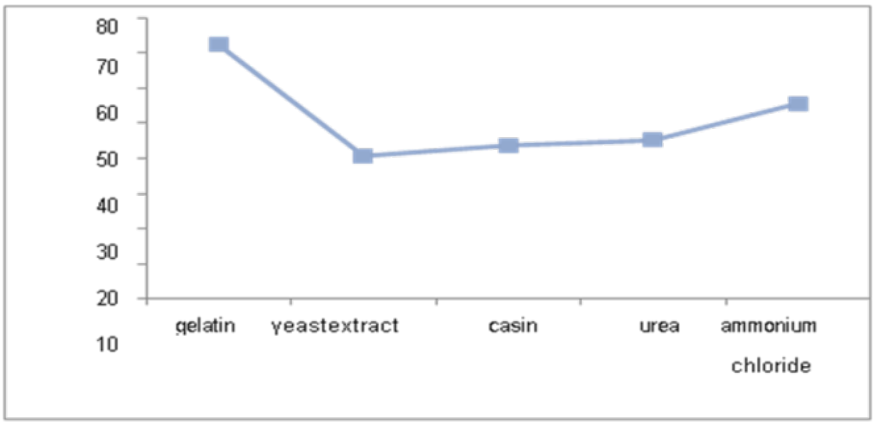


Figure (7):Effect of Sodium chloride on enzyme production.



Figure(8): Production of lipase at different nitrogen source.

DISCUSSION

In the present study, the lipase producing bacterial strains were isolated from oil soil and identified as *Bacillus cereus* and *Bacillus subtilius*. Among the different substrate tested, olive oil was found to be suitable for enhancing the lipase production by the isolated and screened *Bacillus cereus* and *Bacillus cereus* strain.

Rohit *et al.,* (2001) reported that the lipase production was more when vegetable oil, olive oil, soya beanoil, sunflower oil and gingili oil were used as carbon source.

As reported by Nakashima *et al.,* (1988) the presence of olive oil as growth medium greatly enhanced the lipase activity of *Bacillus* strain. Also, some grey Streptomyces were found to be the best lipase producers using 1% olive and palm oil**,** Nasser *et al.,* (2001).

Furthermore, irrespective of the substrate tested, the lipase activity was maximum at pH 7.5, in low and high pH tested, the lipase activity was less. This result is in consistence with the earlier report of Achamma *et al.* (2003) and mates and Sunakevitz (1972).

In the present study, the influence of medium temperature indicated that the lipase production by the isolated strains was higher at 370 C when compared to those at 270 C and 470C. Which is consistence with the earlier reported by Walavalkar and Bapat (2001). Also, Selvamohan and Alavesam (2008) show highest lipase activity of isolated *Bacillus* spp. at 37^{o}C. Immanuel and Essakkiraj (2007) shows lipase production by *Serratia marcescens* was higher at cultivation temperature of 250C compared to 30 and 35 0C. While Pseudomonas aeruginosa MB shows higher lipase production at 300C as reported by Marcin and Katz (1993).

In the present study, in all the tested substrate and also in all the media pH the tested bacillius strain show maximum activity during 48 hrs. of the culture period. On further increase inculture Period to 72 hrs. The lipase activity was decreased. Nasser *et al.* (2001) reported that lipase production by *Staphylococcus spp.* Was greater at 48 hrs. Of incubation period.

The effect of different carbon sources on lipase activity shows glycerol and glucose decreases the lipase activity, which is consistence with the earlier reported by (Mates and Sudakevitz, 1972). Lakshmi *et al.*, reported that the production of lipase was high in medium added with vegetable oil than the medium added with glucose. In contradiction Bannerjee *et al.* (1985) reported that some microorganisms showed higher activities when grown in medium containing glucose. Novotny *et al.* (1988) reported that olive oil in combination with glucose increases lipase

activity and, in most cases, and also the presence of olive oil, together with glucose or glycerol in the medium significantly decreased the both lipase and esterase level. They also inferred that if the olive oil was used as the only carbon source for growth, the enzyme activities of *Candida guillermondii* and yeast spp. showed a four to five-fold increases. This result shows that glycerol act as inducer as well as inhibitor for lipase production.

The effect of nitrogen sources on lipase production shows organic nitrogen (peptone) shows highest lipase activity as compared to other nitrogen sources (Fadilog and Erkman, 2002) also reported that olive oil in combination with other nitrogen sources enhanced the lipase production , but the presence of carbon source in the olive oil significantly decreased the lipase activity and biomass content. They also reported that organic nitrogen sources were found to increase lipase synthesis by Candida rugosa grown in the presence of olive oil. Lima *et al.*, reported that lipase production by Pseudomonas aurantiogriseum was high when using inorganic nitrogen sources, but organic nitrogen sources displayed more lipase production as compared to use of two organic nitrogen sources.

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