International Journal of Current Science Research
www.drbgrpublications.in

Volume: 7; Issue: 6; June 2021; pp 2144-2170; ISSN: 2454-5422

Production of Protease and Lipase Enzyme Producing Microorganism from Tannery Effluent

Somaprabha. A[1*] and Durairaj. K[2]

[1]Department of Plant Biotechnology, School of Biotechnology, Madurai Kamaraj University, Madurai, India.

[2]Department of Zoology, Thiru.Vi. Ka. Government Arts College, Tiruvarur, India.

*Corresponding Author E-mail id: somabt2012@gmail.com

Abstract

Enzymatic dehairing in tanneries has been envisaged as an alternative to sulfides. *Bacillus sp* is industrially important enzymes producing a wide variety of extracellular enzymes (Protease, amylase, cellulose, lipases, pectinases and xylanases) producing microorganisms that are active and stable at high pH values. *Bacillus sp* isolated from local leather samples were collected from Salem. The present investigation on tannery effluent waste sample, was performed the collected tannery industry waste water was serially diluted, spread plated and characterized morphologically and biochemically. Screening of the isolates to detect the ability for extracellular protease and lipase enzyme by conforming measure of zone producing microorganism by *Bacillus subtilis* was studied. In order to enhance the lipase and protease enzyme supplementation of tween20/tween80 was performed. Determinations of protein contain for protease and lipase was carried out. In order to illustrated protease and lipase activity enzyme assay was performed. In order to enhance protease and lipase production tannery waste isolates. Optimization of experiments with pH, temperature, Incubation period, nitrogen source, carbon source, effect of NaCl was demonstrated. The optimum pH was found to be 7.0, optimum temperature was found to be 37 °C. Among the carbon and nitrogen sources were tested glucose and urea was found to be best source for enhanced enzyme activity. Another important study was incubation period for maximum production of enzymes as protease and lipase found at 24-48 hours. The agarose gel electrophoresis was run in order to check the plasmid DNA. The study was further extended to inoculate the lipase activity supplemented with different oils with carbon

source was demonstrated. Besides dehairing activity of the crude protease enzyme were analyzed.

Keywords: Tannery waste, *Bacillus subtilis*, protease, lipase, dehairing activity.

Introduction

Tanneries were constantly concerned with the obnoxious odour and the pollution caused by the extremely toxic sodium sulfide used in the dehairing process. It induces health hazards to human and other aquatic organisms. It also makes the land and soil infertile, the ground surface water turns to be unfit for irrigation and drinking (Sangita *et al*., 2012). Environmental pollution has become a major problem in developing countries in the last few decades. Major source of water pollution is the untreated industrial effluent. The discharge from the tannery industries is becoming a crucial problem to the environment, since the rate of discharge is alarming (Nadeem, 2000). In India, there are more than 3000 tanneries and processes about 7 million tons of hides and skin annually. About 90% of tanneries use chromium salts us tonnage materials because of the excellent properties of the chromium compounds in the tanning (Olsan *et al*., 2000).

Enzymatic dehairing in tanneries has been envisaged as an alternative to sulfides (Chakraborty *et al*., 2008). Tanneries are constantly concerned about the obnoxious odor and pollution caused by the extremely toxic sodium sulfide used in the dehairing process step (Kashayp *et al.,* 2001). Deaths due to this toxic chemical process have even been reported (Kocher and Mishra, 2009). Worldwide, it is estimated that 315 million bovine leathers are produced per year considering a waste treatment cost of $0.30 per m2 of leather produced (A. Klein, personal communication), more than $1 million is spent per day to treat the waste from tanneries around the world. We report here a novel keratinase from *Bacillus subtilis* that has the potential to replace sodium sulfide in the dehairing process.

Bacillus sp is industrially important enzymes producing a wide variety of extracellular enzymes (Protease, amylase, cellulose, lipases, pectinases and xylanases) producing microorganisms that are active and stable at high pH values. Some areas of application for alkaline stable enzymes are leather tanning, Paper pulp bleaching, production of cyclodextrins and waste treatment. A novel keratinase from *Bacillus subtilis* that has the potential to replace sodium sulfide in the dehairing process of leather industry (Kroll *et al.,* 1990).

Lipase produced by *Pseudomonas* play an important role in biotechnology both as hydrolases for detergent additives and synthesis catalyzing the kinetic resolution of ceramic compounds. Large-scale production of pseudomonas lipases needs correct folding and secretion through the bacterial membranes. Lipase from pseudomonas were excellent catalysts in a variety of organic transformations, also provide the selectivity of the hydrolysis of a crystal ester. Although a wide variety of Gram-positive and gram-negative bacteria species produce lipases, the most widely used enzymes originate from the genus pseudomonas. These lipases have been grouped into three categories according to amino acid sequence homolog (Manfred Reetz, 2002).

Enzymes were vitally important to the existence of life itself. Enzyme play crucial roles in food and textile industry. Enzymes were also important in reducing both energy consumption and combating environmental pollution. The use of enzymes in leather manufacturing process particularly for un hairing has not been accepted by the industry to the desired level. This is mainly because: a) enzymes are not effective enough to eliminate the sulfide completely, b) There is an apprehension that the enzymes assisted process needs stringent process control, and c) the cost of enzyme is not encouraging. Hence the present work has focused on screening for Proteolytic enzymes from suitable microorganisms, which is economically viable and effective enough to eliminate the sulfide completely (Arunachalam and Saritha, 2009).

Enzymes have a great industrial potential and are widely found in various sources like plants, animals and microbes. Microbes have undermined plants and animals as sources of enzymes due to their broad biochemical diversity case of mass culture and also due to the ease with which they can be genetically modified of all industrially important enzymes, proteases and lipases were exploited maximally due to their various applications. Lipases are of significant importance in leather industry and alkaline proteases are used in detergent formulations (Fujshige, *et al.,* 1992) for dehairing and bating process in leather manufacturing. Alkaline xylanases have been mainly used in the pre bleaching of paper pulp (Hameed *et al*., 1996). Textile industry is another important application area of alkaline pectinases and amylase (Esakkiraj *et al.,* 2007). Proteases are a group of enzymes whose catalytic function is to hydrolyze peptide bonds of proteins and breakdown them into polypeptides or free amino acid. Source of protease include all forms of life that is plants, animals and microorganisms. Screening of proteases producing *Bacillus sp*

from different ecological environments can result in isolation of new alkaline proteases with unique physio-chemical characteristics (Singh *et al*., 1999).

Lipases were water soluble enzymes which have the ability to hydrolyze triglycerols to release free acid glycerols. Lipases constitute a major group of biocatalyst that have immense biotechnology applications. Lipases have been isolated and purified from fungi, yeast, bacteria, plant and animal sources, of all these, bacterial lipases are more economical and stable (Jensen, 1983). High concentrations of free fatty acid or vegetables oil repress lipase synthesis. Hence many lipase production studies use tween80 as a sole carbon source. Nitrogen sources have varied effect on lipase production and sources like peptone have been reported to augment lipase production (Gunasekaran *et al*., 2006).

In the present study focused on isolation of microorganisms from tannery industrial effluents from Salem region. Screening of microorganisms for enzyme production such as lipase and protease were assayed. Since enzymes are vitally important to the existence of life itself. Besides, enzymes were also important for combating environmental pollution like untreated tannery effluents and in textile industries. Characterization of the isolate was carried out. Studies pertaining to optimization with various factors for enzyme production were performed. Molecular analyses, such as isolation of DNA and agarose gel electrophoresis were studied.

Material and Methods

Sample collection

The tannery effluent sample was collected from leather processing industry, Salem and transported to laboratory in sterile conditions.

Isolation of microorganisms

Samples were serially diluted with sterile distilled water and spread on the nutrient agar plates followed by incubation for 24-48 hrs at 37 °C for the growth of microorganisms.

Screening of protease secreting microorganisms

Casein hydrolysis

This test to detect the ability of microorganisms to hydrolyze the casein by means of the extracellular enzyme like protease. Skim milk agar was prepared and the isolates were streaked

on the centre of the plates for testing the proteolytic/caseinolytic activity of organisms. Incubate the plates at 37 °C for 24hours. Strains that produce clearing zones in this medium indicate a positive result. The isolated organisms from tannery effluent was cultured in casein agar plates containing nutrient agar with 1% casein and incubated at 30 °C for 48hrs.

Gelatin hydrolysis

The isolated organisms from tannery effluent was cultured in gelatin agar plates containing nutrient agar with 1% gelatin and incubated at 30 °C for 48hrs.

Screening of lipase secreting microorganisms

ROA plate assay

A sensitive and specific plate assay for detection o lipase producing bacteria makes use of Rhodamine-olive oil-agar medium (Bornsceuer *et al*., 2002). The growth medium contained (g/l): nutrient broth, 8.0; Nacl, 4.0 and agar agar 20. The medium was adjusted to pH 7.0, autoclaved and cooled to about 60 °C. Then 31.25ml of olive oil and 10ml of Rhodomine B solution (1.0 mg/ml distilled water and sterilized by filtration) was added with vigorous stirring. It was then poured into petri plates under aseptic conditions and allowed to solidify. The bacterial culture was inoculated on to the medium in these plates. Lipase producing strains were identified on agar medium after incubation for 48hrs at 37 °C. The hydrolysis of substrate causes the formation of orange fluorescent halos around bacterial colonies visible upon UV irradiation.

Tween 20/ Tween 80 hydrolysis

The hydrolytic activity of lipase was studied using tween 20 and tween 80 supplemented in medium composed of (g/l): peptone, 10; NaCl, 5; $Cacl_2.2H_2O$, 0.1; agar agar, 20; tween 20 or tween 80, 10ml (v/v). The bacterial culture was inoculated on to the medium in these plates. Lipases producing strains were identified on agar medium after incubation for 48 hours at 37 °C.

Characterization of the Bacterial Isolates

Morphological characterization

Morphological characteristics such as abundance of growth, pigmentation, optical characteristics, size and shape were studied on nutrient agar plates.

Gram staining

The isolates was smeared on the slide and heat fixed. The crystal violet dye was added, kept for 1 min and washed in running tap water. The gram's iodine was added, kept for 1 min and washed in running tap water. It was decolorized with ethanol and them counter-stained with safranine and washed in running tap water. It was observed in compound microscope. The bacteria that retained the crystal violet strain (appear violet) was designated as gram positive those cells that stained with pink colour are called gram negative.

Spore staining

The isolate was smeared in the slide and heat fixed. Then the slide was flooded with malachite green and steam heat the slide for 2-3 min. Cool the slide and washed in running tap water. It was then added with counter strain safranin and kept for 30 sec. wash the slide with running tap water. Air dry the slide and examined under oil immersion, in microscope. The spores appeared green in colour while the vegetative cells appeared red in colour.

Hanging drop method

One loop full of inoculum was kept at the center of the cover slip. The cavity of slide was placed over the coverslip and turned over to prepare a hanging drop. It was viewed under microscope. The motility was determined from the swarming movement of the microorganisms.

Biochemical Characterization

Indole Production test

Indole production test is used to test whether the organism can have the ability to produce indole. Peptone broth was prepared, sterilized and cooled. Inoculate the test organism n to the sterile peptone broth and incubate the tubes 37 °C for 24 hrs. the culture producing the cherry red coloring following the addition of Kovacs reagent indicated as positive. The absence of red coloration indicates a negative result.

Methyl Red test

Methyl red test was employed to detect the ability of microorganisms to oxidize glucose with the production of high concentration of acid end products. The isolated organisms were inoculated

into test tubes containing sterile MR-VP broth and incubate the tubes for 24 hrs at 37 °C. After incubation, add 7-8 drops of methyl red indicator and appearance of red color indicated the positive result.

Voges-Proskauer test

This is known as acetoin production test. This test was used to differentiate the capacity of organisms to produce some non-acidic (or) nautral end product such as acetyl methyl carbinol (or) 2, 3,-butanediol. The isolated organisms were inoculated into sterile MR-VP broth tubes and incubate for 24 hrs at 37°C. Development of deep rose colour following the addition of Barritt"s reagent A and B indicated the positive result. The absence of deep rose color is a negative result.

Citrate utilization test

Some of the organisms were capable of utilizing citrate as the sole carbon source and mono ammonium phosphate at the sole source of nitrogen. As a result, the pH of the medium change, this was indicated by changes in the indicator present in the medium. Simmon's citrate medium was prepared sterilized, and kept in a slanting position and allowed the tubes to solidify. The test organism was streaked on the slant and incubates at 37 °C for 24 hrs. The change of color from green to Prussian blue colored slant indicated the positive result.

Triple sugar iron test

TSI test was used to differentiate the isolate according to the ability to ferment lactose, sucrose and glucose and production of hydrogen sulfide. Trible sugar iron agar medium was prepared and sterilized. The tubes were kept on slant and butt and allow it to solidify. Streak a loop full of test organisms on the surface of the slant and incubate at 37 °C for 24 hrs. Acidification of the medium caused by the isolates attacking one of the sugar causes the phenol red indicator to change to yellow colour. Gas production is indicated by bubble formation in the butt. Hydrogen sulfide production causes the formation of a black precipitate at the junction between the slope and the butt.

Nitrate reduction test

This test was used to detect whether the organisms reduced the nitrates to nitrites or not. Nitrate broth was prepared and sterilized. Inoculate one loop full of test culture and incubate at 37 °C for

98 hrs. Following incubation, add 0.1 ml of test reagent (sulphanilic acid and alpha-napthalamine) to the test culture. A red colour developing within a few minutes indicates the presence of nitrites and hence the ability of the organisms to reduce nitrates.

Gelatin hydrolysis

Gelatin is an incomplete protein lacking the essential amino acids tryptophan and it acts as nutrient sources for many microorganisms. When gelatin is enzymatically hydrolysed into amino acids it loses its stability to become gel even at low temperature. Prepare nutrient gelatin tubes and sterilized. Inoculate the test organisms on to the tubes and incubate at 37 °C for 24-48 hours remain liquefied after 30 min showed positive result and that remained solid showed negative result.

Starch hydrolysis

This test is to detect the ability of microorganisms to hydrolyze the starch by means of the extracellular enzyme like amylase. Starch agar medium was sterilized and make a single line of streak of the organisms across the center of the starch agar plate. Incubate the plates at 37 °C for 24 hrs for sufficient growth. After incubation, flood the plate with iodine solution. Hydrolysis is indicated by clear zones around the growth which indicated the positive result.

Carbohydrate fermentation test

Prepare Sucrose and Dextrose broth (beefextract-3g/l, peptone-5g/l, Sucrose and dextrose-5g/l) and take about 5ml in test tube fill the durham's tube with broth and put it into the test tube taking care that no air bubble enter into it. Sterilized the broth and inoculated with isolated culture and then incubated at 37 °C for 24 hours. After incubation, the tube was observed for acid and gas production. Production of acid was indicated by change in color from green to yellow and production of gas was indicated by formation of bubble in Durham's tube.

Enzyme Assay

Assay of protease activity

Protease activity

The protease activity in the liquid medium was assessed first, by culturing the bacteria in an enrichment medium containing beef extract (0.3%), Peptone (0.5%), NaCl (0.5%), and glucose

(0.5%) at pH 7 for 24 h, and then 10% of enriched culture was inoculated in a 250 ml flask containing 45 ml basal medium containing (g/l): $(NH4)_2SO4$, 2g; K2HPO4, 1g; KH2PO4,1g; MgSO4.7H2O, 0.4g; MnSO4.H2O, 0.01g; FeSO4.7H2O, 0.01g; Yeast extract, 1g and peptone, 10g at pH 7.0. The culture was then incubated for two days by shaking at 32 °C. The cells were then harvested by centrifugation at 10,000 x g for 15 min, and the supernatant was used for further protease assay (Esakkiraj *et al*., 2007).

Protease assay

Protease activity was measured using 0.5 ml of glycine NaOH buffer (pH 10.0, 0.2M) which was added to 0.5 ml of appropriately protease diluted enzyme and then incubated with 1 ml of 1 development. For color development, 5 ml of 0.4M sodium carbonate solution was added to 1ml of the protease filtrate and kept for 10 min. To this, 1:1 diluted% casein solution (prepared in glycine NaOH buffer, pH 10.0) for 15 min at 60 °C. The reaction was stopped by the addition of 4 ml of 5% (v/v) trichloroacetic acid. The contents were centrifuged after 1h at 3000 x g for 10 min, and the filtrate was used to measure protease activity based on the basis of color Folin'sciocalteau phenol reagent was added and kept in the dark for 30 min, and the optical density was recorded at 660 nm using UV-visible spectrometer. The amount of protease produced was measured with the help of a tyrosine standard graph. Based on the tyrosine released, the protease activity was expressed in micrograms of tyrosine relased under standard assay conditions (Meyers and Ahearn, 1977).

Assay of lipase activity

Lipase Assay

Lipase activity was measured by titrimetric method using olive oil as a substrate. Olive oil (10% v/v) was emulsified with gum Arabic (5% w/v) in 100mM potassium phosphate buffer pH 7.0. 100µl of the enzyme was added to the emulsion and incubated for 15 minutes at 37 °C. The reaction was stopped and fatty acids were extracted by addition of 1.0 ml of acetone: ethanol solution (1:1). The amounts of fatty acids liberated were estimated by titrating with 0.05M NaOH until pH 10.5 using a phenolphathelin indicator. One unit of enzyme is defined as the amount of enzyme required to hydrolyze µmol of fatty acids from triglycerides.

Determination of Protein

Protein concentration of the bacteriocin in supernatant was determined by the method of Lowry *et al*., 1951, using bovine serum albumin (BSA) as the standard.

Reagent Required

1. Bovine serum albumin (BSA) (1mg/ml), 2. Analytical reagent:

(a) 50 ml 2% sodium carbonate mixed with 50 ml of 0.1 N NaOH solution, (b) 10 ml of 1.56 % copper sulphate solution mixed with 10 ml of 2.37 % sodium potassium tar tare solution. Prepare analytical reagents by mixing 2 ml of commercial reagent (2 N) with an equal volume of water on the day of use (2 ml of commercial reagent + 2ml distilled water).

Protocal for protein estimation of whole cell extract

1 ml sample of whole extract was taken and 0.2 ml alkaline copper reagent was added, it was incubated at room temperature for 10 minutes, 500 ml distilled water folin's reagent (1:1 with distilled water) was added, Tubes were incubated at room temperature for 30 minutes and sample was read at OD 66o nm (immediately).

$$\text{Protein (mg/ml)} = \frac{\text{OD of the sample x concentration of the standard x OD of the standard}}{\text{Volume of sample}}$$

Optimization Parameters for Profound Protease Enzyme Activity

Effect of medium pH on protease activity

The effect of different pH (5.0, 6.0, 7.0, 8.0 and 9.0) on protease production was determined as each *Bacillus* species culture was inoculated on media with different pH set values. The initial pH of the growth medium was adjusted with 1 M HCL or NaOH before sterilization (121 °C for 15 min) (Nadeem *et al*., 2008).

Effect of temperature on protease activity

The effect of temperature on protease production was studied each *Bacillus* species in fermentation media set at different temperatures (25, 37, 45, 50 and 60 °C). The inoculated

substrates were incubated at different temperatures to determine the optimum fermentation temperature for alkaline protease production (Ranilson *et al*., 2009).

Effect of incubation period on protease activity

50 ml of selected medium was taken in each 100 ml conical flask. All flasks were autoclaved at 121 °C and 15 lb pressure for 20 min. After cooling, the flasks were inoculated with equal quantity of inoculums. The flasks were incubated at 37±1c at 24, 48, 72, 96, and 120 h (Anwar *et al*., 2005).

Effect of sodium chloride for protease and lipase production

Protease Production was also tested by adding different concentration of NaCl (1, 2, 3, 4, and 5%) in the carbon and nitrogen optimized medium (Esakkiraj *et al*., 2007).

Effect of carbon sources on protease activity

The effect of carbon sources on protease production was tested by using a different carbon sources namely, glucose, fructose, sucrose. They were tested individually at a concentration of 0.5% in the nitrogen source optimized basal medium. Then, maximum protease producing carbon source was further optimized by varying concentrations (0.5, 1, 1.5, 2, and 2.5 %) on nitrogen optimized medium (Esakkiraj *et al*., 2007).

Effect of nitrogen sources on protease activity

The growth medium was initially supplemented with different organic nitrogen source on urea at 1% (mass per volume) and inorganic nitrogen sources, NaNO3, NH4Cladditionally at 1% (mass per volume). After screening, maximum protease yielding nitrogen source was further optimized by varying concentration (0.5,1, 1.5, 2, and 2.5%) on basal medium (Muhammad *et al*., 2008).

Factor Influencing Production of Lipase Enzyme Activity

Effect of pH on Lipase Activity

The optimum pH for enzyme production was selected by varying the pH of the production medium from 5 to 9 was as the other parameters were unaltered.

Effect of Temperature on Lipase Activity

For selection of optimum temperature for the production of lipases, the temperatures varying from 25 to 60 °C were selected by keeping the remaining parameters same.

Effect of Incubation Period on Lipase Activity

Production medium containing yeast extract, NaCl, Peptone and 1% (w/v) olive oil at 36 °C in an in orbital shaker at agitation speed of 150 rpm. The culture broth was harvested at 8h intervals by centrifugation at 10,000g, 30min at 4 °C. The supernatant collected was used as crude enzyme solution and was assayed for enzyme solution and was assayed for enzyme activity.

Effect of carbon source on lipase activity

The effect of carbon source on lipase activity. The major factor for expression of lipase activity always reported as carbon source. The production medium for lipase was Fructose, olive oil, and dextrose was tested.

Effect of nitrogen source on lipase activity

The effect of nitrogen source in the production medium for lipase enzymes. Among the nitrogen sources tested, peptone and yeast extract exhibited on the prominent lipase activity.

Dehairing activity of crude alkaline protease

Goat skin was cut into 3 cm^2 pieces and incubated with 10.0 ml of crude protease for 24 h at 37 °C and lipase 48 h at 37 °C. The skin pieces were then virtually analyzed for alkaline protease-dehairing activity (Mukherjee and sundhir, 2009)

Separation of Chromosomal DNA by Agarose Gel Electrophoresis (AGE): DNA isolation and purification

The culture was grown overnight in 10ml nutrient broth at 37 °C. 1.5ml of broth culture was centrifuged at 8000 rpm for 10 minutes and the resultant pellet was suspended in 200µl Tris EDTA (TE) buffer and incubate for 30 minutes in a water bath (37 °C). then 300µl freshly prepared NaOH (3M) was added, the contents were mixed gently for three times. Then 600µl of 2M Tris was added and again the content was mixed gently for three times. Then 1ml of freshly prepared 5M NaOH was added. Then the mixture was allowed to incubate at refrigeration condition for 30 minutes. After incubation the contents were centrifuges at 10000 rpm for 10

minutes. The aqueous phase (400µl) was transferred to a fresh eppendroff tube and 800µl of ice-cold ethanol was added and kept for incubation at 4 °C for 10-15 minutes. After incubation, DNA was pelletized by centrifugation at 10000 rpm for 20 minutes. The pellet was allowed to dry and dissolved in 50µl of TE buffer.

Results and Discussion

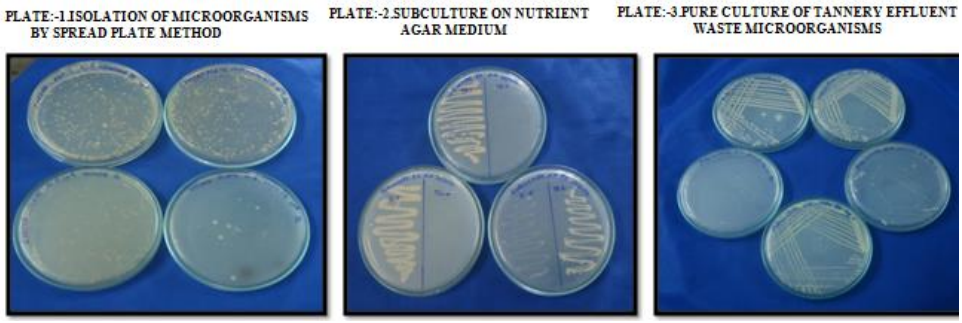
The present study was focused with much attention in isolating a potential bacterial strain which can produce lipases. These enzymes have great industrial potential that are widely found in microorganisms, plants and animals. Proteases and lipases have common application best used in tannery industrial. Tannery effluent samples were obtained from tannery industries in Salem. Alkaliphilic bacteria, especially *Bacillus* strains have the ability to produce industrially extracellular enzymes. Alkaliphilic *Bacillus* sp can be found mostly in alkaline like soda soils, soda lakes and deep-sea sediments.

Isolation and identification of protease producing organisms

1ml of tannery effluent sample was dissolved in 100ml distilled water and suspension were serially diluted from 10^{-3} to 10^{-7}. An aliquot of 0.1 ml was drawn from $10^{-3}$to 10^{-7} dilutions and streaked into sterile petri plates. The plates were incubated at 37 °C for 24 hours (Table: 1 and Plate:1, 2 and 3). The isolated was maintained as pure culture. In the present study 5 isolates were isolated from tannery effluent and all plates were screened for protease and lipase production. Isolation of microorganisms from tannery industrial of the carried out. The total cultivable bacterial counts were found to be approximately. The results were coincides with Adinarayana and Ellaiah, 2003.

Table:1. Isolation of microorganisms from tannery effluent

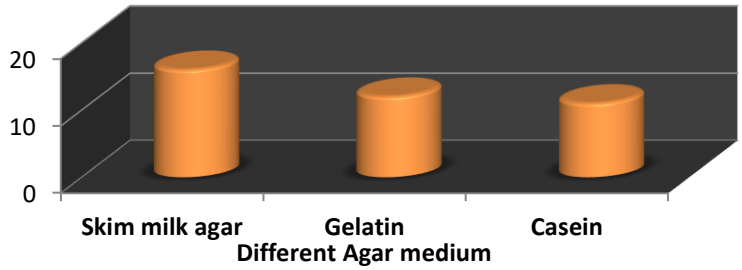
S.No	Dilution factor	Number of colonies	Colony forming unit (CFU/ML)
1	10^{-3}	137	$1.37x10^{6}$
2	10^{-4}	107	1.07×10^{7}
3	10^{-5}	86	0.86×10^{8}
4	10^{-6}	38	0.38×10^{9}
5	10^{-7}	13	0.13×10^{10}
6	Control	-	-



Screening of Protease and lipase activity

Screening of protease and lipase production were performed by inoculating the five isolates into different agar medium for proteolytic were performed by inoculating the four isolates into different agar medium for proteolytic and lipolytic activity. TE-1 exhibited large zone of hydrolysis with gelatin agar followed by skim milk agar. The results were recorded in Fig:1 and Plate:4. Screening of alkaline proteases ecological environment can result in isolation of new alkaline proteases with unique (Kumar *et al*., 1999). Proteases are commercially most viable enzymes and microbial use dominated the worldwide enzyme marketing, accounting for two-third share of industry Gupta and Roy, 2002.

Fig:1. Screening of the isolates for proteolytic activity TE-1



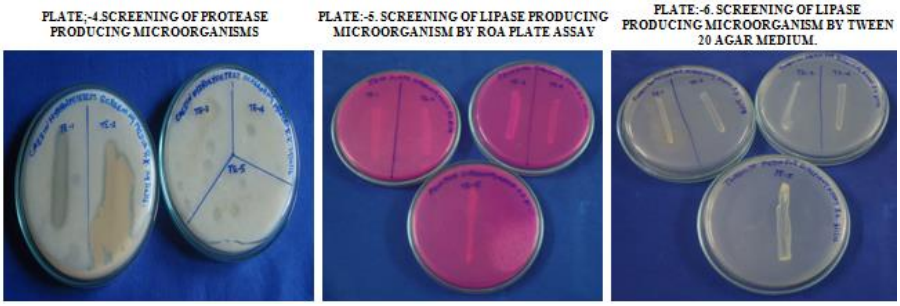
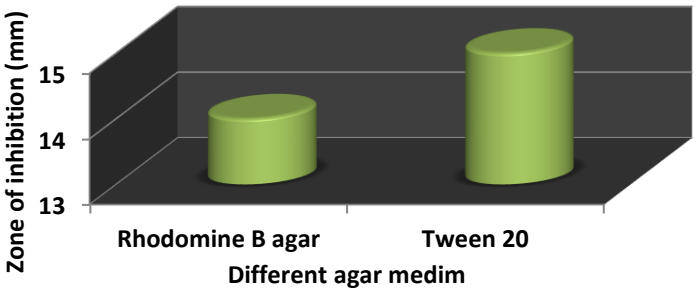


Fig:2 and Plate:5 and 6, depicted screening of isolated for lipolytic activity. In these studies, out of five isolates were subjected to qualitative screening on Rhodamine B agar and tween 20 agar

medium for lipolytic activity. Zone of hydrolysis produce was by the isolated were screened in tween 20 agar medium which showed that isolate at TE-1 produced zone of hydrolysis 15mm.

Fig:2. Screening of the isolates for lipolytic activity TE-1



In our results were similar with the protease leave proteolytic and keratinolytic activity used in dehairing and is tannery industry and in processing feathers from poultry slaughter houses (Esyaki raj, 1999).

Morphological characterization of the isolates

Morphological characterization of the isolated (TE-1) such as gram staining revealed gram positive long rods, motile absence of pigmentation with cream colour, circular with undulate margins with sporulation. Biochemical characterization of the isolate of TE-1 conformed as *Bacillus* sp on the basis of biochemical investigations. The organism was indole test, Methyl red, were found to be negative besides the TSI production was found to be alkaline sland. It fermented sucrose and dextrose. Above all these results isolate TE1 suggested it belonged to the genus *Bacillus.* The results were noted in Table:2 and Plate:7, 8 and 9.

Table: 2. Morphological and Biochemical characterization of tannery effluent waste

S.No	TEST	RESULT [TE-1]
	Morphological Characterization	
1	Gram staining	Positive
2	Endo spore staining	Positive
	Biochemical Characterization	
3	Indole test	Negative
4	Methyl red test	Negative
5	Voges praskaur test	Positive
6	Citrate utilization test	Positive
7	Triple sugar iron test	Positive

8	Starch hydrolysis test	Positive
9	Nitrate reduction test	Positive
10	Gelatin hydrolysis test	Positive
	Carbohydrate fermentation test	
12	Sucrose	Positive
13	Dextrose	Positive

Biological characterization of the isolates TE-1 revealed the following, rods, exhibiting motility, absence of pigmentation with sporulation (Lesuisse *et al.*, 1993).

PLATE:-7.BIOCHEMICAL CHARACTERIZATION

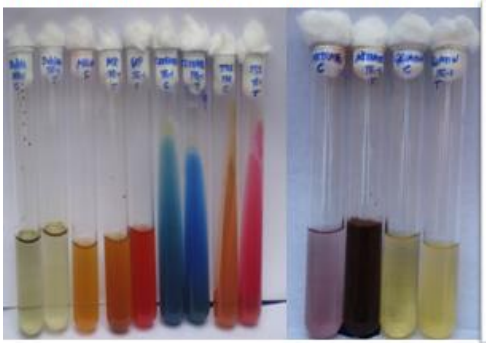


PLATE:-8.STARCH HYDROLYSIS TEST

PLATE:-9. CARBOHYDRATE FERMENTATION TEST

Assay of protease and lipase activity

Table:3 and Plate:10 and 11, denotes the assay for protease and lipase. The maximum protease activity was observed for TE-1 (0.82 Unit/ml). The maximum lipase production was seen in TE-1 with the enzyme unit of (0.4 unit/ml). our results were similar with Davendrakumar, 2012.

Table: 3. Assay of protease and lipase activity

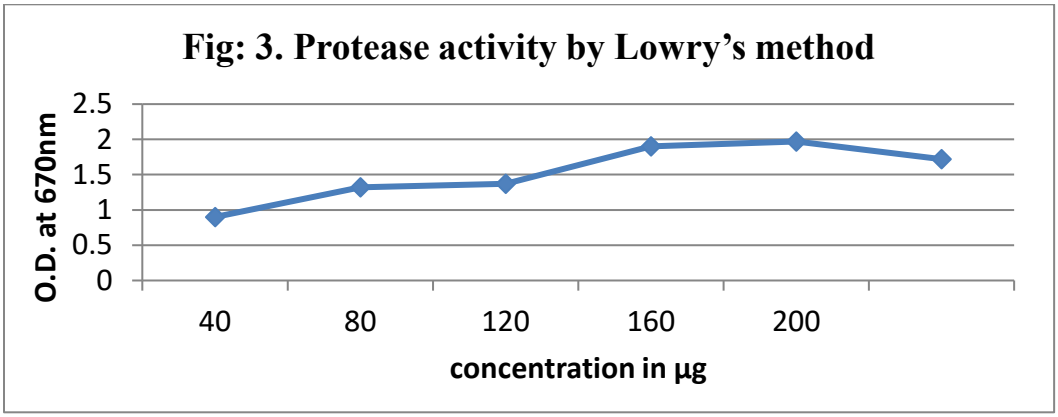
S.No	Incubation period (hours)	Protease activity (U/ml) TE-1	Lipase activity (U/ml) TE-1
1	48	46.75	38.92

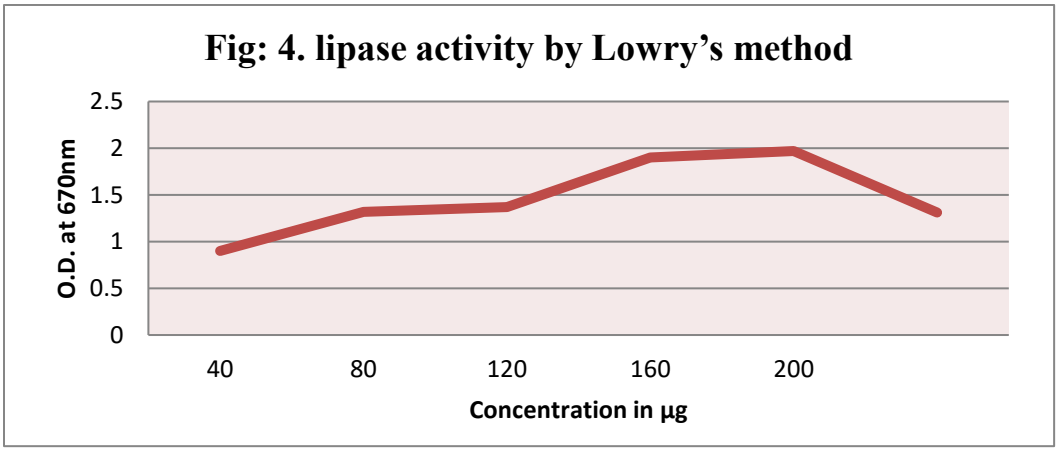
PLATE:-10. PRODUCTION MEDIA FOR PROTEASE ENZYME

PLATE:-11 PRODUCTION MEDIA FOR LIPASE

Protein estimation by Lowry's method

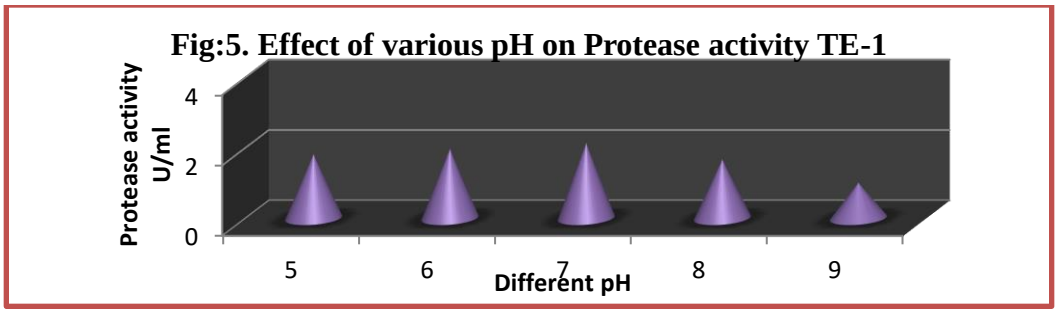
The total protein present in the sample of silkworm larvae was quantitatively estimated by Lowry's method. The amount of protein content of sample was found to be 1.72mg/ml for protease and 1.31 mg/ml for lipase (Lowry *et al*., 1951 and Hanan, 2012). The results were noted in Fig: 3 and 4.





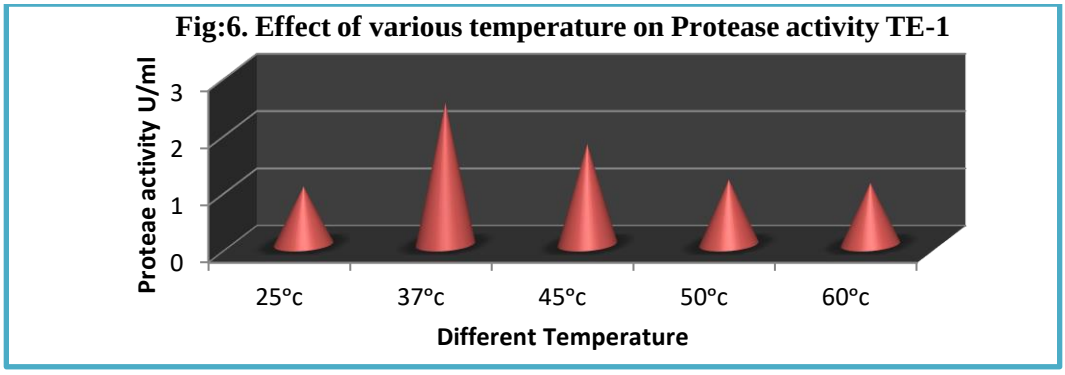
Effect of medium pH on protease activity

Microorganisms are sensitive to the change in the H^+ ion concentration of their environment. Therefore, to detect the optimum medium pH the selected organisms were incubated at different pH and their production of protease was recorded. The strain TE-1 showed a maximum production of protease at pH 7 (2.74 unit/ml). The least enzyme production was noted at pH 9 (1.01 unit/ml). The results were depicted in Fig: 5. Our research findings were consequence with Beg and Sahai, 2003.



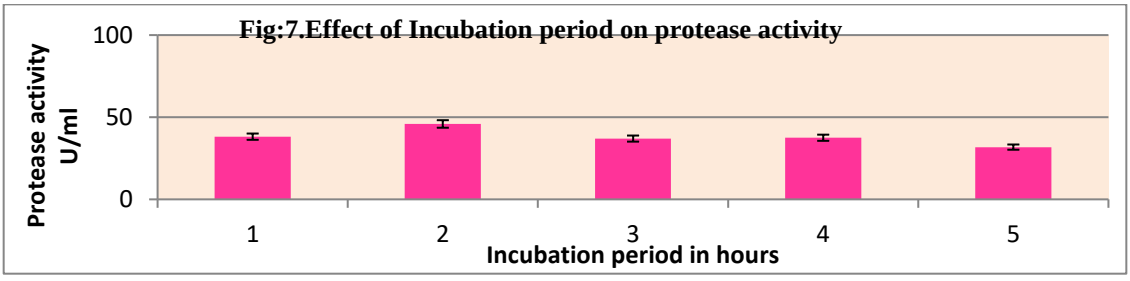
Effects of Various Temperatures on protease activity

The growth and enzyme activity of microorganisms are greatly influenced by different incubation temperature. The highest protease production temperature after incubation was analyzed and reported in Fig:6. The highest protease activity was recorded at 37 °C, for TE-1 (2.47 unit/ml). The least activity was observed at 25 °C for the strain TE-1 exhibited 1.01unit/ml (Bhosale *et al.*, 1995).



Effect of incubation period on protease activity

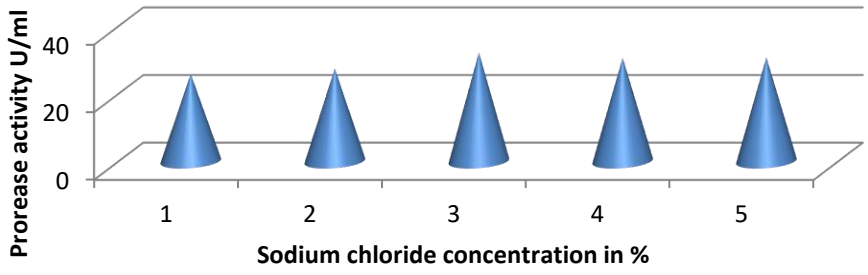
Fig:7, revealed that both the organisms show considerable variation at different incubation period when the isolated were grown in selective media strain TE-1 showed maximum protease production (45.9 unit/ml) at 48 hours of incubation. The least enzyme activity was noted in 120 hours for TE-1 (Patel *et al.*, 2006).



Effect of NaCl concentration on protease activity

Fig:8, investigated whether the concentration of affected the production of protease. For that the inoculated strains TE-1 was grown on a medium added with different concentration of Nacl (1-5%). A maximum protease production of NaCl concentration was recorded at 3% for both the strains (34.25 Unit/ml). At 1% NaCl both the strains revealed less enzyme activity 25.85 Unit/ml (Pedersen *et al.*, 2003).

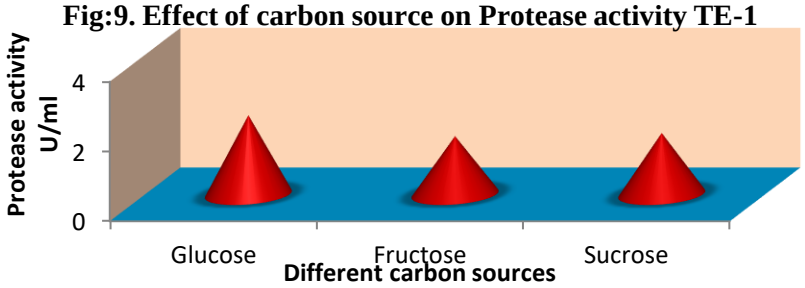
Fig:8. Effect of Sodium chloride concentration in protease activity



Effect of carbon source on protease activity

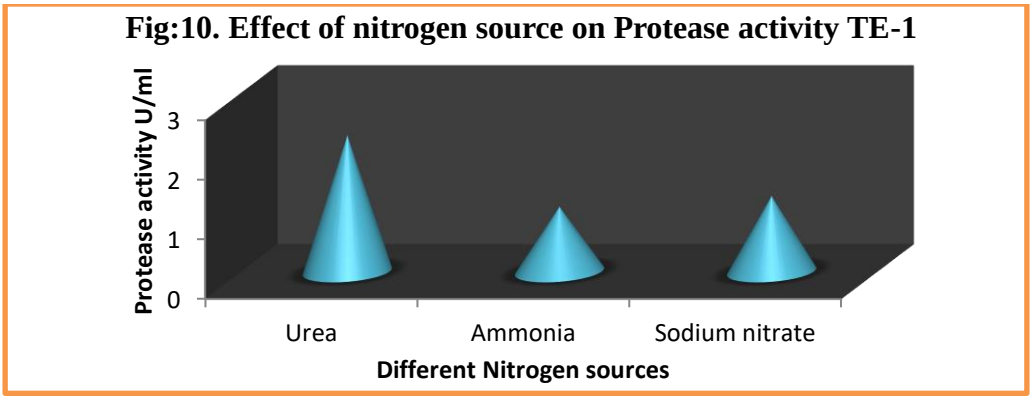
The effect of different carbon source on the yield of protease production was observed in Fig:9. The result indicated that the strain TE-1 revealed enhanced protease activity (2.27 unit/ml) when glucose was given as its sole carbon source. The strains produced least amount of protease when fructose was supplemented with carbon source (Zheng *et al.*, 2001).

Fig:9. Effect of carbon source on Protease activity TE-1



Effect of nitrogen sources

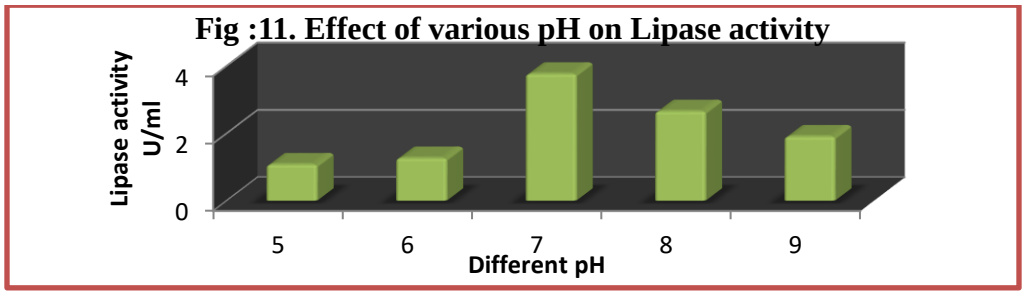
Among the different nitrogen sources tested, the strain TE-1 exhibited highest protease activity (46.75unit/ml) when urea was supplement as its sole nitrogen source. The strain TE-4 utilized peptone as its nitrogen source. Both the strain revelaed least enzyme activity when NH4cl was supplement as nitrogen source. The results were denoted in Fig:10 (Zheng *et al.*, 2001).



Optimization of lipase production

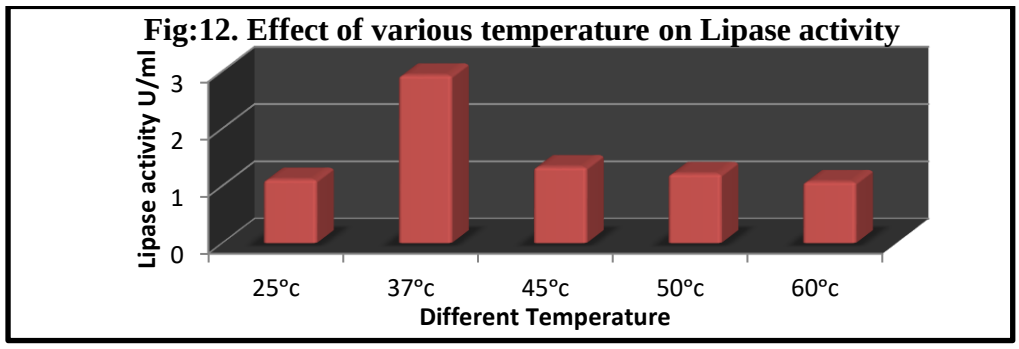
Effect of growth medium pH on lipase activity

Fig:11, denotes the effect of Growth medium pH on lipase activity. The effect of pH on lipase production was determined by growing *Bacillus* sp (TE-1) at different pH (5.0, 6, 7, 8 and 9). At alkaline pH 7, the enzyme activity for the isolate TE-1 exhibited 3.77 U/ml (Srisha *et al.*, 2010).



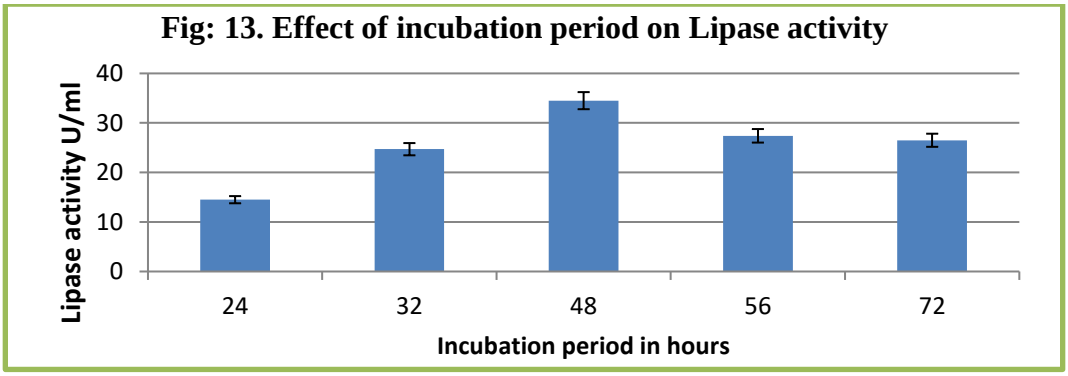
Effect of temperature on lipase production

Fig:12, denoted the effect of incubation temperature on lipase activity. In order to determine the optimum temperature for the isolate, it was grown in the production medium at various temperatures like 25 °C-60 °C. It showed the ability to grow in the respective temperature especially at 37 °C for TE-1 value for 2.93 U/ml (Ghori *et al.*, 2011).



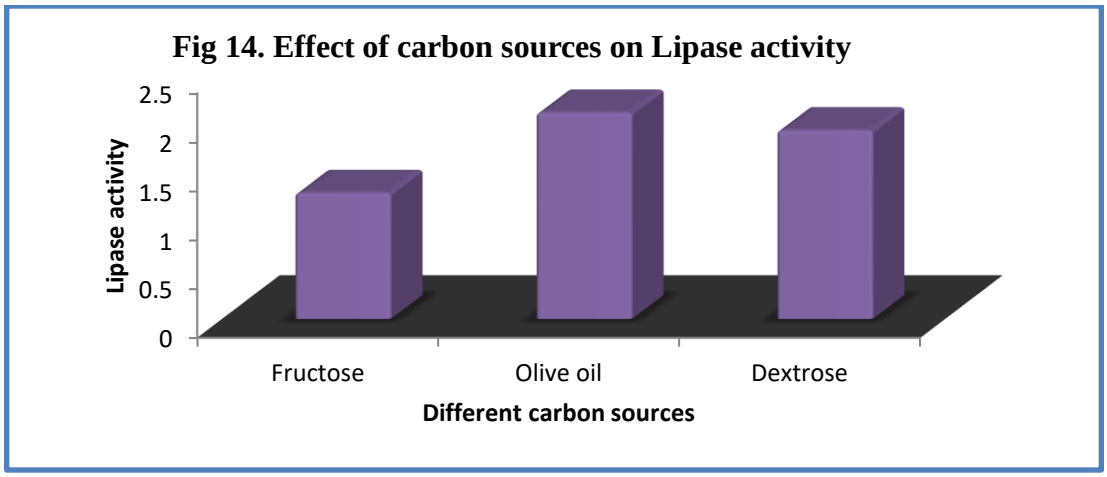
Effect of incubation period on lipase activity

Effect of incubation period on lipase period on lipase production was tested with different incubation temperature ranging from 24-72 °C and the results were noted in Fig: 13. The result suggested that 48 hours was the optimum incubation period for the strain TE-1, simultaneously, considerable enzyme production was observed between 48-72 hours of incubation (Gupta *et al.*, 2004).



Effect of carbon source on lipase activity

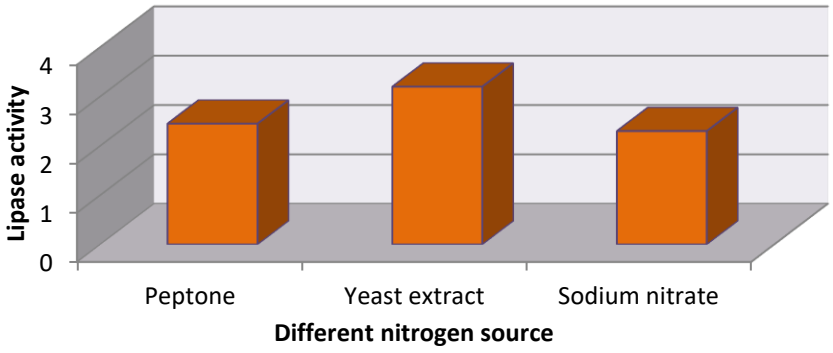
Fig:14, depicted the effect of carbon source on lipase activity. The major factor for expression of lipase activity always reported as carbon source. The production medium for lipase was Fructose, olive oil, and dextrose was tested. Among this olive oil was found to be effective carbon source for lipase production exhibited 2.11 unit/ml for *Bacillus* strain (Hanan *et al.*, 2001).



Effect of nitrogen source on lipase activity

Fig: 15, illustrated the effect of nitrogen source in the production medium for lipase enzymes. Among the nitrogen sources tested, peptone and yeast extract exhibited on the prominent lipase activity (Higaki *et al.*, 2003 and Sangeetha *et al.*, 2011).

Fig:15. Effect of nitrogen source on Lipase activity



Plasmid DNA Isolation by Alkaline Lysis Method

Bacillus subtilis was found to be has a good degree of resistance for tannery effluent waste water treatment. In the present investigation, plasmid DNA from industrial effluent strain was isolated by alkaline lysis method (Rao *et al.*, 1998). Agarose gel electrophoresis was performed to get plasmid profile of the industrial effluent isolate *Bacillus subtilis*. It was observed that the strain exhibited single band plasmid DNA with different molecular weight with 12 and 15 kb. The results were noted in Plate:12.

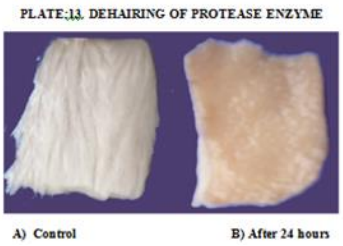
PLATE:12. ISOLATION OF PLASMID DNA BY ALKALINE LYSIS METHOD

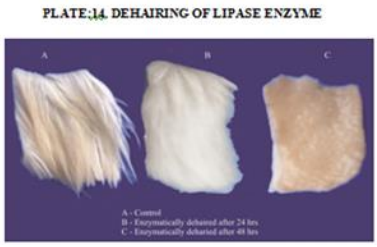
Lane: 1. DNA Marker, Lane:2. Protease enzyme, Lane:3. Lipase enzyme

Dehairing activity of crude alkaline protease

The new enzyme technology would make it possible to obtain good dehairing without using chemicals such as sulfide. Therefore, enzymatic dehairing is an efficient method to produce quality leather without causing pollution to the environment. In the present investigation the action of the enzyme on raw goat skin showed that the enzyme can remove the hair from the skin within 24 hours, when the protease enzymes from TE-1 were used. The results were depicted in

Plate:13 and 14. The enzymatic deharining and bating of hides have been widely accepted as an alternative to the chemical process (Alissara *et al*., 2006). Although, microbial proteases may be an alternative eco-friendly strategy to replace the use of eco-hazardous chemicals for the dehairing purpose. Similar work was carried out by Hun *et al.*, 2003.





Application and highlights of current research findings were revealed the enzymes found huge applications and various industries. Our study made much attention on isolation of protease and lipase from tannery industrial effluent. These enzymes are involved in deharing and bating of skin and leather is one of the major export and its quality processing by adopting these techniques. From the foregoing discussions microbial protease may be eco-friendly to replace the use of hazards chemical for dehairing. Lipases and protease enzymes are great demand. Owing to numerous biotechnological applications. *Bacillus* was known to secrete two major types of protease that is serine and protease. With have wide applications in industries. It is concluded that as a result of this studies *Bacillus* and *Pseudomonas sp* isolated capable of production proteolytic enzymes giving optimum performance under high alkaline condition making it potential candidate for industrial applications.

Conclusion

Environment pollution has become a major problem in developing countries in the large few decades. Major sources of water pollution are the untreated industrial effluents. The discharge from the tannery industries is becomes a crucial problem in the environment, since the rate of discharge is alarming. Alkaliphilic enzymes are alkaline stable enzymes which were useful for leather tanning, paper pulp bleaching, and production of cyclodextrins and waste treatment. In the present investigations alkaliphlic microorganisms were inoculated in to different agar medium of the four isolates screened only two isolates exhibited protease and lipase activity, to screen the isolates for proteolytic and lipolytic activity and investigation the action of protease enzyme of *Bacillus subtilis* effectively removed the hairs from the skin within 24 hours.

References

Adinarayana, KP., Ellaiah, DS. (2003). Purification and parital characterization of thermostable serine alkaline prohease from a newly isolated *Bacillus subtilis* PE-11. AAPS. Pharm. Sci. Tech. 56(4): 1-9.

Alford JA, Steinle EE. (1967). A double layered plate method for the detection of microbial lipolysis. J. Appl. Bacteriol. Dec: **30**(3): 488–494.

Alissara R, Siriporn Y, Masaaki Y. (2006). Purification and Characterization of Alkaline Protease from *Bacillus megaterium* Isolated from Thai fish sauce fermentation process. Science Asia 32: 377-383.

Anwar MN, Sharmin S, Towhid H. (2005). Isolation and characterization of a protease producing bacteria *Bacillus amovivorus* and optimization of some factors of culture conditions for protease production. J. Biol. Sci 5(3): 358-362.

Arunachalam, C., Saritha, K. (2009). Protease enzyme: an eco-friendly alternative for leather industry. 2(12)

Beg, AK., Sahai, V. (2003). Statistical media optimization and alkaline protease production from *Bacillus mojavesis*in a bioreactor. Proc. Biochem. 39: 203-207.

Bhosale, S.H., Roa, M.B., Deshpande, V.V., Srnivasanm M.C. (1995). Thermostability of high activity alkaline protease from conidioboluscoronatus [NCL 86.8.20]. Enzyme Microbiology and Technology. 17: 136-139.

Chakraborty S. JL, G. Hollaway, A. Freeman, R. Norton, K.A. Garret, K. Percy, A. Hopkins, C. Davis and D.F Karnosky. (2008). Impacts of Global Change on Disease of Agricultural Crops and Forest Trees. CAB Reviews: Prespectives in Agriculture, Veterinary science, Nutrition and Natural Resources. 3: 1-15.

Davenderkumar. (2012). Screening, isolation and production of lipase/protease producing *Bacllius sp*. strain DVL2 and its potential evaluation in esterificaton and resolution reactions. Archves of Applied Science Research. 4(4): 1763-1770.

Esakkiraj PG, Immanual S, Sowmya M, Iyapparaj P, Palavesam A. (2007). Evaluations of protease producing ability of fish gut Isolate *Bacillus cereus* for Aqua Feed. Food Bioprocess Technol. Springer Sci. 2: 383-390.

Fujshige, A., Smith, K.R, Silen, J.L., Agard, D.A. (1992). Correct folding of a lytic protease is required for its extracellular secretion from *Esherchia coli*. J. Cell Biology. 118: 33-42.

Ghori, M.I., Iqbal, M.JardHameed, A. (2010). Characterization of a novel lipase from *Bacillus sp*. isolated from tannery wastes. Indian J. Microbiology. 42: 22-29.

Gunasekaran, A., C. Patel, and R.E. McGaughey. (2006). A Framework for supply Chain Performance Measurement. Int. J. Production Economics. 87 (3):333-347.

Gupta, R., Gupta, N.,Rathi, P. (2004). Bacterial lipases: An Overview of production, Purification and biochemical properties. Appl. Microbiol. Biotechnol. 64: 763-81.

Gupta., R., Beg Q.K and Lorenz, P. (2002). Bacterial alkaline proteases, molecular approaches and industrial applications. Appl. Microbiol. Biotechnol. 59: 15-32.

Hameed,A., Natt,M.A. and Evans,C.S. (1996). Short communication: Production of alkaline protease by a new *Bacillus subtillis* isolated use as a bating enzyme in leather treatment. World J. Microbiology and Biotechnology. 12: 259-291.

Hanan,F.Shah,A.A, Hameed,A. (2001). Optimization of lipase production from *Bacillus sp*. Pak. J. Bot.16: 33-37.

Hanan,S.Alnahdi. (2012). Isolation and Screening of extracellular proteases produced by new isolated *Bacillus sp*. J. Applied Pharmaceutical Sci. 2(9): 071-074.

Higaki,S., Morohashi,M. (2003). Propionibacterium acnes lipase in seborrheic dermatitis and other skin disease and unseri-in. Drugs Exp. Res.,29:157-9.

Hun CJ, Rahman R, Salleh AM. (2003). A newly isolated organic solvent tolerant Bacillus sphaericus 205y producing organic solvent-stable lipase. Biochem. Eng. J. 15 (2):144-151.

Jensen, R.G. (1983). Detection and determination of lipase (acylglycerol hydrolase) activity from various sources. Lipids, 18:650-657.

Kashayp, D.R., P.K., Soni, S.K and Tewari, R. (2001). Degumming of the buel (Grewiaoptiva) bast fibers by pectinolytic enzyme from *Bacillus sp*. DT7. Biotechnology letters 23, 1297-1301.

Kocher, G.S., Mishra,S. (2009). Immobilization of *Bacillus circulans* MTCC 7906 for enhanced production of alkaline protease under batch and packed bed fermentation conditions. Int. J. Microbiol. 7: P2.

Kroll, R.G. (1990). Alkalophiles' in microbiology of extreme environments, edited by C.Edwardst McGraw-Hill publishing Co., (New York, 1990), PP.55-92.

Kumar,C.G., Takagi, H. (1999). Microbial alkaline protease from a bioindustrial view point, Biotechology advances17: 561-594.

Lesuisse, E., Schanck,K., Colson,C. (1993). Purification and preliminary characterization of the extracellular lipase of *Bacillus subtillus* 168, an extremely basic pH-Tolerant enzyme. Eur. Biochem. 216: 55-60.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurement with the folin phenol reagent. J. Biol. Chem.193: 265-275.

ManfredReetz T. (2002). Lipase as practical biocatalysts. Curr. Opin. Chem. Biol. 6-45.

Meyers, S.P. and Ahearn, D.G. (1977). Extracelllular proteolysis by Candida lipolytica. Mycologia 69: 646- 651.

Muhammad N, Javed IQ, Shahjahan B, Qurat-ul-ain S. (2008). Effect of medium composition on commercially important alkaline protease production by *Bacillus licheniformis* n-2. Food Technol. Biotechnol. 46(4): 388-394.

Mukherjee AK, Sudhir KR. (2009). Ecological significance and some biotechnological application of an organic solvent stable alkaline serine protease from Bacillus subtilis strain DM-04. Bioresour. Technol. 100: 2642-2645.

Nadeem A, Raj HG, Chhabra SK. (2008). Effect of vitamin E supplementation with standard treatment on oxidant- antioxidant status in chronic obstructive pulmonary disease. Ind. J. Med. Res. 128:705-711.

Olsen, H.S (2000). Cleaning-in-place process equipment in dairy or slaughter house by circulating a solution containing a protease and a lipase united states patent 6071 13:56.

Patel, R: Dodia, M,: Singh, S. P. (2006). Purification and characterization of alkaline protease from a newly isolated Haloalkaliphilic *Bacillus sp*. Proc. Biochem. 41 (9): 2002-2009.

Pedersen, N.R., Wimmer, R., Matthiesen, R., Pedersen, L.H and Gessesse, A. (2003). Synthesis of sucrose laurate using a new alkaline protease. Tetrahedron:Asymmetry 14, 667-667.

Ranilson SB, Talita SE, Ian PG, Amaral, Diego SB, Givanildo BO, Luiz Jr BC (2009). Fish processing waste as a source of alkaline proteases for laundry detergent. Food Chem. 112: 125-130.

Rao M.B, TanksaleA.MMohiniS.G, Despanae.V.V. (1998). Molecular and Biotechnological aspects of microbial protease. Microbiology Review. 62:597.

Sageetha R, Arulpandi I and Geetha A. (2011). Bacterial lipase as potential industrial biocatalysts. An overview. Res. J. Microbiol. 6,1-24.

Sangita, P.I., Kakde, A.U. and Maggirwar, R.C. (2012). Biodegradation of tannery effluent by using tannery effluent isolate. Int. Multidisciplinary Res. J. 2: 43-44.

Singh J., Vohra, R.M and Sahoo, D.K. (1999). Alkaline protease from a new obligate alkalophilic isolate of *Bacillus sphaericus*. Biotechnology Letters. 21: 921-924.

Sirisha, E, N. Rajasekar and M. Lakshmi Narasu. (2010). Isolation and Optimization of Lipases Producing Bacteria from Oil Contaminated solsadvances in Biological Research 4 (5): 249-252.

Zheng, L., Du, Y and Zhang, J. (2001). Degumming of ramie fibers by alkalophilic bacteria and their polysaccharide- degrading enzymes. Bio resource technology. 78 (2001) 89-94.