DISCUSSION
5. DISCUSSION

5.1. SCREENING AND SELECTION OF LIPASE PRODUCING BACTERIA

Soil is a reservoir of a large and diverse microbial population. Lipase producers have been isolated mainly from soil that contains vegetable oil (Sharma et al., 2011). Lipase production from microbial source, in general, and from bacteria, in particular, has always been a matter of study for finding lipases with novel and specific properties along with lower production and processing cost. Considering the ever increasing demand for the better lipases in the industry and the need to understand the mechanisms of lipase actions and the scope to research for novel sources of lipase, the present research has been carried out to isolate and characterize the novel lipase producing bacteria from oil spilled soil samples.

Soil Microorganisms as potential source of novel and or improved products of commercial importance have gained utmost prominence in microbial technology and bioprocess engineering. Microbial enzymes are generally cheaper to be produced and are obtained in high yields. Their contents are more predictable and reliable supply of products of constant composition is more easily available from microorganisms as their growth rate is comparatively fast and enzymes with identical substrate profile produced by different microorganisms vary significantly in the optimal conditions for their reactions like temperature, pH, substrate concentration etc.(Vuppu and Mishra, 2011).

Many microorganisms growing in polysaccharide rich environments display among their hydrolytic enzymes a wide range of esterases, including lipases, responsible for the degradation of the diverse lipids found in vegetable biomass (Cruz Ramírez et al., 2011). The oily environment such as oil-contaminated soils, wastes around oil processing factories, cooking waste are used as a source for the isolation of lipase producing bacteria since they comprise of long chain triacylglycerols which are the natural substrate for lipases(Sagar et al., 2013). In fact, soil strains with lipolytic activity are very frequent. For example, screening of several thousands of microbial soil strains, including filamentous fungi, yeast and
bacteria revealed that about 20% of them were lipase producers (Jaeger and Eggert, 2002). A numerous number of bacteria were reported for the production of lipase (Gupta et al., 2004b; Mahanta et al., 2008).

Lipases are by and large produced from microbes and specifically bacterial lipases play a vital role in commercial ventures. Bacterial lipases are mostly extracellular and are produced by submerged fermentation. Most bacterial lipases can act in a wide range of pH and temperature, though alkaline lipases are more common (Gupta et al., 2004b). Bacterial lipases are generally preferred among microbial enzymes as they offer higher activities compared to yeasts and fungus and tend to have neutral or alkaline pH optima and are often thermostable (Cadirci and Yasa, 2010; Aysun and Alper, 2013). Genetic and environmental manipulation to increase the yield of cells (Gao et al., 2009; Sanchez and Demain, 2008), to increase the enzyme activity of the cells by making the enzyme of interest constitutive, or by inducing it, or to produce altered enzymes (Soliman et al., 2007), may be employed easily using bacterial cells because of their short generation times, their relatively simple nutritional needs, and since screening procedures for the desired characteristic are easier (Hasan et al., 2006).

In the present study, primary screening was done in Tributyrin agar plate, which gave satisfactory results. The most common methods reported for measuring the activity of lipases. These are non-quantitative methods. Lipase activity is usually detected by the appearance of degradation haloes on culture media supplemented with mechanical emulsions of the desired substrates: tributyrin, triolein, olive oil etc (Jaeger et al., 1999). Many reports are available for screening lipase activity using Tributyrin agar plate assay (Selvin et al., 2012; Prasad, 2014).

Although, different screening strategies have been proposed for the determination of lipase activity, assays using agar plates are highly recommended, because it is an easier method with lower cost. Assay using agar plates are performed due to the fact that activities for lipases are hard to determine because of the water-soluble enzyme acting on substrates which are insoluble (Kanchana et al., 2011).
To isolate microorganisms from the oil-spill contaminated soils, screening studies were performed by Gopinath et al. (2005) using tributyrin and different substrates on agar plates. Tributyrin, is convenient because it is easily dispersed in water by shaking or stirring without the addition of any emulsifiers. Tributyrin is a very strong surface-active substance, and its hydrolysis can be followed by measuring the increase in the diameter of the clear zone.

Ertugrul et al. (2007) isolated 17 bacterial strains that could grow on media based on olive mill wastewater (OMW) and selected the most promising strain for lipase production. After screening in tributyrin agar medium, a strain of Bacillus sp. was identified as the best lipase producer. Kiran et al. (2008) isolated 57 heterotrophic bacteria from the marine sponge Dendrodoris nigra, of which 37% produced a clear halo around the colonies on tributyrin agar plates for lipase production. Particularly, the strain Pseudomonas MSI057 exhibited large clean zones around the colonies.

Takaç and Marul (2008) isolated microbial cultures from soil enriched by periodic sub-culturing of samples in nutrient broth containing 1% (v/v) tributyrin. The isolation process was performed by serial dilution samples on tributyrin agar (TBA) plates. Bacillus sp. was selected based on the production of the largest opaque halo.

Since both esterases and lipases can hydrolyze tributyrin (Bjurlin et al., 2002), rhodamine B-olive oil-agar plate assay was performed in further screening in order to select efficient lipase producing bacteria. The 32 bacterial isolates which showed high lipolytic activity in the TBA plate were screened further in rhodamine B-olive oil-agar plate. By this technique 20 isolates having large fluorescent zone on ROA plates was selected for further screening. Similar results was reported by Cadirci and Yasa (2010), isolated 38 solvent-tolerant strains from different environment and 20 of them were lipase positive in tributyrin agar plates. They further screened the lipase positive isolates in rhodamine B plate method among which 12 strains having bright pink zones were selected as true lipase producing bacteria.
Bouaziz et al. (2011) initially screened 120 strains isolated from various Tunisian biotopes using ROA plate assay and selected putative lipase producer by the formation of fluorescent zone around the colonies. Similarly Anbu et al. (2011) isolated fifty three distinct morphological bacterial strains from oil contaminated soil collected in South Korea using serial dilution. The isolated bacteria were screened for the extracellular lipase using spirit blue agar and rhodamine-B agar media. Two of the isolates produced larger clear areas of deep blue color around the colonies on spirit blue agar and fluorescent zone on rhodamine-B agar medium.

Through primary screening of 22 oil contaminated soil samples, hundreds of colonies with fluorescent color circle were grown on the plates, among which, 45 strains with larger fluorescence diameters on Rhodamine plate were selected (Fan et al., 2013).

In order to select best lipase producer for enzyme purification and characterization the isolates screened as lipase producer by plate assay was further selected in liquid screening medium and lipolytic activity was determined by colorimetric method. The search for the most lipolytic strains began with the selection, of 200 isolates producing the highest activity on olive oil- and/or tributyrin-supplemented plates and rhodamine olive oil supplemented plates. Among the isolates analysed, BLP141 was the most active in general terms since it was the highest lipase producer in liquid screening medium. Therefore, from these highly lipid-degrading bacterial isolate BLP141 was selected for further analysis.

In this study lipase assay was conducted by copper soap colorimetric method. Copper soap colorimetry measures color after fatty acids are converted to copper soaps by copper reagent. This procedure was originally developed by Duncombe . (1963). Lowry and Tinsley. (1976) used cupric acetate–pyridine as the copper reagent. This method was further modified by Kwon and Rhee. (1986) by replacing benzene with isooctane, and solvent evaporation and centrifugation steps were avoided. The use of isooctane made the estimation very positive, since the use of benzene is not recommended due to its toxicity and hazardous nature. The method is
specific for fatty acids and more sensitive in comparison to titrimetric method (Stoytchev et al., 2012).

Saisubramaniyan et al. (2004) used a simple and sensitive method for the determination of lipase activity in soils by copper soap colorimetric method. In that method soil was incubated with emulsified substrate olive oil and the fatty acids liberated are treated with cupric acetate–pyridine reagent, and the color developed is measured at 715 nm. Use of olive oil leads to an estimation of true lipase activity in soil. They reported that this assay is sensitive and it could be adopted to screen for lipase producers from enriched soils and oil-contaminated soils before resorting to isolation of the microbes by classical screening methods.

Rahman et al. (2005a) studied and characterized organic solvent tolerant lipase from Pseudomonas sp. S5 and performed colorimetric method for the measurement of purified lipase activity. Zhou et al. (2011) performed copper soap colorimetric method using an n-hexane-olive oil microemulsions with slight modifications to investigate the lipase from Aspergillus oryzae CJLU-31 isolated from oily waste soil sample. Jermsuntiea et al. (2011) have purified and characterized lipase from the fungus Mortierella alliacea YN-15 and determined lipase activity by colorimetric method. They reported that the purified lipase retained its activity over wide ranges of pH (2-12) and temperature (20-80 °C). Nisha et al. (2013) isolated Micrococcus roseus from vembanattu lake. They investigated production and optimization of lipase with different parameter and measured lipase activity by colorimetric method.

5.2. IDENTIFICATION OF A POTENT LIPASE PRODUCING BACTERIA

The isolate BLP 141 formed yellowish-white, smooth, mucoid and large colonies. Taxonomical studies on lipase-producing strain showed that the strain is gram negative, rod-shaped, aerobic, catalase and oxidase-positive. From these results, the isolate BLP141 was identified as Pseudomonas sp. (Holt et al., 1994).

Molecular techniques utilizing amplification of target DNA provide alternate methods for diagnosis and identification (Kurtzman and Robnett, 1997). To identify
the experimental strain exactly according to 16S rRNA sequence analysis as well as
taxonomical studies, genomic DNA of the strain was used as template to amplify
partial 16S rRNA using universal bacterial primer pairs 27F and 1492R . Finally, the
obtained partial 16S rRNA sequence of this strain was analyzed with BLAST. It was
found to have 95- 99% identity with different strains of Pseudomonas. Among them,
it showed high similarity (99%) with Pseudomonas gessardii strain CIP 105469.
Therefore, it could be concluded that the BLP141 strain was Pseudomonas gessardi.
A phylogenetic tree was also constructed based on the homology of known 16S
rRNA sequences.

The genus Pseudomonas includes polar flagellated strictly Gram negative,
non-spore forming aerobic rods with a respiratory type of metabolism in which
oxygen is used (Anzai et al., 2000). Other characteristics which tend to be associated
with Pseudomonas species (with some exceptions) include secretion of pyoverdin
(flurescein) under iron limiting conditions (Meyer et al., 2000), pyocyanin by
Pseudomonasa aeruginosa (Lau et al., 2004) and thioquinolobactin by
Pseudomonasa fluorescens (Matthijs et al., 2007).

The genus Pseudomonas is a diverse group of bacteria that are common
inhabitants of soil and freshwater ecosystems (Park et al., 2006). The Pseudomonas
genus demonstrates a great deal of metabolic diversity, and consequently
Pseudomonas sp. are able to colonise a wide range of niches (Gupta et al., 2008).
Their ease of culture and the availability of an increasing number of Pseudomonas
strain genome sequences have made the genus a focus for scientific research (Zhang
et al., 2009a).

Pseudomonas strains are of considerable environmental and biotechnological
importance due to their broad metabolic diversity and array of unique enzymatic
capabilities (Jaeger and Eggert, 2002; Nojiri et al., 2003; Huertas et al., 2006). They
exhibit the ability to bio-transform a wide range of organic compounds and are able
to degrade various chemical pollutants such as simple hydrocarbons, aromatic
hydrocarbons, nitroaromatics, chlorinated polycyclic aromatics and other recalcitrant
or toxic pollutants (Hong et al., 2004; Huertas et al., 2006; Onaca et al., 2007) of interest to the pharmaceutical, environmental, chemical and energy sectors. Therefore, there is a high interest in isolating novel hydrolytic strains belonging to this genus, such as *Pseudomonas gessardii*.

A few reports available earlier on production of lipase from *P. gessardii* and they are focused on the utilization of slaughterhouse lipid waste (Ramani et al., 2010a) and the isolation of lipase producing bacteria from oil spilled soil of Dharmapuri and Salem districts has been rarely cited in literature. Thus, the present study was focused to isolate lipase degrading bacteria *P. gessardi* from the oil spilled soil and to investigate the ability of this bacterium to produce lipases.

### 5.3. Lipase Production by Pseudomonas Gessardii

Microbial lipases are produced mostly employing submerged fermentation system (Ito et al., 2001), although solid state fermentation equally holds potential for industrial production of enzymes (Chisti, 1999). Lipase production by *P.gessardii* under submerged fermentation was optimized for various physicochemical parameters that influence lipase production. Lipase production is influenced by the type and concentration of carbon and nitrogen sources, the culture pH, the growth temperature, and the dissolved oxygen concentration (Elibol and Ozer, 2001). Many studies have been carried out to define the optimal culture and nutritional requirements for lipase production by submerged culture. However submerged fermentation for production of lipase at large scale demands high energy demand, higher capital and recurring expenditure (Satyanarayana, 1994). Lipid as carbon sources seem to be generally essential for obtaining a high lipase yield (Shimada et al., 1992).

The incubation time for enzyme production is governed by the characteristics of the culture and is based on growth rate. In the present study, lipase production was observed to commence after 24 hr of incubation and the activity declined after 48 hr of incubation, indicating that incubation for 48 hr is optimal requirement for maximal enzyme production. The optimum incubation time for maximum lipase
production of pseudomonas starts from the late log phase of growth to early stationary phase of growth (Ramani et al., 2010a). Similar results were reported showing highest lipase activity at 48 hr of incubation for Pseudomonas gessardii (Ramani et al., 2010b), Staphylococcus sp (Sirisha et al., 2010) and Trichoderma viride (Kashmiri et al., 2006) respectively. Maximum lipase production was observed at 72hr for Pseudomonas (Tembhurkar et al., 2012) and Bacillus coagulans (Prasanth and Valsa, 2007), 96 h for Penicillium roquefortii (Petrovic et al., 1990) and Aspergillus niger (Ellaiah et al., 2004) respectively.

The decrease in lipase production after long fermentation times could be due to inactivation of the enzyme by extracellular proteases, as was observed for other lipase-producing microorganisms (Sánchez et al., 1999). Swift et al. (1996) reported that once cell densities have reached a certain threshold level, generally in the late logarithmic phase, the expression of genes encoding exoproteins and a secretion system is induced.

Temperature is a critical parameter that has to be controlled and it varies from organism to organism. Temperature influences secretion of extra cellular enzymes by changing the physical properties of the cell membrane. The optimal incubation temperature for maximal enzyme production by P.gessardii was recorded as 37°C. Nevertheless, there was considerable level of enzyme production at all the lower temperatures studied. Generally the temperature required for lipase production corresponds with the growth conditions of most microorganisms (Salihu and Alam, 2012). 37°C was reported as the best temperature for maximum lipase production by Pseudomonas xinjiangensis (Khemika et al., 2012), 36°C for Pseudomonas fluorescens (Kulkarni and Gadre, 2002), 30°C for Pseudomonas aeruginos (Padhia et al., 2011; Zouaoui and Bouziane, 2011) and Serratia marcescens (long et al., 2007). Whereas 22-30°C was observed to be optimum for maximum lipase production for Pseudomonas putida (Pabai et al., 1995), Acinetobacter sp. (Barbaro et al., 2001) and Pseudomonas sp. (Haba et al., 2002).
The initial pH of the growth medium is important for lipase production (Rathi et al., 2001). Lipase activity and growth were significantly affected by the initial medium pH. The maximum lipase activity of P. gessardii was obtained when the initial medium pH was 7.0. Most of the bacteria reported for lipase production grown at neutral initial pH for optimal growth and lipase production (Cardenas et al., 2001; Large et al., 1999) as evidenced in Pseudomonas fluorescens HU380, Pseudomonas sp, P. aeruginosa (Kojima and Shimizu, 2003; Narasimha et al., 2011; Zouaoui et al., 2012). However, maximum lipase activity at higher initial pH by various thermophilic Bacillus sp. has also been reported (Ghanem et al., 2000).

Most lipases that are active at extremely acidic pH are mainly from mammalian sources e.g. gastric lipase. However, Pseudomonas sp. MS1057. (Kiran et al., 2008), P. gessardii (Ramani et al., 2010b), Pseudomonas sp. (Kavitha and Shanthi, 2013) shows high level of extracellular lipase when cultured at acidic pH 3 to 6. Also, high lipase producing microorganisms such as Bacillus sp., Pseudomonas sp., Burkholderia sp.(Gupta et al., 2004b) and Yarrowia lipolytica (Vakhlu and Kour, 2006) grow and produce lipases at pH ranges from 6 to 8.

The molecular charges and consequently molecular interactions and functions are directly related to medium pH; thus, any change in medium pH affects many biological functions (Viviani et al., 2008). Thus, medium pH is very important in nutrient absorption and growth of bacteria, stimulation of enzyme production via signaling pathways and release of extracellular enzymes (Paetzel et al., 2002). Also, the pH change observed during growth of the organism may affect the enzyme stability in the medium (Gupta et al., 2003a).

It is inferred from the results obtained in the present study that the agitation is required for the bacteria to produce lipase since there was no lipase production at stationary condition. Maximum enzyme production was recorded at 160 rpm. Further, the rate of agitation above 160 rpm led to a decrease in enzyme production. In submerged fermentation, the production rate of a microbial metabolite is also influenced by the agitation speed. Maximum enzyme production was achieved at
150 rpm for *P. aeruginosa* and *C. albicans* (Padhiar *et al.*, 2011), whereas, speed of 200 rpm is favorable for *A. flavus, Y. lipolytica, Rhodotorula mucilaginosa* (Padhiar *et al.*, 2011; Alonso *et al.*, 2005; Potumarthi *et al.*, 2008). The increase in lipase production could be attributed by increased oxygen transfer rate, increased surface area of contact with the media components and better dispersability of the oil substrate during fermentation under agitated condition. However, at higher agitation rates, there was a reduction in growth as well as lipase production (Gulati *et al.*, 2000; Sirisha *et al.*, 2010). Agitation rate not only affects oxygen availability but it also exerts influence on the availability of other nutrients in the medium. Low enzyme activity at higher agitation rates may be attributed to the effect of shear stress on bacterial cells as well as on the enzyme structure (Sooch and Kauldhar, 2013).

It is important to provide an optimum inoculum level in fermentation processes. At a suitable inoculum size, the nutrient and oxygen levels are enough for sufficient growth of bacteria and therefore enhance the lipase production. If the inoculum size is too small, insufficient biomass will lead to reduced levels of secreted lipase. High inoculum size can result in the lack of oxygen and nutrient depletion in the culture media resulting in poor product yield (Dheeman *et al.*, 2010). Variation in the level of inoculum concentration from 1 % to 10 % did not greatly influence the rate of enzyme production by *P. gessardii*. The data clearly showed that there was no direct relation between inoculum concentration and enzyme activity. Similar result was reported by YU *et al.* (2009) that highest lipase was produced when the inoculum concentration was 6% for *Pseudomonas Lip35*. Maximum lipase production was reported at 10% (v/v) for *Serratia marcescens* (Zhao *et al.*, 2010), 5% (v/v) for *Thermus thermophiles* HB27 (Dominguez *et al.* 2005). It is desirable to produce maximum enzyme activity with lower concentration of inoculums for industrial application.

In the present study sugars supplied as additional carbon source did not enhance lipase production by *P. gessardii* compared to control. Galactose, maltose and sucrose inhibited lipase production. Whereas, all the other sugars tested led to a
reduced level of enzyme production when compared with that of the control. Significantly decreased lipolytic enzyme levels were obtained with starch, which is similar to the effect of starch on lipase production in *Issatchenka orientalis* (Costas *et al*., 2004). Whereas Sztajer and Maliszewska. (1988) reported that starch was the best carbon source for lipase production by *P. fluorescens*. Reduction in the lipase production in the presence of sugars as carbon sources could be due to catabolite repression by readily available carbon sources in the medium. (Kiran *et al*., 2008; Yu *et al*., 2009). Glucose used as a sugar additive showed increased lipase production from *Burkholderia cepacia* in the presence of different oils (Rathi *et al*., 2001).

Aminoacid as an additional nitrogen source has influenced the lipase production. Among the various aminoacid investigated histidine and lysine has greatly influenced lipase production. On the other hand asparatic acid, tryptophan, valine, glutamine, leucine and alanine caused a considerable reduction in enzyme production. Arginine, threonine and lysine were found to give best lipase production by *Pseudomonas fluorescens* 2D (Makhzoum *et al*., 1995). Arginine, lysine and glutamic acid in medium was observed to be effective for lipase production by *P. fragii* (Alford and Pierce, 1963). Alanine, glutamic acid, methionine, tryptophan, tryptophan, glycine and valine appeared to stimulate lipase production in *Pseudomonas aeruginosa* (Kathiravan *et al*., 2012).

Generally, microorganisms provide high yields of lipase when organic nitrogen sources are used. Nitrogen sources play an important role in the biosynthesis of lipase by microorganisms. Inorganic nitrogen sources can be used quickly, while organic nitrogen sources can supply cell growth factors and amino acids needed for cell metabolism and enzyme synthesis (Tan *et al*., 2004). All the nitrogen sources tested enhanced lipase production. Proteose peptone and peptone enhanced maximum level of lipase production. Similar reports were made earlier where peptone enhanced maximum lipase production by thermophilic *Bacillus sp.* and various *Pseudomonads* (Sugihara *et al*., 1991; Sharma *et al*., 2002b), *Pseudomonas fluorescens* NS2W (Kulkarni and Gadre, 2002), *Pseudomonas aeruginosa* (Mobarak-Qamsari *et al*., 2011).
Likewise, higher lipase production was reported using yeast extract as a nitrogen source for *Saccharomyces cerevisiae* (Shirazi et al., 1998). In addition, maximum lipase production from *Pseudomonas* sp. KWI-56 (Izumi et al., 1990), *Candida cylindracea* (Muralidhar et al., 2001) and *R. arrhizus* (Rajendran and Thangavelu, 2009) was obtained in media containing peptone and yeast extract as organic nitrogen sources.

It is clearly evident from the result that all the inorganic nitrogen sources tested have a positive effect and enhanced the lipase production. Maximum enzyme production was supported by ammonium sulphate and ammonium chloride followed by ammonium nitrate and ammonium dihydrogen phosphate. Similar reports were made earlier that inorganic nitrogen source in form of ammonium chloride was reported to be the best for *P. citrinum* (Miranda et al., 1999) and *C. cylindracea* NRRL Y-17506 (Brozzoli et al., 2009). Similarly diammonium phosphate showed a significant increase in lipase production by *Bacillus cepacia* (Rathi et al., 2001).

Poor yield of lipase and biomass in medium containing urea was reported earlier for *Aspergillus* sp. (Cihangir and Sarakaya, 2004). In the present study also supplementation of urea and nitrate salts did not increase lipase yields. The requirement of the type of nitrogen varies among microorganisms, some prefer inorganic form while others prefer organic nitrogen (Nahas, 1988). Higher production of lipase was observed in the presence of diammonium hydrogen orthophosphate with *Burkholeria cepacia* (Rathi et al., 2001). Whereas, *P. fragi* utilized ammonium sulphate for maximum lipase production (Alford and Pierce, 1963).

Lipases are mostly inducible enzymes and inducers such as oils are necessary for lipase production (Maia et al., 2001), although their role in lipase synthesis and stimulation is poorly understood (Large et al., 1999). In some cases, lipases were produced constitutively and showed a significant increase in activity on addition of oil to the medium (Messias et al., 2009; Ohnishi et al., 1994). Among the various oils used in the present study, maximum lipase production was achieved using
sesame oil followed by gingelly oil, sunflower oil and coconut oil. Natural oils such as soybean, corn, sunflower, olive, palm and cotton seed oils are cited as inducers for lipase production, comprising at times the sole source of carbon in the medium (Lin and Ko, 2005; Rathi et al., 2001; Tan et al., 2004). The variation in preferential utilization of specific oil as substrate for maximal enzyme may be attributed to the diversity in nutritional requirement of each species of bacteria.

Among the various inorganic salts tested, only magnesium sulphate, calcium chloride and combination of magnesium sulphate with calcium chloride enhanced lipase production. Whereas, all the other inorganic salts potassium chloride, zinc sulphate, copper sulphate and ferrous sulphate inhibited lipase production. Kulkarni and Gadre. (2002) reported similar results that magnesium sulphate and calcium chloride individually or in combination, increased lipase production of *P. fluorescens* NS2W by 26%. Addition of 0.02% magnesium sulphate and ferrous sulphate to the fermentation medium stimulated lipase production by *Pseudomonas aeruginosa* MTCC 10,055 (Bist et al., 2012). Magnesium chloride in presence of calcium chloride was reported to tremendously increase lipase production in *Burkholderia cepacia* (Rathi et al., 2001). Generally, magnesium salt is required by most microorganisms due to its ability to play some regulatory functions associated with increased adenosine triphosphate metabolism and nucleic acid synthesis (Bankar et al., 2009). Others needed for microbial growth include potassium in yeast strains, which was found to be essential for osmoregulation. Irons are essentially used for heme and cytochrome synthesis (Venkateshwar et al., 2010) and calcium was found to be necessary for effective lipase stabilization and activity in *Acinetobacter* sp. (Snellman and Colwell, 2004).

It has been reported that salt enhance lipase production by modifying the lipase structure. Hence the effect of salt on lipase production was studied. The presence of sodium chloride in the medium significantly affected lipase production. Addition of sodium chloride at concentrations 1% to 2% (w/v) enhanced lipase production, recording a maximum activity with 1%. Sodium chloride concentrations above 2% led to a gradual decline in lipase production compared to control (without
NaCl). Similar result was reported that maximum lipase production occurred at 0.5M NaCl by *Pseudomonas* sp (Kavitha and Shanthi, 2013), 0.5% NaCl by *B. coagulans* (Kumar and Valsa, 2007) and *Pseudomonas* sp. MSI057 (Kiran et al., 2008).

**5.4. STATISTICAL OPTIMIZATION OF BIOPROCESS VARIABLES FOR LIPASE PRODUCTION**

In fermentation processes, where the operational variables interact and influence each other's effect on response, it is essential that the optimization method account for these interactions, so that a set of optimal experimental conditions can be determined (Sen and Swaminathan, 1997). Optimization through factorial design and response surface analysis particularly fulfils this requirement (Elibol and Ozer, 2002). In recent years, use of statistical approach involving Plackert Burman (P-B) designing and response surface methodology (RSM) has gained lot of impetus for medium optimization and for understanding the interactions among various physico-chemical parameters using a minimum number of experiments (Gupta et al., 2004; Rathi et al., 2002). Medium that could support maximal lipase production by *P.gessardii* was optimized employing statistical approach.

From the results of Plackett-Burman design it is inferred that among the eleven variables screened during P-B design, five factors viz. incubation period, temperature, proteose peptone, ammonium sulphate and calcium chloride were found to be the most significant variables. The statistical significance of the model equation was evaluated by the *F-test* analysis of variance (ANOVA), which revealed that this regression is statistically significant. The Model F-value of 24673.85 implied that the model is significant. Values of "Prob>F" less than 0.05 indicate that the model terms are significant. This fit of the model was checked with the coefficient of determination R2, which was calculated to be 1.0000, Adj R-Squared 1.0000 and Pred R-Square 0.9994. This model can be used to navigate the design space.

The effect of individual parameters studied in P-B design evidenced that proteose peptone and ammonium sulphate had a positive effect in enhancing enzyme production along with increase in their concentration, followed by calcium chloride...
while incubation time and temperature had a negative effect on enzyme production along with increase in the variable.

Response surface methodology (using Box-Behnken design experiment) adopted towards selection of optimal level of the significant variable was analyzed ANOVA and shows that showed that Prob > F value was less than 0.05, which indicate that the model is significant. A high F-value and a very low probability (PF = 0.0001) indicated that the present model was in a good prediction of experimental results (Dutta et al., 2004). R², or determination coefficient, is the proportion of variation in the response attributed to the model rather than to random error (Heni, 1972). The R value always lies between 0 and 1 and for a good fit of model, R2 should be at least 0.80 (Joglekar and May, 1987). Similarly, Doddapaneni et al. (2007) suggested that closer the value of R² to 1.0, the stronger the model and the better its prediction efficiency of the responses. The model predicted maximum lipase production up to 396.7 U/ml. An overall 2.4 -fold increase in lipase production was achieved after validation of RSM in shake flasks.

Validation of the deduced response surface model based on the previous experiments was carried out in shake flasks under conditions predicted by the model. The experimental values were found to be very close to the predicted values and hence, the model was successfully validated. The statistical approach employed in process optimization led to the identification of the variables for obtaining maximum lipase production by P. gessardii.

5.5. PURIFICATION OF LIPASE ENZYME

The number of commercially available lipases has increased considerably in recent decades, along with the demand for these biocatalysts (Jaeger and Eggert, 2002). The characterisation of new lipolytic enzymes, the development of new purification procedures and the increased number of studies, mainly on lipases of microbial origin, are all factors that influence the novel biotechnological applications of these enzymes (Silva et al., 2009; Trincone, 2010). Lipolytic enzymes are subdivided into different groups including carboxyl esterases, lipases and sterol
esterases. Some of these enzymes show very wide substrate specificity and it is not always possible to decide the group to which they belong (Calero-Rueda et al., 2002).

Lipases were reported to be extensively purified and in terms of their activity and stability profiles relative to pH, temperature, and effects of characterized metal ions, oxidizing agents, reducing agents and organic solvents. In many cases, lipases have been purified to homogeneity and crystallized (Sharma et al., 2001b). After an initial screening of bacteria for lipolytic activity P.gessardii was shown to produce a potent lipase. In the present investigation, a unique electrophoretically homogeneous lipase was purified from the liquid cultures of P.gessardii using conventional methods and a 12.3 fold purified enzyme was obtained after ion exchange chromatography with a specific activity of 1872.0 U/mg could be achieved.

Several authors reported the purification of Pseudomonas lipase enzyme. A lipase from Pseudomonas aeruginosa KKA-5 was purified by ammonium sulphate precipitation followed by separations using chromatography on hydroxyl appetite and achieved 516 fold pure enzyme (Sharon et al., 1998). Similarly Kukreja and Bera. (2005) partially purified lipase enzyme produced by Pseudomonas aeruginosa MTCC 2488 by ammonium sulphate precipitation, showed 20.79 fold increase in specific activity (U/mg) and 1.7% reduction in carbohydrate content as compared to crude enzyme. Likewise an organic solvent-tolerant lipase from newly isolated Pseudomonas aeruginosa LX1 has been purified by ammonium sulfate precipitation and ion-exchange chromatography leading to 4.3-fold purification and 41.1% recovery (Ji et al., 2010).

SDS-PAGE analysis of the purified enzyme and the molecular mass of lipase, estimated by comparison with the electrophoretic mobility of marker proteins indicate that the P.gessardii lipase has an apparent molecular mass of ~ 75kDa. The denatured molecular mass (75 kDa) of P.gessardii lipase is in the range reported for other enzymes with lipolytic activity that generally bacterial lipases range in size from about 30-75 kDa (Kawasaki et al., 2002).
Similar results on bacterial lipase having the molecular mass of more than 60KDa \textit{P. fluorescens} HU380 (Kojima and Schimizu, 2003), \textit{Pseudomonas} sp. S5 (Rahman \textit{et al.}, 2005), \textit{P. aeruginosa} PseA (Gaur \textit{et al.}, 2008), \textit{Geobacillus stearothermophilus} strain-5. (Sifour \textit{et al.}, 2010) are reported. However several reports are available on the molecular mass of less than 50KDa \textit{Pseudomonas} and \textit{Bacillus} bacterial lipases (Kumar \textit{et al.}, 2005; Shaixin \textit{et al.}, 2007; Borkar \textit{et al.}, 2009 ; Cadirci and Yasa, 2010 and Bora and Bora, 2012). Most of the lipase purification schemes described in the literature focused on purifying small amounts of the enzyme to homogeneity to characterize it. Whereas little information has been published on large-scale processes for commercial purification of lipase. May be the reason is that most commercial applications of lipase do not require highly pure enzyme and excessive purification is expensive and reduces overall recovery of the enzyme (Chisti, 1998).

The zymogram showed a single fluorescent band obtained with ion exchange purified sample. Results confirm the purity and activity of the purified lipase obtained from \textit{P. gessardii}. An important advantage of the zymographic technique is that after activity detection, the same gels can subsequently be stained with a conventional dye in order to determine the molecular mass of the active proteins (Prim \textit{et al.}, 2003).

5.6. CHARACTERIZATION OF PURIFIED LIPASE ENZYME

The lipase enzyme was active over a wide range of temperature while recording maximal activity at 45°C. Temperatures above 50°C led to a sharp decline in enzyme activity. The enzyme retained 52% and 67.2% of its maximum activity at 25 and 55 °C, respectively. Similarly Rahman \textit{et al.} (2005) reported that optimum activity of thermostable purified lipase of \textit{Pseudomonas} sp was at 45°C and retained 86 and 52% of its maximum activity at 37 and 50 °C, respectively. Sharma \textit{et al.} (2001b).reported that their \textit{Pseudomonas} sp. AG-8 lipase had optimal activity at 45 °C. Lin \textit{et al.} (1996) reported that \textit{P. pseudoalcaligenes} F-111 lipase had an optimal temperature of 40°C. The optimum temperature of \textit{Pseudomonas} lipases,
such as those from *P. fragi* (Mencher *et al.*, 1967), *P. fluorescens* 2D (Makhzoum *et al.*, 1996), *P. fluorescens* HU380 (Kojima and Shimizu, 2003), *P. mendocina* (Jinwal *et al.*, 2003) and *P. aeruginosa* PseA (Gaur *et al.*, 2008) were found to be optimally active at 35–45°C which is similar to this result.

Temperature stability studies conducted using lipase showed that the enzyme was stable at 45°C for more than 180 minutes and retained up to 100% activity, suggesting the thermostability of enzyme. The lipase enzyme retained more than 57% activity between 30°C and 50°C for at least 180 mins. Apart from the species of *Bacillus* and *Pseudomonas* genera, there are only a few examples of bacteria reported to produce lipases that are active and stable above 50°C (Lima *et al.*, 2004). Thermostability is a desirable characteristic in lipases used for applications in different industrial processes operating at high temperatures (Nawani and Kaur, 2007; Sharma *et al.*, 2002a). Thus, the high activity and stability of the new lipase from *P. gessardii* in the temperature range of 40–50°C points to its suitability for applications in biocatalytic processes at high temperatures.

The purified lipase exhibited an optimum pH between 6.0 and 9.0 and the maximal maximum activity was observed at pH 7.0 in agreement with most other lipases from the *Pseudomonas* sp. (Baharum *et al.*, 2003; Boran and Uğur, 2010; Cadirci and Yasa, 2010; Ji *et al.*, 2010; Li *et al.*, 2011; Ogino *et al.*, 2004; Wang *et al.*, 2009; Zouaoui *et al.*, 2012). It was also noted that the enzyme was totally inactive at pH below 4.0 and above 10.0. More than 70 % of maximal enzyme activity was recorded at pH in the range between 6.0–9.0. The optimal pH of lipase from *P. gessardii* was significantly different from that of other *Pseudomonas* reported, such as *P. fluorescens* NS2W (Kulkarni and Gadre, 2002), *P. fluorescens* HU380 (Kojima and Shimizu, 2003), *P. aeruginosa* PseA (Gaur *et al.*, 2008) and *P. aeruginosa* CS-2 (Peng *et al.*, 2010) whose optimal pH were 9.0, 8.5, 8.0, and 8.0 respectively.

The lipase enzyme showed good pH stability retaining 100% activity for 180 minutes at pH 7.0. In the acidic range, significant reduction in enzyme activity was
observed. For instance, at pH 5.0 the lipase retained only 15% of its maximum activity. According to previous reports, *Pseudomonas* lipases do have some special characteristics, such as thermophile, thermostability and wide pH range (Cadirci and Yasa, 2010; Gao *et al.*, 2000; Ji *et al.*, 2010). Thus, the high activity and stability of this lipase in neutral conditions suggests its usefulness in a range of industrial applications.

Effect of oxidizing agent on enzyme activity was studied using hydrogen peroxide. It is evident from the data that 100 % of enzyme activity was retained in the presence of 1 % hydrogen peroxide and 53 % of enzyme activity was retained even upto 4% concentration studied. The enzyme was inactive at concentration of above 9% hydrogen peroxide. Thus it is concluded that the enzyme stability declined along with increase in concentration of hydrogen peroxide. The current thrust for novel enzymes that tolerate oxidative stress makes the present lipase of high commercial value.

Effect of reducing agents on lipase activity was evaluated using β- mercaptoethanol. Results indicate that the reducing agents have a positive effect on enzyme activity except at highest concentration tried. β- mercaptoethanol led to an enhancement in enzyme activity along with increase in concentration from 0.2 % to 1 % upto 30 mins. At concentration higher than 1% the residual enzyme activity was declined. A similar effect of reducing agents was observed for *A. mediterranei* DSM 43304 and *G. thermoleovorans* YN lipase (Dheeman *et al.*, 2011; Soliman *et al.*, 2007).

The stability in surfactant and bleach oxidants is desirable for lipases to remain active in detergent formulations, and has been achieved by protein engineering (Sangeetha *et al.*, 2011). Surfactants facilitate the access of substrate to the enzyme by stabilizing the interfacial area where catalytic reaction of lipase takes place (Sing and Banerjee, 2007a). Among the surfactant lipase was stable in Tween 20 and Triton X- 100 for 30 mins and activated enzyme activity. Sodium deodecyl sulphate completely inhibited lipase enzyme activity. The residual enzyme activity was reduced with surfactant Tween 80, Brij 35 and Gum Arabic. In agreement with
the present study thermostable lipase from *Burkholderia cepacia* ATCC 25416 was apparently inhibited by PMSF, EDTA and also DTT with SDS (Wang et al., 2009b).

In contrast Gaur et al. (2008) reported that Non-ionic detergents Tween-80 and Brij-35 stimulated the lipase activity and anionic surfactant sodium deoxycholate caused only 10% reduction in activity of *Pseudomonas aeruginosa* PseA lipase. Likewise *B. cepacia* and *G. thermoleovorans* YN lipases were reported to be stable with significant residual activity in SDS (Soliman et al., 2007; Yu et al., 2009b). Besides direct activation or inactivation, detergents may alter the hydrophobicity of the enzyme and affect micelle formation, thus the availability of substrate to the enzyme. Therefore, the effects of detergents on enzyme activity are the sum of many parameters (Aloulou et al., 2007; Helistö and Korpela, 1998). Thus, the stability in non-ionic surfactants add novel properties to this lipase for applications in detergent formulations.

The stability or enhancement of activity in presence of organic solvents is generally considered a desirable feature, as it is a prerequisite for synthetic applications in non-aqueous solvents (Klibanov, 2001). Lipases are diverse in their sensitivity to organic solvents, but there is general agreement that water miscible hydrophilic solvents are more destabilizing than water immiscible hydrophobic solvents (Doukyu and Ogino, 2010). Lipases are known for their ability to work in aqueous as well as in organic solvents. Effect of organic solvents on lipase activity was tested using different organic solvents up to 2 hrs. Lipase produced by *Pseudomonas gessardii* was stable in the organic solvents benzene, hexane, methanol, diethyl ether and ethanol for 2 hrs at 10% concentration while the enzyme activity was inhibited by butanol and isopropanol.

Similar to this result relative activity of purified *Pseudomonas* S5 lipase increased with organic solvent cyclohexane, benzene, and n-hexane after 30 minutes of exposure (Rahman et al., 2005a) and *Pseudomonas* sp. AG-8 lipase was activated in the presence of ethanol and methanol (Sharma et al., 2001a). In contrast, the stability of LST-03 lipase was lower in the presence of alcohol such as butanol,
ethanol, and 1,4-butanediol (Ogino et al., 2000) and the relative activity of purified acidic lipase of \textit{P.gessardii} was decreased when treated with 10\% organic solvents like methanol, ethanol, acetone and isopropanol for 2 hrs (Ramani et al., 2010b).

At higher concentration of 20\% all other organic solvent except hexane and benzene reduced the enzyme activity. Similarly several authors reported that the higher concentration of organic solvent significantly reduced the enzyme activity (Rahman et al., 2005a; Ramani et al., 2010b). This phenomenon is due to the high toxicity of the organic solvent to the enzyme (Sikkema, 1994). Similarly, Sugihara et al. (1992) reported that \textit{Pseudomonas cepacia} lipase was inactivated at all concentrations of benzene and hexane after 30min incubation under assay conditions. In contrast Gaur et al. (2008) studied the effects of various organic solvents at 25\% concentration for 24 hour and reported that the \textit{P. aeruginosa} lipase was stable in the presence of polar solvents like DMSO, methanol, ethanol and isopropanol as well as non-polar hydrophobic solvents.

Though solvent stability is reported for other \textit{Pseudomonas} lipase (Rahman et al.,2005a) the stability/activation in polar solvents like methanol and ethanol has rarely been observed (Ogino et al., 2001). It is reported that polar solvents strip off the essential water molecules from the active site of enzymes. For this reason, use of polar solvents is avoided and hydrophobic solvents are more often employed in non-aqueous enzymology. The polar solvent tolerant lipases therefore appear promising for catalysis in low water medium.

The property of the retaining enzyme activity at 10\% and 20\% organic solvent concentrations is observed as a novel attribute of \textit{P. gessardii} lipase produced in the present study and similar attributes are also reported in few cases viz. \textit{Bacillus megaterium}, \textit{P. mendocina}, \textit{Serratia marcescens}(Zhao et al., 2008) and \textit{P.aeruginosa} (Ogino et al., 2000; Gaur et al., 2008). The enzyme activity retainability of \textit{P. gessardii} lipase in organic solvents and its hydrolytic activity makes it a potential catalyst to be employed for many practically industrial important applications.
Lipase activity measurement was made for different substrate concentrations at constant temperature and pH to determine kinetic parameters, maximum reaction rate (Vmax) and Michaelis–Menten constant (Km). Lineweaver–Burk plots were linear and indicated that hydrolysis of various triglyceride esters by the lipase followed Michaelis–Menten kinetics. The graph gave two kinetic parameters, Km and Vmax, which show the substrate affinity of enzyme. The smaller Km value indicate the higher enzyme affinity to substrates. It was reported that the Km values of most industrial enzymes are varied in the range of 10⁻¹ to 10⁻⁵ M when acting on biotechnologically important substrates.

Spectrophotometric determination of lipase using p-NPL has been reported by several authors (Nawani et al., 2006; Bisht and Panda, 2011; Chen et al., 2011). Lipase activity is determined by hydrolysis of p-nitrophenyl esters of fatty acids with various chain lengths. p-nitrophenyl derivatives of fatty acids (generally lauric or palmitic acids) have been popular. p-nitrophenyl laurate appears to be stable at elevated temperatures (Hasan et al., 2009).

In this study kinetic parameters Km and Vmax values were determined, as a function of p-nitrophenyl laurate concentration for the purified neutral lipase calculated from Michaelis–Menten plot. The purified acidic lipase showed lower Km value (0.24 mM) and higher Vmax value (0.35 mM/min) than the other Pseudomonas sp. Lipase reported in literature (Gaur et al., 2008; Ramani et al., 2010b). Borkar et al. (2009) determined kinetic parameters for P. aeruginosa and reported that Km values of p-NPL and p-NPP were 0.11 and 0.037 and Vmax values were calculated to 161.3 and 188.6 mmol/L/min respectively for p-NPL and p-NPP. Similarly Ghor et al. (2011) determined the Km and Vmax of Bacillus sp lipase using pNPL as a substrate. The Km and Vmax values for the free enzyme was estimated from Lineweaver-Burk plot were 0.345 Mm and 7.61 μM /ml / min. These results reveal that the lipase from the bacteria displayed high affinity for p-nitrophenyl laurate. The lower value of Km represents higher affinity between
enzymes and substrates while Vmax represents the higher catalytic efficiency of lipase (Zhou et al., 2012).

The characterization of purified lipase produced by P.gessardii showed stability in organic solvents, oxidizing agent and reducing agents, and hydrolytic activity. These properties make this lipase an ideal candidate for biocatalysis in organic media for the production of novel compounds.

5.7. MOLECULAR CLONING AND TRANSFORMATION OF LIPASE GENE

In the present study, amplification of the genomic DNA with degenerate primers yielded an amplicon of 1748bp was cloned into the T/A vector, transformed in E.coli DH5a, screened by blue/white selection. Presence of recombinant plasmid with the insert was reconfirmed by amplification of the insert with specific primer. Based on the Blastx analysis it was found that partial coding sequence revealed high similarity to that of other Pseudomonas lipase genes deposited in the GenBank. However, more sequencing will be needed to obtain the full length lipase gene in an attempt to express this gene and characterize the lipase in term of kinetic properties towards different substrates and to classify the enzyme. Many lipase genes have been cloned and sequenced (Cho et al., 2000; Rahman et al., 2005; Saeed et al., 2006). However, recombinant enzyme production has been limited to a few microbial lipases (Houde et al., 2004).

5.8. CONCLUSION

The main purpose of this study was investigation, production, optimization, purification and characterization of lipase enzymes which was isolated from oil spilled soil. Pseudomonas gessardii gave positive results to lipase activity in different lipase assay protocols. The results obtained from the present study indicate the scope for the utilization of the soil bacteria Pseudomonas gessardii for extracellular lipase production employing submerged fermentation. To the best of our knowledge this is the first report on the production of thermotolerant neutral lipase by a mesophilic bacteria isolated from oil spilled soil employing statistical
modeling towards industrial production. The characterization of purified lipase produced by \textit{P. gesssardii} showed stability in organic solvents, oxidizing agent and reducing agents. These properties make this lipase an ideal candidate for biocatalysis in organic media for the production of novel compounds. There is ample scope for further research on the biochemistry of the enzyme, structure elucidation and enzyme engineering towards wide range of further applications.