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PARTIAL PURIFICATION OF LIPASE FROM *STREPTOMYCES VARIABILIS* NGP 3 AND ITS APPLICATION IN BIOREMEDIATION OF WASTE WATER

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ABSTRACT: Partial purification and bioremediation of waste water by lipase from the marine actinomycete *Streptomyces variabilis* NGP 3 (Accession no: (JX843530)) were carried out. The optimum incubation period, pH, temperature and agitation speed for enzyme production were fifth day (61.2 U/ml), 9.0 - 9.5 (105 U/ml), 35ºC (39.4 U/ml) and 120 rpm (38.7 U/ml) respectively. Lactose (2.0 g/l) and peptone (0.6 and 0.8 g/l) proved to the best carbon and nitrogen sources respectively for lipase production. The partially purified lipase showed a specific activity of 1440.97 U/mg protein, 7.63 fold pure and yielded 3.19 per cent of protein. The enzyme activity was maximum at the pH and temperatures were 8.5 and 45ºC respectively. The molecular weight of the first and second isoenzymes was found to be 55.0 and 56.0 KDa respectively. Bioremediation of automobile effluent and slaughter house waste water were carried out by the isolated actinomycetes isolate *S. variabilis* NGP 3. The chemical oxygen demand (COD), total organic chloride (TOC) and fat/oil content of the effluent were analyzed. The COD and fat/oil degradation rate were increased by the simultaneous reduction of TOC in the treated effluent.

INTRODUCTION: Lipases (triaycl glycerol acyl hydrolases, EC 3.1.1.3) catalyze the hydrolysis and the synthesis of esters from glycerol and long chain fattyacids[1]. Lipases are obtained from microorganisms which produce a wide variety of extracellular lipases [2].

Many lipases are active in organic solvents where they catalyst a number of useful reactions including esterification, transesterification, regioselective acylation of glycerols, synthesis of peptides and other chemicals [3].

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Lipases are mostly inducible enzyme and require some form of oil, fatty acid, fatty acid alcohol or fatty acid ester and surfactant for induction [4]. Recently, the interest in microbial lipase production has increased [5], because of its large potential in industrial applications as additives for foods (flavor modification), fine chemicals (synthesis of esters), waste water treatment (decomposition and removal of oil substances), cosmetics (removal of lipids), pharmaceuticals (digestion of oil and fats in foods), leather (removal of lipids from animal skins) and medical (blood glyceride assay) fields [6, 7, 8]. Other useful features are such as broad substrate specificity, the versatility of the molecular structure and stability in organic solvents [9].

In view of the variety of applications, there has been renewed interest in the development of sources of lipases.

Numerous species of bacteria, yeasts and fungi produce lipases with different enzymological properties and specificities [10]. The enzyme lipase is produced by a variety of microorganisms including *Fusarium oxysporum* [11], *Pseudomonas aeroginosa* [12], *Aspergillusoryz*ae CJLU-31 [13], *Bacillus pumilis* RK 31 [14], *Geobacillus thermo-dentrificans* IBRL-nra [15], *Streptomyces griseus* [16] and *Streptomyces aurofaciens* [17].

Lipids (fats, oils and greases) are major organic matters in municipal and some industrial waste water can cause severe environmental pollution. Waste water produced from edible oil refinery, slaughter house, wool scouring and dairy products industry contains a high (> 100 mg/l) concentration of lipids [18].

Higher concentration of these compounds in waste water often causes major problems in biological waste water treatment process (Bioremediation) is difficult due to the formation of monolayer on water surfaces and decrease oxygen transfer rate into the aerobic process. Bioremediation is interested by lipase producing microorganisms because lipid residues are converted to carbon-di-oxide, water and biomass [19].

Another well recognized problem in today's world is releases of oil into the environment. Contamination of soil by oil causes the loss of soil fertility, water holding capacity, permeability and binding capacity.

Many species of microbes such as bacteria, fungi, yeast and actinomycetes are involved in the bioremediation process by obtaining energy and tissue binding material from petroleum and other automobile effluents [20].

MATERIALS AND METHODS: For the determination of optimum culture conditions for growth and lipase enzyme production, the actinomycetes isolate *S. variabilis* NGP 3 at the concentration of 10^{-4} spores/ml (inoculums volume - 10% v/v) was inoculated in 50 ml of production medium [21] in an orbital shaker (120 rpm). The production medium consists of starch 20.0 g, Peptone 20.0 g, NH_4Cl 3.8 g, $MgSO_4$ 1.0 g, K_2HPO_4 5.0 g, Olive oil 10.0 g, pH 7.0, Water 1000 ml. The growth and enzyme production were determined.

Incubation period: The actinomycete isolate *S. variabilis* NGP3 were grown for various incubation period (1 to 10 days) at room temperature; the growth and enzyme production were determined at the end of each incubation period.

pH: The activity of L- asparaginase was evaluated at different pH values. The flask containing 50 ml of the production medium was mixed with acid/alkali solution to obtain required pH. The growth and enzyme production were determined at the pH range of 5.0 to 12.5 at room temperature.

Temperature: The effect of temperature for growth and enzyme production were determined in the range between 25 and 70ºC.

Agitation: The effect of agitation in the range of 90 to 130 rpm was performed for growth and enzyme production.

Carbon sources: Galactose, lactose, mannose and sucrose (0 to 3.0 per cent) were tried as carbon sources for growth and enzyme production.

Nitrogen sources: The nitrogenous compounds ammonium sulphate, peptone, potassium nitrate and yeast extract (0.2 to 1.0 per cent) were tried as nitrogen sources.

Assay of lipase activity: Lipase assay was done using olive oil as substrate in Tris-HCl buffer pH 8.5 [22]. A volume of 1.0 ml culture filtrate was added to 10 ml buffer and 10 ml olive oil emulsion. The reaction mixture was incubated at room temperature for 20 min. Then the reaction was terminated by adding 2.0 ml of acetone-ethanol mixture (1:1 v/v). The amount of fatty acid liberated was determined by titration with 0.05 M NaOH. One unit of lipase activity was defined as the amount of enzyme required to release 1µmol of fatty acid per ml per min under above assay conditions.

Partial purification of lipase enzyme: The purification procedure was referred to [23] method. The culture filtrate was collected through Whatman No. 1 filter paper and the filtrate was centrifuged at 4000 rpm for 10 min to obtain crude enzyme. Then the ammonium sulphate was added to the filtrate to give final concentration of 60 - 80 per cent (w/v) saturation at 4ºC.

The precipitate was collected by centrifugation at 4000 rpm for 4 min. The precipitate was then dissolved in 20 ml of double distilled water and dialyzed for 24 hrs at 4ºC against double distilled water and concentrated by lyophilization.

Sephadex G 100 column chromatography: An aliquot of the lyophilized sample (1.0 ml) was loaded on to a sephadex G-100 column (45 X 1.5 cm) previously equilibrated with 0.02 M phosphate buffer (pH 7.8). Elution of lipase was performed by a linear gradient of NaCl (0-1.0 mol/l in equilibrating buffer) at 30 ml/h. The fractions of 5.0 ml each were collected for every one hour (30 fractions) and assayed for lipase and protein activity.

Determination of Enzyme properties:

1. **Optimum pH:** To determine the optimum pH for maximum enzyme activity, the activity was estimated at different pH levels (3 to 10) at 30ºC. A graph was plotted by taking pH range in X axis and enzyme activity in Y axis. From this graph, the optimum pH for enzyme activity was extrapolated.

2. **Optimum temperature:** The optimum temperature for each enzyme activity was determined by estimating the enzyme activity at various temperature levels (30-70ºC) and plotting the values in a graph by taking temperature range in X axis and enzyme activity in Y axis and extrapolating the optimum temperature levels.

3. **Determination of molecular weight:** The molecular weight of the sample was determined by SDS-PAGE[24] with standard marker proteins in the range of 14,000 to 97,100 KDa.

4. **Bioremediation of waste water by lipase producing marine actinomycetes:** Bioremediation of automobile effluent and slaughter house waste water by the isolated actinomycetes isolate *S. variabilis* NGP 3 were carried out for 10 days. Total organic chloride (TOC), Chemical oxygen demand (COD) and fat/oil content of the sample were analyzed an alternative days [25].

5. **Preliminary characterization of Automobile effluent and Slaughter house waste water:** The automobile effluent and slaughter house waste water were collected from automobile workshop and slaughter house respectively in Coimbatore, India and their characteristic features such as total organic chloride (TOC), chemical oxygen demand (COD) and fat/oil content were analyzed.

The above mentioned parameters were determined again after the effluents were treated with actinomycetes isolate *S. variabilis* NGP 3.

Experimental set up for automobile effluent and slaughter house waste water treatment: A volume of 1000 ml sample was taken in a conical flask which was inoculated with 1 per cent (v/v) actinomycete culture. After vigorous shaking, it was divided in to 20 portions of 50 ml effluent each in 250 ml conical flasks. All the flasks were incubated at room temperature at 120 rpm. Samples were taken at regular intervals of 24 hrs for the determination of TOC, COD and fat/oil content of the effluent.

Total organic chloride (TOC): Total organic chloride content was estimated[26]. The culture filtrate (10 ml) was digested with few drops of 0.3 N NaOH and added with 0.1 g of sodium bicarbonate in a porcelain basin. To this digested sample, 0.1 to 0.2 ml of 5 per cent potassium chromate was added and titrated against 0.02 N silver nitrate. Appearance of persistent chocolate brown colour precipitate was the end point. A blank was maintained with chloride free distilled water.

Chemical oxygen demand (COD): Chemical oxygen demand was estimated for the waste water [26]. The diluted sample (1:1 v/v-50ml) was taken in a refluxing flask and added with 25 ml of 0.25 N potassium dichromate solution. To this, a pinch of silver sulphate and mercuric chloride (in the ratio of 10:1 to the chloride content of the sample) and 30 ml of concentrated H_2SO_4 were added and mixed thoroughly. A few anti bumping granules were added. The contents of the flask were refluxed for two hours cooled and make up to 350 ml with distilled water.

To this, 2-3 drops of ferroin indicator (0.695 g ferrous sulphate and 1.485 g/l, 10 phenonthronine monohydrate in 100 ml distilled water) was added and titrated against 0.25 N ferrous ammonium sulphate solution. The end point is the sharp color change from blue green to reddish brown. A blank was run simultaneously with distilled water instead of sample.

Estimation of fat/oil degradation: Fat/oil degradation is defined as the process by which oil is converted to a form that is no longer extractable by benzene [20]. Oil conversion was determined by extracting 50 ml of incubation mixture with 10 ml of benzene in a separating funnel. The aqueous phase was extracted again second time with 10 ml of benzene and the combined benzene fractions were filtered through Whatman No. 1 filter paper in order to clarify the extract. The benzene was evaporated at 37ºC to constant weight in a tared large petri dish. The rate of degradation was expressed in milligrams (mg) as well as in per cent (%).

RESULTS AND DISCUSSION:

Incubation period: The effect of incubation period on culture conditions and enzyme production by *S. variabilis* NGP 3 were carried out. The maximum growth (20.0 mg mycelial dry weight) of the isolate was observed on seventh day; lipase production was maximum (61.2 U/ml) on fifth day; but after the optimum period the enzyme productions were gradually decreased. In an earlier study, the maximum production of lipase was obtained after 96 hrs of incubation [27]; whereas in an earlier again [28] the production was obtained maximum on 8 days of incubation using *A. niger*. The maximum production of enzyme from *S. aurofaciens and S. griseus* were obtained on 24 and 72 hrs of incubation period[17&16]

pH: Effect of pH on culture conditions and enzyme production were studied in the pH range from 5.0 to 12.5. The maximum growth (26.5 mg dry weight) was observed at pH 9.0; the production (105 U/ml) was maximum at pH 9.0 and 9.5.These data are in agreement with that of previous study [11], who reported that, maximum lipase activity from *Fusarium oxysporum* was detected at pH 9.5. In another report, the lipase production by *Staphylococcus* sp. Lp 12 required alkaline pH [21]; whereas for *Bacillus* sp SS-1 the maximum production was found at 8.0 [29]. The maximum production of enzyme from *S. aurofaciens and S. griseus* were obtained in the range of pH 6.0 – 90 [17 & 16].

Temperature: The effect of temperature on culture conditions and enzyme production were studied in the temperature ranges from 25 to 70°C. In *S. variabilis* NGP 3, the growth (30.0 mg) was favoured at 30 and 35°C. The enzyme production (39.4 U/ml) was found to be maximum at 35°C. The present result was similar with the previous study that, the production of lipase was optimum at the temperature of 37°C by *Bacillus* [30]. In a study, the temperature 35°C favored the lipase production of *Pseudomonas aeruginosa* [31].

Agitation: The effect of agitation on culture condition and enzyme production was studied in the agitation speed ranges from 90 to 130 rpm. The speed of 110 rpm favoured mycelial growth (30.0 mg dry weight) of the isolate but the production was favored by 120 rpm (38.7 U/ml).In the previous study, the agitation speed of m125 rpm favored lipase production [32]. Agitation speed of 200 rpm favored the lipase production in the previous study [15].

Carbon sources: In the present study, galactose, lactose, mannose and sucrose were supplemented as carbon sources. By the amendment of In Galactose maximum growth (29.0 mg) was observed at 2.0 g/l, the production level was found to be 52.5 U/ml at 2.0 g/l substrate concentration. In lactose supplement, the mycelial growth (31.0 mg) was observed at 1.5 g/l substrate concentration. Lipase production was maximum at 1.5 g/l (92.5 U/ml) substrate concentration. In mannose amendment, 1.5 g/l substrate concentration favoured mycelial growth (25.0 mg dry mycelium) and the production was 43.75 U/ml at 1.0 g/l substrate concentration. In sucrose variation, the mycelial growth (32.0 mg dry weight) and the production (50.0 U/ml) were maximum at 1.5 and 2.0 g/l concentration respectively. In an earlier report, lipase production from *Amycolatopsis mediterranei* DSM 43304 was influenced by the amendment of lactose in the medium [33]; similarly lipase induction in the presence of sugar sources were optimum in the actinomycetes [34].

Nitrogen sources: Nitrogen sources used in the present study were ammonium sulphate, peptone, potassium nitrate and yeast extract. In ammonium sulphate amendment, maximum growth of the mycelium was found to be 21.0 mg at 0.8 g/l concentration; the production was found to be 71.2 U/ml at 0.8 g/l concentration. In peptone amended medium, maximum growth (23.0 mg) and the production of enzyme (108.7 U/ml) were found maximum at 0.8 and 0.6 g/l concentration respectively.

In potassium nitrate supplementation, maximum growth of the mycelium and lipase (25.0 mg and 56.2 U/ml) were favored at 0.6 g/l concentration; in yeast extract variation, at 0.6 g/l concentration favored mycelial growth (27.0 mg) and the concentration of 0.8 g/l brought the maximum production of lipase (58.7 U/ml). The nitrogen source peptone favored the lipase production and enhanced the cell mass of the fungus *Fusarium oxysporum* [11]. Peptone has also shown to support the maximum production of lipase in the case of *Aspergillus*sp [35], *Staphylococcus* sp. Lp12 [21].

Enzyme purification: The data presented in **table 1 and figure 1**, revealed that the culture filtrate of *S. variabilis* NGP 3 had 0.17 mg/ml protein. The total activity and the specific activity of the lipase were 16250 and 188.95 U/mg protein respectively. In ammonium sulphate precipitation, protein (0.16 mg/ml) and total activity (1187.5) were decreased, but increased specific activity (296.88 U/mg) was noted. The purification fold was 1.57. The enzyme recovery was 7.30 per cent. In sephadex G 100 column, fractions 21 and 20 exhibited lipase activity. In fraction 21, the specific activity was 1440.97 U/mg protein and protein content was 0.07 mg/ml. The recovery yield and purification fold were 3.19 and 7.63 respectively. In fraction 20, the specific activity, protein content, purification fold and recovery were 472.52 U/mg protein, 0.08 mg/ml, 6.25 and 2.90 per cent respectively. The purification of lipase was carried out with ammonium sulphate precipitation followed by dialysis and sephadex G100 column chromatography [36]. In earlier, the yield and purification factor were determined by column chromatography was 2.4 per cent and 3.45 respectively from *Alternaria brassicicola* [37].

TABLE 1: PARTIAL PURIFICATION OF LIPASE

Sample	Volume (ml)		Activity (U/ml)	Protein (mg/ml)	Total Activity (U)	Specific Activity (U/mg)	Recovery Yield (%)	Purification factor
S. albogriseolus **NGP**								
Crude extract		500	32.5	0.172	16250	188.95	100	1.00
Ammonium sulphate precipitation Column Chromatography		25	47.5	0.160	1187.5	296.88	7.31	1.57
Sephadex G – 100	21	5	103.75	0.072	518.75	1440.97	3.19	7.63
Fraction	20	5	94.50	0.080	472..50	1181.25	2.91	6.25

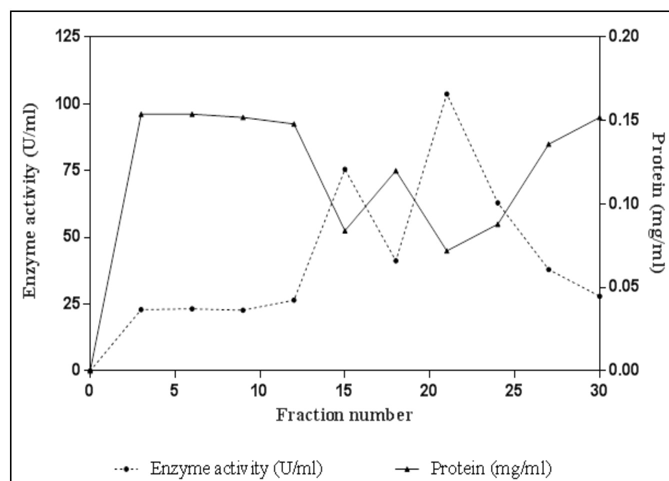

FIG. 1: PURIFICATION OF LIPASE OF *S. VARIABILIS* NGP 3 ON SEPHADEX G 100 COLUMN

Determination of Enzyme properties:

Optimum pH and temperature: To determine the optimum pH and temperature for maximum enzyme activity, the specific activities of the enzyme at various pH levels and temperature were determined (**Table 2**).

The optimum pH level for lipase of *S. variabilis* NGP 3 was found to be 8.5; beyond the pH 8.5 the enzyme activity was decreased. However, the enzyme was active in the pH range of 5.0 to 8.0. The specific activity was observed to be 1658.33 U/mg protein (**Fig. 2**). The optimum temperature for lipase production was 45°C; the enzyme was

active in the range of 25 to 40ºC, after that the enzyme activity was decreased which was presented in **figure 3**. The specific activity was found at the optimum temperature (45ºC) was 1757.81 U/mg protein. According to the previous research, the optimum pH and temperature of the partially purified lipase were 8.0 and 40°C respectively [38 & 39]. Lipase from *Pseudomonas* sp strain S5 was stable at 45°C and pH 6-9 [40].

TABLE 2: PROPERTIES OF LIPASE

Parameters	Enzyme source
	Streptomyces albogriseolus NGP
Optimum pH	8.5
Optimum temperature (°C)	45
Molecular weight (kDa)	55
SDS PAGE	56

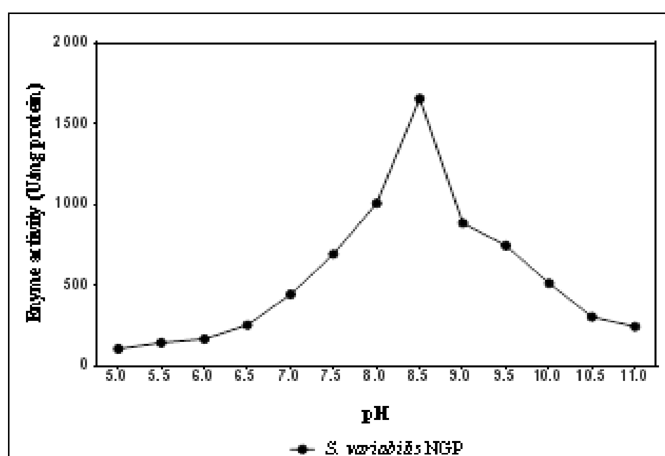

FIG. 2: EFFECT OF PH ON LIPASE ACTIVITY BY *S. VARIABILIS* NGP 3

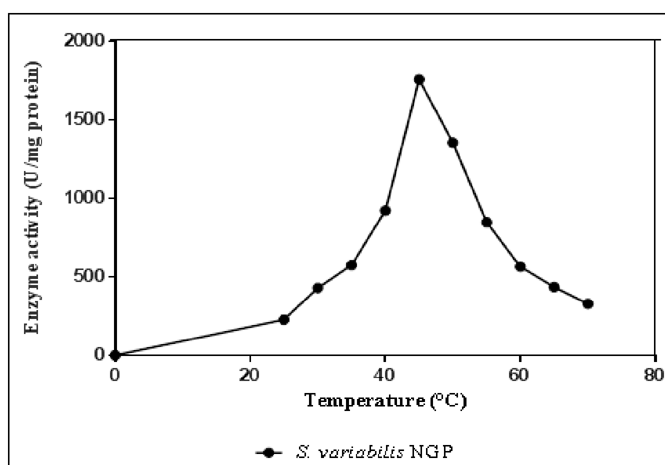

FIG. 3: EFFECT OF TEMPERATURE ON LIPASE ACTIVITY BY *S. VARIABILIS* NGP 3

Molecular weight: The molecular weight of the enzyme was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

Lipase exhibited two bands which had the molecular weight of 55.0 and 56.0KDa respectively (Table 2; **Plate 1**). The molecular weight of the lipase from *Aeromonas*sp. LPB 4 was determined as 50.0 kDa by SDS-PAGE [41]; the apparent molecular masses of LipA and LipB determined by SDS-PAGE were 50.0 and 57.0 kDa respectively from *Thermosyntropha lipolytica* [42].

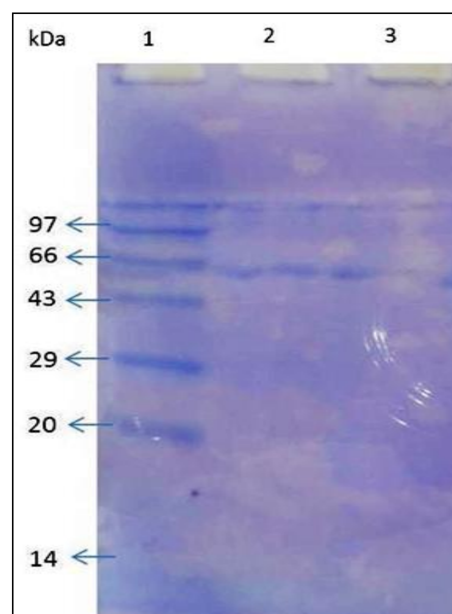

PLATE 1: MOLECULAR WEIGHT OF LIPASE (SDS - PAGE) Lane 1: Molecular weight markers; Lane 2-3: Lipase

Bioremediation of waste water by lipase producing marine actinomycetes:

Bioremediation of automobile effluent: A preliminary work was carried out to treat the auto mobile effluent using *S. variabilis* NGP 3. The general properties of the waste water such as TOC, COD and fat/oil degradation percentage were carried out (**Table 3 and figure 4**). The results showed that, the initial TOC and COD content of the effluent were 115 and 2530 mg/l. After the treatment with *S. variabilis* NGP 3 the chloride content of the effluent was increased up to 58.0 per cent on tenth day of incubation. Maximum reduction in COD (83.99 %) was observed on tenth day after the treatment with *S. variabilis* NGP 3. Another parameter such as fat/oil degradation percentage was analyzed in the effluent. Initial degradation percentage of fat/oil in the effluent by *S. variabilis* NGP 3 was 69.33 per cent. It was increased up to 97.08 per cent on fifth day. After fifth day, the degradation percentage was decreased.

In the earlier study, degradation of oil from automobile effluent by *Pseudomonas* sp. was the best degrader with the combination of glycerol and inorganic salts. The oil and grease containing wastes were treated with lipase, in that scale the removal of COD was increased and the fat degradation ratio was also decreased rapidly [20, 43].

Bioremediation of slaughter house waste water: Bioremediation of slaughter house waste water was carried out by *S. variabilis* NGP 3. The results were showed in **table 4 and figure 5**. The general parameters such as TOC, COD and fat/oil degradation percentage were analyzed. The TOC content of the waste water before the treatment was 185 mg/l; it was gradually increased up to 77.0 per cent on tenth day. The Initial content of the COD was 4150 mg/l, on tenth day the COD content of the waste water was decreased up to 44.44 per cent. In the fat/oil degradation, the initial degradation was found to be 64.60 per cent. It was increased up to 93.91 per cent on fourth day. The degradation percentage was decreased drastically after fourth day of incubation.

In the previous report, the biodegradation of slaughter house waste water was carried by lipase producing bacterial species. The degradation studies were continued for 12 days of incubation period for the reduction of BOD and lipid content [25]. Similar studies were carried out with the combined culture of *P. aeruginosa, B. subtilis* and *A. calcoaceticus* to facilitate the bioremediation process [44].

TABLE 3: LIPASE ACTIVITIES OF *S. VARIABILIS* NGP 3 IN THE BIOREMEDIATION OF AUTOMOBILE INDUSTRY EFFLUENT

Incubation period (days)	Total organic chlorine (mg/l)	COD (mg/l)	Fat/Oil degradation (%)
0	115	2530	-
1	183	1800	69.33
2	226	1560	81.22
3	278	1440	92.42
4	307	1160	96.54
5	392	1000	97.08
6	426	880	82.61
7	467	840	81.30
8	498	680	76.49
9	533	600	49.36
10	590	480	28.05

TABLE 4: LIPASE ACTIVITIES OF *S. VARIABILIS* NGP 3 IN THE BIOREMEDIATION OF SLAUGHTER HOUSE WASTE WATER

Incubation period (days)	Total organic chlorine (mg/l)	COD (mg/l)	Fat/Oil degradation (%)
0	185	4150	-
1	244	3960	64.60
2	284	3840	80.66
3	298	3560	93.47
4	356	3320	93.91
5	425	3120	86.25
6	496	2960	54.00
7	567	2800	59.07
8	638	2560	43.10
9	709	2440	36.18
10	801	2400	35.98

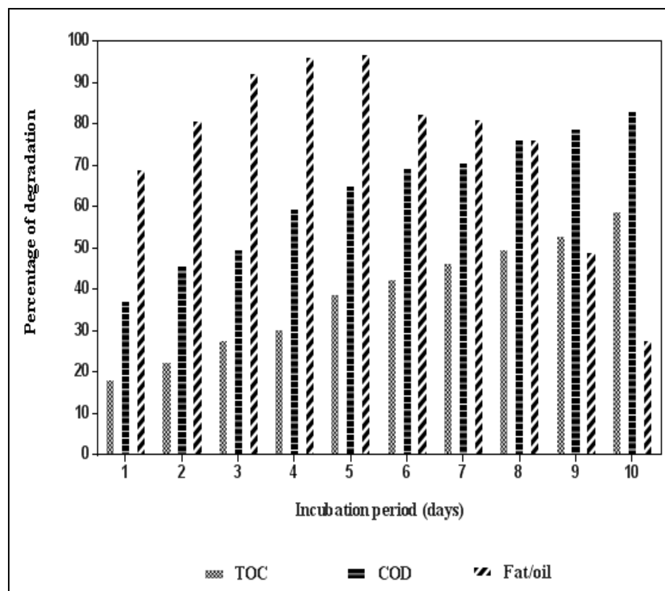

FIG. 4: BIOREMEDIATION OF AUTOMOBILE EFFLUENT BY LIPASE PRODUCING *S. VARIABILIS* NGP 3

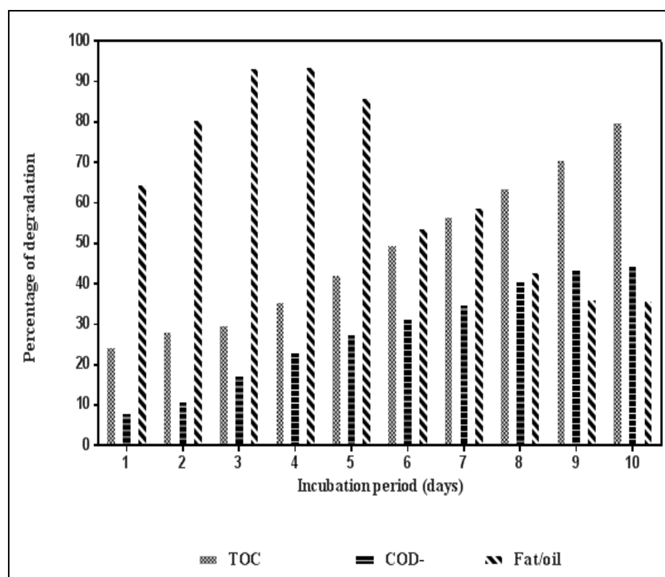

FIG. 5: BIOREMEDIATION OF SLAUGHTER HOUSE WASTE WATER BY LIPASE PRODUCING *S. VARIABILIS* NGP 3

CONCLUSION: The present work was concluded that, the treatment of automobile industry effluent and slaughter house waste water by the marine actinomycete isolate *S. variabilis* NGP 3. In this treatment, various parameters such as total organic chlorine (COD), chemical oxygen demand (COD), fat/oil degradation were analyzed up to 10 days of incubation period. In this two different waste water treatment, the isolate efficiently treated the automobile industry effluent than slaughter house waste water.

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