Materials and Methods

3.1 Survey and Collection of Samples

Periodical survey was undertaken during 2000 to 2004 throughout Chitradurga and Davangere districts of Karnataka state from different natural sources like, ground nut processing unit samples from oil mills. Ground cake samples, uncleaned wastes of mills, the processing unit effluents, oil contaminated soil and also soil from oil ghana premises. The groundnut cake samples collected from different commercially available fodder shops. The groundnut kernels and processing unit samples with high oil content were procured from the oil refinery industries were shown in Table 3.1. Oil contaminated soil samples from groundnut crushing industries were collected by using sterile spatula and transferred to clean sterile containers. The samples were collected into clean, dry and sterile polythene bags under aseptic conditions. Thus collected samples were brought to the laboratory for further process and investigations. Each sample was subjected to various standard microbiological techniques as described by Whittaker (1992) for the isolation of microorganisms.

Table 3.1: Details of Groundnut industry samples.

Samples	During the period of study				Total
	2000-2001	2001-2002	2002-2003	2003-2004	
Groundnut cake	9	9	6	6	30
Processing unit samples	2	2	8	8	20
Spoiled Groundnut kernels	18	18	16	16	68
Groundnut oil spilled floor samples	5	5	5	5	20
Groundnut effluents industry samples	8	8	8	8	32
Oil contaminated soils	15	15	15	15	60
Scrapings of Groundnut oil storage utensils	8	8	8	8	48
				Total	**278**

3.2 Isolation and Primary Screening of Microorganisms

3.2.1 Fungi

One gram of each representative soil sample was taken into a test tube and mixed up with 9 ml of sterile saline water and shaken vigorously for 5 minutes to prepare a suspension. One ml of soil suspension was serially diluted up to 10^{-6} and 0.1 ml of the aliquot of this diluent was spread onto potato dextrose agar (PDA) plates (pH 5.6) for isolation of fungi (Benson 1994; Collins and Lyne, 1998). To discourage the growth of bacteria and other unwanted microorganisms and to encourage the fungal growth exclusively, antibiotic streptomycin (1mg/100ml of media) was incorporated in the medium.

The groundnut cake samples collected from different commercially available fodder shops. They were further cut into small pieces (about 10 mm square), pressed onto PDA plates and incubated at room temperature for mould growth. The processing unit samples with high oil content (turbid liquid) were directly streaked on to PDA plates.

Damaged groundnut kernels (10 grams) collected from oil refineries were immersed in 10 ml potato dextrose broth in a test tube for enrichment. The test tube was gently shaken for over 10 minutes, 1ml of this enriched culture was serially diluted up to 10^{-7} and 0.1ml of diluent was inoculated onto PDA plates by spread plate method (Svenson, 1994). The PDA plates inoculated with samples from various natural sources were incubated at 25$\pm$2°C for 4-8 days. At the end of every 24 hours the plates were observed for the growth, observations were made up to 6 to 7 days of incubation.

The colonies were selected and isolated by picking from the margins were sub cultured onto fresh PDA plates for obtaining pure cultures. These isolates were separately maintained on PDA slants and refrigerated at 4°C for preservation for further characterization (Domasch *et al.,* 1990 and Barnett, 1990).

3.2.2 Bacteria

The samples collected were air-dried for one week and kept at 45°C for 1 hr to minimize bacterial contamination. About 1gm of representative soil sample was transferred to an Erlenmeyer's flask containing 99ml of sterile nutrient broth (pH 7.2). The suspension was further diluted to 10^{-5} dilution level. One ml of the diluted suspension

was spreaded over the nutrient agar to encourage the bacterial growth. The petriplates were then inoculated at 37°C and colonies were observed after 24 hours. These colonies were picked up and pure cultures were established on Tributyrin Agar. Simultaneously the pure cultures were also maintained in nutrient agar slants and preserved at 4°C.

3.2.3 Yeasts

One gram of each representative soil sample was taken into a test tube and mixed up with 9 ml of sterile saline water and shaken vigorously for 5 minutes to prepare a suspension. One ml of soil suspension was serially diluted up to 10^6 and 0.1 ml of the aliquot of this diluent was spread onto yeast peptone dextrose agar (YPDA) plates (pH 5.6) for isolation of yeast (Collins and Lyne, 1998).

3.3 Screening of Lipase Producing Fungi

The isolation of molds after primary screening was plated on to modified CZA supplemented with olive oil (Table 3.2). The mold grown on olive oil medium (CZA)/MEA after four to five days incubation was considered as a positive for lipolytic activity.

Table-3.2: Composition of the modified CZA supplemented with olive oil.

$NaNo_3$	-	5 gm
$MgSO_4.7H_2O$	-	0.5 gm
KCl	-	0.5 gm
KH_2PO_4	-	1 gm
Na_2HPO_4	-	3 gm
$ZnSO_4.$ 7 H_2O	-	0.01 gm
$MnSO_4$.H2O	-	0.01 gm
Sucrose	-	5
Olive Oil	-	(1% v/v)

CZA medium containing olive oil (5 v/v) and Chloromphenicol (100 mg/ml)

Malt extract Agar

KH_2PO_4	-	6 g/l
Urea	-	49/l
$MgSO_4$ $7H_2O$	-	1 g/l
Micronutrients	-	$Fecl_3$ 0.6 H_2O
Inositol	-	0.008 mg/l
biotin	-	0.2 mg/l
Carbon Source	-	10 gm/l of Olive oil/Glycerol/ Oleic acid

3.4 Screening of lipase producing bacteria

Interest on microbial lipases has been increased markedly in last two decades (Maarteen, 1989). Certain bacteria produce and secret lipases that hydrolyze triglycerides, diglycerides, monoglycerides, glycerol and fatty acids of long chain acylglycerols. Many microbiologists generally use simple and reliable plate assays for primary screening,

Initial screening for the lipolytic bacterial species was carried out by enrichment culture at 30°C in yeast extract liquid medium supplemented with olive oil with constant shaking at 120 rpm for five days described by Sugihara *et al,* (1991). The composition of the medium is given in Table 3.3. The colonies which developed were further purified by streaking on the same medium. The lipase producing colonies were detected by plating on tri-butyrin agar medium (Table 3.4). The colonies showing larger and clear zones were identified as lipase produces. The strain showing maximum size of clear zone was selected as potent lipolytic enzyme producer.

Table 3.3: Composition of the yeast extract liquid medium supplemented with olive oil.

Yeast extract - 4.0 gm

Peptone - 2.5 gm

Glucose - 1.0 gm

Starch - 1.0 gm

Olive oil - 10.0 gm

KH_2PO_4 - 0.5 gm

$MgSO_4.7H_2O$ - 0.5 gm

Nacl - 0.25 gm

Distilled water- 1000 ml (pH - 7.5)

Table-3.4: Composition of Tributyrin agar

Fat-splitting organisms cause spoilage in buffer etc., most of these bacteria split glycerol tributyrate (Tributyrin).

Peptone	-	5 gm
Yeast extract	-	3 gm
Tributyrin	-	10 gm
Agar	-	20 gm
Water	-	1000 ml

Heat to dissolve, adjust pH 7.5 tube or bottle autoclave at 115°C for 15 minutes. Colonies of lipolytic microorganisms Clear the medium.

3.5 Screening of lipase producing yeast

Yeasts obtained from the plates with serial dilution of 10^4 were transferred to test the ability to produce lipases, tested by using a medium supplemented with 5% v/v olive oil, emulsified by vigorous shaking. After 10 days of incubation, the plates were flooded with saturated copper sulphate and appearance of colony with a bluish colour was considered as positive for lipolytic yeast.

3.6 Identification and characterization of fungal isolates

Twenty eight moulds were isolated from different samples soil, groundnut cake and processing industry wastes were plated on PDA for primary screening. These cultures were further subjected for morphological studies by low and high power microscopy. Characterization was done by staining technique to observe the mycelial structures, color, shape, size and ornamentations of spores and also many other details of special structures etc. The cultures were stained with lactophenol cotton blue and were observed under10X and 100X under oil-immersion for more detailed observations for some of the structural specialties such as mycelial attachment, ornamentation of spores etc., (Koneman, *et al.*, 1972) The dimensions of the spores and sporogenous structures were determined by micrometry (Pathak, 1984). They were identified as per the key suggested by Simmons (1967), Raper and Funnel (1965), Samson (1974; 1979), Ellis (1971), Christensen and Raper (1978), Alexopoulos and Mims (1988). Finally, 13 molds belonging to four genera-*Aspergillus, A. niger, A. fumigatus, Aspergillus sydowii, A. nidulans, A. terreus, A. glaucans, Mucor meihei, Fusarium - F. oxysporum, F. monaliformae, Penicillium*

marnefii, Cladosporium sp. *Nuerospora* sp. *Histoplasma capsulatum* were subjected for further screening to evaluate their lipid degrading ability.

3.7 Identification and characterization of bacterial isolates

3.7.1 Gram staining

A thin bacterial smear was made on a clean slide which was air dried and fixed by flaming. The smear was flooded with crystal violet solution for 1 min, decolorized with 95% ethanol for a few seconds and then finally counter-stained with safranin solution. The slide was blot dried and observed under microscope under oil immersion (Schaad, 1988).

3.7.2 Biochemical tests

Various biochemical tests were performed by taking 48 hour old cultures grown on nutrient agar medium.

3.7.2.1 KOH *solubility test*

A drop of freshly prepared 3% KOH solution was taken on a clean slide and it was mixed with a loopful of test bacterial culture with the help of a tooth pick for not more than 10 sec. Tooth pick was raised a few centimeters from the slide and was observed for the formation of a mucoid thread (Schaad, 1988).

3.7.2.2 Casein hydrolysis

24-48 hours old cultures were inoculated onto skimmed milk agar medium, incubated at 37°C for 24 hours and observed for clear zone formation around the bacterial colony (Benson 1994).

Skim milk agar medium

Peptone	-	5gm
Skim milk powder	-	100gm
Agar	-	20gm
Distilled Water	-	1 ltr.
pH	-	7.2

3.7.2.3 Litmus milk reactions

Litmus milk (0.4%) was prepared in distilled water. The medium was transferred to test tubes and autoclaved at 121°C for 20 minutes. After cooling the tubes were inoculated with test isolate. Negative controls were maintained. The tubes were incubated at 26°C for three days and change in the color of litmus was observed (Schaad, 1988).

3.7.2.4. Carbohydrate fermentation

The fermentation ability of carbohydrates such as glucose, sucrose and lactose by bacterial cultures was carried out in a fermentation tubes (Table 3.5), that contains Durham's tube placed in an inverted position for the detection of gas production. The observations were made for any colour change in the medium due to change in the pH (Collie and Miles, 1996).

Table 3.5: Composition of carbohydrate fermentation medium:

Component		Amount
Beef Extract	-	3gm
Yeast extract	-	3gm
Peptone	-	20gm
Glucose	-	1gm.
Lactose	-	10gm.
Sucrose	-	10gm.
Ferric citrate	-	0.3gm
Sodium Chloride	-	5gm.
Sodium thiosulphate	-	0.3gm
Agar	-	12gm.
Phenol red, 0.2% solution	-	12ml
Distilled Water	-	1 ltr.
pH	-	7.2

3.7.2.5. H_2S production

Test tubes containing peptone solution (1%) were sterilized at 121° C for 20 min. Filter paper strips (0.5 x 7.5cm) soaked in saturated solution of lead acetate were sterilized and dried in an oven at 60°C, and placed at the mouth of the test tube in such a way that one half of the strip was hanging below the cotton plug and the other half

remained outside. Tubes were inoculated in duplicates. Negative control were maintained without culture medium. Tubes were incubated at 26°C for three days and observed (Schaad, 1988).

3.7.2.6. Nitrate reduction

The Nitrate Reduction Test demonstrates the presence of the enzyme nitrate reductase, which causes the reduction of nitrate to nitrite. It was tested by the addition 0.1 ml MR test reagent to the culture and incubated at 37°C for 96 hrs. A red color development within few minutes indicates the presence of nitrite (Schaad, 1988).

Nitrate Reduction (NR) Medium

Potassium nitrate	-	0.2g
Peptone	-	**5g**
Distilled Water	-	1 Ltr.
pH	-	7.0

NR Test Reagent:

Solution A: 8 gms of sulphanilic acid in 1 L of acetic acid.

Solution B: 5 g of a-naphthylamine in 1 L of acetic acid. Mix 1:1 A and B solutions before use.

3.7.2.7. Indole production

Indole test was conducted to test the ability of certain bacteria to decompose the amino acid tryptophan to indole. Tryptone broth inoculated with 24-48 hrs old bacterial culture and incubated for 48 hrs. at 37°C. Indole production was then tested by the colored reaction with the addition of Kovac's reagent. The red colour in the alcohol layer indicates a positive reaction (Collie and Miles, 1996).

Kovac's reagent

p-dimethylaminobenzaldehye	-	5gm
Amyl alcohol	-	75ml
Concentrated HCl	-	25ml

(Dissolve the aldehyde in alcohol slowly add acid)

3.7.2.8. Methyl red test

Many bacteria produce large amounts of lactic, acetic, succinic and formic acids when they ferment sugar these acids results in lowering of pH of the medium to 5.0 or less. MR – VP broth (Table 3.6) was inoculated with bacteria and incubated at 37°C. After incubation, about five drops methyl red was added to cultures and observed for color change, if it turns red suggesting that the organism is mixed acid fermenter indicating the positive test (Cuppuscino, 1994).

3.7.2.9. Voges-Proskauer test

Bacteria may utilize sugar and produce 2, 3-butanediol and ethanol instead of acids. Presence of butanediol in the culture broth cannot be directly tested. However, acetion (acetyl methyl carbinol), a precursor of butanediol is easily detected with barritt's reagent. MR – VP broth (Table 3.6) was inoculated with bacteria and incubated for 24-48 hrs. A positive reaction was confirmed by the development of a pink or red color.

Table 3.6: Composition of MR - VP broth

Component		Amount
Peptone	-	5gm
di-potassium hydrogen orthophosphate	-	5gm
Glucose, 10% solution (sterilized separately)	-	500ml
Distilled water	-	1Ltr.
pH	-	7.6

Barritt's reagent:

Solution A: 6g a-naphthol in 100ml 95% ethyl alcohol

Solution B: 16g potassium hydroxide in 100 ml water.

3.7.2.10. Citrate utilization

Certain bacteria are capable of using citrate as a carbon source. To test for the ability of an organism to utilize citrate as the sole carbon and energy source for growth Simmon's citrate agar medium (Table 3.7) is used. The medium was inoculated with 24-48 hrs. Old bacterial cultures and incubated up to 96 hrs. at 37°C. Observations were

made for the color change from green to blue was due to change of pH in the medium (Collie and Miles, 1996).

Table 3.7: Composition of Simmon's citrate agar medium

Ingredient		Amount
Ammonium dihydrogen phosphate	-	1gm
Dipotassium phosphate	-	1gm
Sodium chloride	-	5gm
Sodium citrate	-	2gm
Magnesium sulphate	-	0.2gm
Agar	-	20gm
Bromothymol blue	-	0.08gm
Distilled water	-	1 Ltr.
pH	-	6.9

3.7.2.11. Urease activity

The presence of urease enzyme can be tested for by growing the organism in the presence of urea and testing for alkali (NH_3) production by means of a suitable pH indicator. Bacterial cultures was inoculated to urease medium and incubated at 37°C for 24-48 hrs. any change in the medium colour to purple pink was noticed for positive cultures.

Composition of urea broth:

Ingredient		Amount
Peptone	-	1gm
Sodium Chloride	-	5gm
K_2HPO_4	-	2gm
Phenol red (aqueous)	-	6ml
Distilled Water	-	1ltr.
10% Glucose solution	-	100 ml

(Sterilize the glucose and urea solution by filtration. Prepared the basal medium without glucose or urea, adjust to pH 6.8-6.9)

3.7.2.12. Catalase activity

1-2 drops of 3% hydrogen peroxide solution was taken on a clean glass slide, to this a loopful of 24-48 hrs. Old culture to hydrogen peroxide solution and mixed. Observation was made for the emergence of bubbles due to release of oxygen (Schaad, 1988).

3.7.2.13. Starch hydrolysis

Starch agar was prepared by the addition of 0.2% soluble starch to the nutrient agar, sterilized and dispensed into sterile petriplates. The plates were point inoculated with the test isolates. After incubation at 26°C for four days, the culture plates were flooded with Gram's iodine. The plates were observed for the formation of a clear zone of hydrolysis around the bacterial growth (Schaad, 1988).

Starch agar medium

Peptone	-	5gm
Starch (Soluble)	-	10gm
Beef extract	-	3gm
Agar	-	20gm
pH	-	7.0

3.7.2.14. Oxidase test

p-phenylene-diamine discs were placed in a plate and moistened with distilled water, and loopful of bacterial cultures was transferred onto the disc. This test was carried out to determine the presence of oxidases that catalize the transport of electrons between electron donors in the bacteria to a redox dye-tetramethyl-p-phenylene-diamine. Observations were made for the deep purple colour formation within 1 min (Schaad, 1988).

3.7.2.15. Gelatin liquefaction

Gelatin agar medium was stab inoculated and incubated at 37°C. Liquefaction of medium was tested at different time intervals by removing the nutrient gelatin cultures from the incubator and holding them in cool temperature by keeping in refrigerator for 30 min. or on ice cubes, Bacteria which are capable of producing the exoenzyme

gelatinasees hydrolyze the gelatin. Observations were made for the liquification of the medium after cooling (Benson 1994).

Gelatin liquification medium

Beef extract	-	3gm
Peptone	-	3gm
Sodium chloride	-	5gm
Gelatin	-	120gm
Distilled Water	-	1 ltr.
pH	-	7.0

3.7.2.16. β-Galactosidase test

The β-Galactosidase Test determines the presence of the enzyme β-galactosidase by utilizing o-nitrophenyl-β-D-Galacto-pyranoside (ONPG). The bacterial suspension was prepared in saline using 24-48 hrs old pure cultures. ONPG discs were immersed in bacterial suspension and incubated for 24 hrs at $37^{O}C$. cultures were observed for development of yellow color (Benson 1994).

3.8 Identification and characterization of yeast isolates

Yeasts are isolated from Groundnut wastes by plating on to YPD media. Yeast are fungi whose basic means is by budding, unlike moulds these fungi one generally identification by biochemical and morphological Criteria. The methods of identification of yeasts relay on a combination of morphological and biochemical characteristics. The principle morphological Criteria and tests include appearance colour of colonies (production of pigment), size and shape of cells, Presence of capsule Production of psuedohyphae, ability to produce germ tubes, ability to produce chlamydoconidia (Chlamydospores).

Biochemical Tests

These tests are used for testing the assimilation of carbohydrates and nitrate.

Assimilation of a Carbohydrates:- Prepared yeast Nitrogen base (Free from carbon some) Potassium di-hydrogen orthophosphate -1g, Magnesium sulphate –0.5g, Ammonium sulphate –5g, Agar 20gms, distilled H_2O 1 liter. Dissolved the ingredients

and boiled to melt the Agar dispensed into conical flask and autoclaved at 115°C for 15 min. whenever require4d melted the allowed to cool to 45°C and added 5 drops from a heavy suspension of yeast being identified (about 1 loopful of yeast colony in 5ml of distilled water) from the seeded Agar plates and placed the filter paper discs.

Petri plates are inoculated at 27-30°C for up to 48 hours. Growth of yeast around the disc is the indication of the assimilation of carbohydrate or individual compound when compound is not utilized no enhanced growth.

3.9 Determination of lipase activity

All the identified organisms (fungi, bacteria and yeasts were subjected for p-nitro-phenol palmitate assay to confirm their ability to produce the lipases.

3.9.1 p-nitro- phenol palmitate assay

Lipase assay was carried out on the basis of colorimetric principle. Lipases are known to hydrolyze triglycerides and give rise to free fatty acids and glycerol. The assay method for esterolytic activity was by employing either p-Nitro phenolpalmitate (pNPP) or p-nitrophenyl acetate (pNPA) assay (Winkler and Stuckmann, 1979). The reaction mixture consiststed of 900 µl buffer (100mM sodium phosphate- 150mM NaCl 0.5% v/v Triton X 100 (0.5% v/v) adjusted pH 7.0 with 1molar NaOH). 100 µl of enzyme sources during different schemes of fermentation withdrawn at the time of enzyme activity determinations. 10 µl of 50mM substrate in acetonitrile, the mixture was incubated for 5 minutes at 37°C. The colorimetric estimation of P-nitrophenol (pNp) released as a result of enzymatic hydrolysis of pNPA absorbance by the culture supernatant read at 410nm. Each assay was performed in triplicate, activity was quantified by using MEC of P-nitrophenol. One unit of enzyme activity was defined as the amount of enzyme liberated in µMol of p-nitrophenol/min released under assay condition.

3.10 Fermentation studies of lipase extraction

3.10.1 Fungi

3.10.1.1Submerged fermentation

The spores from 5 days old cultures were grown on PDA at 30° C, were suspended in (approximately 10^{10}-10^{12} spores ml^{-1}) in 5 ml of sterile 0.05 M phosphate buffer (pH 6.8) containing 0.1% Tween-80. 1 ml inoculum (2%) was used for inoculating

50 ml of culture medium in 250 ml Erlenmeyer flask containing (g/L) Olive oil – 5 gms; $(NH_4)_2 PO_4$ – 1 gm; KH_2PO_4 – 0.5 gms; $MgSO_4.7H_2O$ – 0.15 gms; $CaCO_3$ – 0.15 gms; pH was adjusted to 6.5 $\pm$ 0.1 with continuous shaking at 150 rpm on gyratory shaker for 7 days. 2 ml of samples were withdrawn for every 24 hrs and subjected for lipase assay.

3.10.1.2 Solid substrate fermentation (SSF) of molds

Solid substrates like wheat bran, rice bran, coconut oil cake, groundnut cake, coconut cake, ground nut kernel sugarcane bagasse, coconut oil cake : groundnut cake (1:1) were taken individually (20 grams) in an Erlenmeyer flask (250 ml) and moistened with mineral salt solution. The substrate was sterilized at 27°C for 1 hr.

20 gm of individual substrate sample was weighed and dried at 100° C for 6 hrs and taken in 250 ml conical flasks, the level of moisture was adjusted by adding 70% mineral salt solution. The sterilized substrate was inoculated with 2 ml of pre-culture prepared in CZA and incubated at room temperature 37° C for 2-8. 200 ml of 0.2 M phosphate buffer (pH 6.9) was added to the fermented broth and kept on orbital shaker at 200 rpm for 1 hr. The suspension was filtered through two layers of cheese cloth and the filtrate was centrifuged 10,000 rpm at 4° C for 15 min (REMI C-24). Supernatant was saved and used as source of lipase for performing the assay (Mulimani and Patil, 1995).

3.10.1.3 Influence of 1% carbon source on lipase production

The studies pertaining to the lipase production *Aspergillus terreus* GCN 8 supplemented with 1% starch, glucose, sucrose, fructose and lactose along with substrates of finely grind coconut cake: ground nut cake 1:1 ratio the carbon sources supplemented to the substrate examined for lipase yield. Incubated for seven days, after every 48 hours by taking two grams of substrate and dissolved in phosphate buffer 1:1 and assayed for lipase activity.

3.10.1.4 Influence of 3% nitrogen source on lipase production

The studies pertaining to the lipase production *Aspergillus terreus* GCN 8 supplemented with 3% yeast extract, peptone, yeast extract + peptone 1:1, tryptone, casein and beef extract along with substrates of finely grind coconut cake: ground nut cake 1:1 ratio the carbon sources supplemented to the substrate examined for lipase yield.

Incubated for seven days, after every 48 hours by taking two grams of substrate and dissolved in phosphate buffer 1:1 and assayed for lipase activity.

3.10.2 Bacteria

Submerged fermentation of bacterial isolates

Among lipase producing bacteria 8 species of bacteria are selected for fermentation studies. The bacterial strains cultured by submerged fermentation using 250 ml conical flasks one ml of 24 h growth having 10^6 CFU Ml-1 was inoculated in 100ml production medium. The medium used for submerged fermentation.

Production Medium

Olive oil	-	10gms
Peptone	-	5 gms
Yeast Extract	-	3 gms
Glucose	-	5 gms
NaCl	-	0.25 gms
$MgSo4.7H_20$	-	0.5 gms

Fermentation flasks incubated in room temperature. The conical flasks were continuously shaken using rotary shaker adjusted 110-140 rpm. The samples are withdrawn from the fermentation flasks after 24, 36, 48, 60, 72 and 96 Hours of growth. The culture fluid was centrifuged at 4000 rpm for 15 minutes, the cell free supernatant was used to measure the crude extracellular activity using spectrophotometer using p-nitro phenyl acetate as substrate.

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