Available online at www.derpharmachemica.com

ISSN 0975-413X
CODEN (USA): PCHHAX

Der Pharma Chemica, 2016, 8(20):298-306
(http://derpharmachemica.com/archive.html)

Optimization and purification of lipase through submerged fermentation by *Alcanivorax* sp. GI-CMST1 from the gut of marine fish *Sardinella longicepsis*

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ABSTRACT

The present study was aimed at optimization and purification of lipase from Alcanivorax sp. GI-CMST. Various nutritional and physical parameters induce more production of lipase production as 2g lactose (w/v), 1.25g yeast extract (w/v), 0.5µl olive oil (v/v), pH 8.0, Temperature 50ºC and incubation time 72h. The lipase was purified by 75% $(NH_4)_2SO_4$ fractionation followed by simultaneous desalting and concentrating by ultrafiltration, then chromatography as DEAE-Cellulose and Sephadex G-75 gel filtration. The molecular weight and activity of the enzyme were 42kDa as determined by crude ammonium sulphate, DEAE-Cellulose, and Sephadex G-75 gel filtration through SDS and native polyacrylamide gel electrophoresis. Detailed results on optimization and purification of lipase are discussed here with.

Keywords: *Alcanivorax* sp. GI-CMST1, lipase, optimization, purification, submerged fermentation.

INTRODUCTION

Enzymes are proteins with highly specialized catalytic functions, produced by all living organisms [1]. Lipases are produced by many microorganisms [2]. Several authors have reported the lipase - producing microorganisms are basically diversified in different environments including marine environment [3]. The major advantage of bacterial species present in the gut of fish can influence the health, the robustness of the host and induce extracellular enzyme production [4]. Lipase - producing bacteria produce orange fluorescent halos around their colonies under UV light, but lipase negative bacteria do not show orange fluorescence upon UV irradiation [5]. Akanbi [6] evidenced that, the lipases-producing *Bacillus* species were identified by the conventional Gram-staining technique, Biochemical tests, and Biologic Micro- station system by using 16S rRNA gene sequencing.

The lipases produced by the identified microbes are through different culture conditions and it was mostly by submerged culture [7], however, solid state fermentation method can also be used for lipase production. Many studies have been undertaken to define the optimal culture and nutritional requirements for lipase production by submerged culture [8] Chartrain [9] reported that the lipase produced by *Pseudomonas aeruginosa* MB5001 was purified by using the three-step procedure, which includes concentration by ultrafiltration, followed by ion exchange chromatography and gel filtration. This work reports on the isolation, optimization of lipase production with different cultural conditions and purification of lipase secreted by *Alcanivorax* sp. GI-CMST 1.

MATERIALS AND METHODS

2.1.Microorganism and initial cultivation condition

The bacterium used in the current study was isolated from the gut of marine fish *Sardinella longiceps* collected from the Colachal coast of Kanyakumari District, Tamil Nadu. Totally 13 different suspected strains were isolated based on the maximum clear zone when streaked on Rhodamine B agar plate supplemented with 1% olive oil. Simply one strain, i.e. AI performed the highest lipase production, it was confirmed through the maximum zone formation. The 16S rRNA sequence of *Alcanivorax* sp. GI-CMST 1 was compared with other similar bacterial groups by NCBI-BLAST data base program and then it was deposited in NCBI data bank (Accession no: HM133642). Founded on the screening and identification results, the lipase positive strain (*Alcanivorax* sp. GI-CMST 1) was streaked on Rhodamine B agar medium and incubated for 3 days at 37ºC. Then the lipase producing ability of the strain was identified based on the formation of clear orange halo around the colony under UV light at 350 nm [5].

2.2. Lipase production by submerged fermentation

The medium optimization experiment was initiated with culturing the candidate bacterium (*Alcanivorax* sp. GI-CMST 1) in lipase enrichment medium. The enrichment medium contained (w/v): peptone - 0.5 g; K_2HPO_4 - 0.15 g, yeast extract - 0.2 g, NaCl - 1.0 g and pH 7.4. The medium with a loopful of candidate bacterium was incubated at 37ºC for 24 h at 150 rpm. After incubation, 5 ml of the enrichment culture was transferred into production medium containing glucose : 0.5g ; yeast extract : 0.25g ; olive oil : 0.225µl ; $CaCl_2.2H_2O$: 0.003g ; $MgSO_4.7H_2O$: 0.001g and $FeCl_3.6H_2O$: 0.05g. The production medium was then incubated in a shaker incubator (at 150 rpm) for 72h at 37ºC. The cells were then harvested by centrifugation at 10,000 rpm for 30 min and the supernatant was further used for lipase assay.

2.3. Lipase assay and protein Measurement

Lipase assay was carried out by the method of [10] using 10mM *P*-nitro phenyl palmitate as a substrate with slight modification. The amount of lipase produced was measured by using *P*-nitro phenol standard graph. The protein contents in the all the samples were estimated by following method [11] and for this sigma, the USA ready to use bovine serum albumin was used as the standard.

2.4. Media optimization for lipase production

The selected bacterial strain was used for lipase production through supplying different chemical and physical parameters such as different carbon, nitrogen sources, triglycerides, different concentrations of NaCl, pH, temperature and incubation time.

2.5. Effect of different carbon sources on lipase production

The effect of carbon sources on lipase production was investigated by using different carbon sources namely glucose, sucrose, fructose, maltose, xylose, and lactose. They were tested individually by replacing the basal formulated production medium at the concentration 1.0 g (2%). The effect lipase production was determined after 72h of incubation by using assay method. Later, the maximum enzyme- inducing carbon source was further optimized by varying its concentration (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 g).

2.6. Effect of different nitrogen sources on lipase production

To test the effect of nitrogen sources on lipase production six different organic and inorganic nitrogen sources *viz*, yeast extract, tryptone, peptone, ammonium nitrate, potassium nitrate and ammonium sulphate were used. They were supplied individually at the concentration of 0.75g (1.5%) in the lipase production medium. The effect of lipase production by these nutrients was determined after 72 h of incubation by assay method. Different concentrations of best nitrogen source i.e. 0.25, 0.50, 0.75, 1.0, 1.25, 1.50, 1.75 and 2.0g were studied to determine the optimum concentration for maximum lipase production.

2.7. Effect of different triglycerides on lipase production

The lipase production was accelerated by incorporation of different lipid sources namely olive oil, sunflower oil, palm oil, neem oil and coconut oil. They were supplied at the concentration of 0.5µl (1%) in individual lipase production medium. Then the medium was allowed for 72 h of incubation at 37 ºC. After that, the lipase production was estimated by assay method. The oil sources producing maximum lipase were optimized by varying their fraction (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 and 0.8µl).

2.8. Effect of different concentrations of NaCl on lipase production

As the bacterium was isolated from the gut of marine fish *Sardinella longiceps*, so the NaCl is a very important nutrient to maintain the osmo regulation problem . Due to its importance, various concentrations of NaCl were tested for lipase production. The concentrations tested were 1.0, 1.50, 2.0, 2.50, 3.0, 3.50 and 4.0g. All

these concentrations of NaCl were individually supplied in the lipase producing basal medium and the effect of lipase production was determined after 72 h incubation.

2.9. Effect of different pH on lipase production

The pH widely influences the growth and production of microorganisms. Optimum pH for lipase production was determined by using different pH ranges (3, 4, 5, 6, 7, 8 and 9) in the basal medium and inoculated with 2 ml of *Alcanivorax* sp. GI-CMST1 seed culture and allowed incubation. After 72h of incubation, the maximum lipase production was determined by assay method.

2.10. Effect of different temperature on lipase production

The effect of temperature was studied by incubating the test bacterial strains at different temperatures. Individual Erlenmeyer flasks (250 ml) containing 50ml of the basal medium was inoculated with 2 ml seed culture of *Alcanivorax* sp. GI-CMST1 and incubated over a period of 72h at different temperatures (10, 20, 30, 40, 50, 60 and 70°C). After 48h incubation, the lipase production was estimated from the culture supernatant by assay method.

2.11. Effect of different incubation time on lipase production

The incubation time is an important factor for the production of extracellular lipase by *Alcanivorax* sp. GI-CMST1. In the present study, 50 ml of individual basal medium was prepared. The contents of the flask were mixed and autoclaved at 121^0C for 15 min. Then 2 ml of seed culture (*Alcanivorax* sp. GI-CMST1) was inoculated in the individual basal medium and incubated at different time intervals (24, 48, 72 and 96 h) in a shaker at 150 rpm. After incubation, the lipase production was estimated by assay method.

2.12. Purification of Alcanivorax sp. GI-CMST 1 lipase

After 72h of *Alcanivorax* sp. GI-CMST 1 fermentation broth was filtered on Whatman paper No. 1 to eliminate all biomass. Benzamidine, a serine protease inhibitor, was added to the fermentation broth to a final concentration of 2 mM to prevent proteolytic degradation occurring during the purification procedure. The filtrate was centrifuged at 10,000 rpm for 30 min at 4ºC. After centrifugation collect the supernatant and discarded pellet. Then allowed for precipitation with 75% ammonium sulphate fractionation. The precipitates were dissolved in 1 ml of Tris-HCl buffer (pH 7.2) and dialyzed (10 kDa dialysis membrane) overnight against 4 L of the 5mM Tris- HCl buffer (pH 7.2). The dialysis sample was checked for enzyme activity as well as protein content. 2 ml of dialysis sample was applied to 15 ml of preactivated DEAE -Cellulose column (Length of column 35 cm and length of packing 15 cm). The enzyme was eluted with linear gradient of 50 ml of 5 mM Tris-HCl buffer, pH 7.2 with a flow rate of 5 ml/2min. All the fractions were checked for enzyme activity. The active fractions were pooled and applied on Sephadex G-75 for separation of protein (Based on the size of the molecule). In the same buffer at a flow rate of 5ml/2min. Then each fraction was tested lipase and protein content through Spectrophotometric assay method Syed *et al.,* (2010) [12].

2.13. Determination of molecular weight and active compound of lipase protein by SDS and Native - PAGE

The Sephadex G-75 high lipase active fraction was determined the molecular weight of *Alcanivorax* sp. GI-CMST 1 lipase by 10% SDS-PAGE as described by Laemmli, [13] A low molecular mass marker kit (Amersham) was used to determine the molecular mass. The gels were stained with silver nitrate staining to reveal the proteins. Lipase active compound was determined through Non-denature polyacrylamide gel electrophoresis by using Sephadex G-75 high lipase active fraction Hiol [14]. After electrophoresis, the gel was taken out from two glass plates by the help of distilled water and place into the shallow staining tank. Coomassie colloidal blue staining solution was applied on the gel for 2h with slow shaking. After 2h added destaining solution. Then show the lipase active compound it indicated the zone of inhibition in the presence of lipase protein presented area.

2.14. Statistical analysis

The results obtained in the present study were subjected to relevant statistical analysis using Microsoft Excel 2007. Tests for significant differences were analyzed using one- way analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Most marine and estuarine organisms are commercially produced a higher amount of lipases such as bacteria, fungi, and other microorganisms. Similarly, in the present study the selected bacterial strain was isolated from the gut of marine fish *Sardinella longicepsis* and the strain was screened for lipase producing ability on Rhodamine B agar plate (Figure 1). The bacterial strain was produced orange color clear zone after 72 h of incubation under the UV light at 350 nm and it was due to the hydrolysis of olive oil. Similarly, [15] had evidenced that the lipase producing *vibrio* Sp.-A was isolated from the gut of Tripod fish. In accordingly Rhodamine B agar, the strains of *V. fischeri* showed 12 mm of orange halos around the bacterial colonies at the 10- day incubation [16].

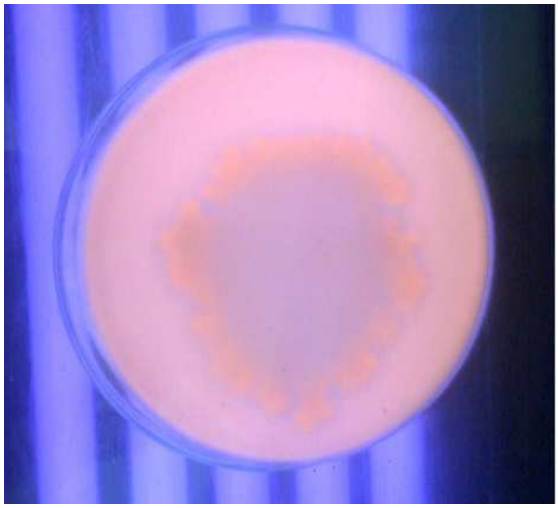

Figure. 1: Screening of *Alcanivorax* sp. GI-CMST 1 for extracellular lipase production on Rhodamine B agar

Based on the morphological, physiological, biochemical characteristics the candidate bacterial strain was identified as *Alcanivorax* sp. Further, the BLAST search of the 16S rRNA of the candidate strain showed 99% similar identity with that of FJ937898; *Alcanivorax* sp. LS45. Then the sequence of the candidate bacterial strain *Alcanivorax* sp. GI-CMST 1 was submitted to GenBank in NCBI data base under the accession no HM133642 (Figure 2). The same [17] had reported that the confidence level (99% identity) was also identified as *Aeromonas* sp. EBB-1 found in other species including *Aeromonas veronii* strain YA090911 (accession no. GU735964.1), *Aeromonas* sp. MK2 (accession no. GU566308.1).

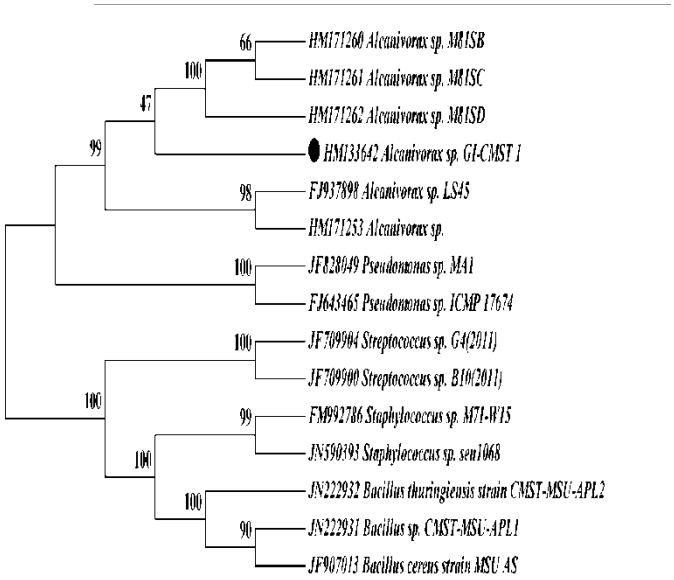

Figure.2: Identification of *Alcanivorax* sp. GI-CMST 1 by using 16S rRNA through phylogenetic construction

● – Candidate strain of *Alcanivorax* sp. GI- CMST 1

The carbon source is an important substrate for energy production in microorganisms. Among the carbon sources tested, lactose has the highest influence on maximum lipase (286.40 U/ml) production than others. (Table 1). The one -way ANOVA for the data on lipase production as a function of variation due to different carbon sources is statistically more significant (F = 960224; P< 0.0001). Among the different concentrations (0.5 to 5.0 g) of lactose tested, 2.0 g was the optimum concentration to produce maximum lipase activity (186.97 U/ml) (Figure 3). The one way ANOVA for the data on lipase production as a function of variation due to different concentration of lactose is statistically more significant (F = 517074; P< 0.0001). Similarly, [18] who reported that the lactose has maximal enzyme production 29.32 UmL^{-1} at 2% concentration by *Enterobacter agglomerans* through submerged fermentation.

Table 1: Efferent of different carbon, nitrogen sources, triglycerides and different concentration of NaCl on lipase production by *Alcanivorax* sp. GI-CMST 1

S. No	Carbon sources (1g)	Lipase production (U/ml)	Nitrogen sources (0.25g)	Lipase production (U/ml)	Different Triglycerides (5µl)	Lipase production (U/ml)	NaCl (g)	Lipase production (U/ml)
1	Glucose	116.86± 0.0572	Peptone	96.530 ± 0.1715	Olive oil	461.71 ± 0.2449	0.5	170.41 ± 0.1715
2	Sucrose	225.34± 0.0490	Casein enzyme hydrolysate	112.94 ± 0.0163	Sunflower oil	66.459 ± 0.0114	1.0	171.24 ± 0.0980
3	Fructose	200.25± 0.0898	Yeast extract	625.30 ± 0.1633	Palm oil	73.977 ± 0.1723	1.5	187.66 ± 0.2694
4	Maltose	160.73± 0.0980	Ammonium nitrate	138.50 ± 0.1633	Neem oil	65.306 ± 0.0841	2.0	200.34± 0.0980
5	Xylose	236.23± 0.0898	Potassium nitrate	81.864 ± 0.1731	Coconut oil	108.24 ± 0.0163	2.5	259.61 ± 0.2531
6	Lactose	286.40± 0.0980	Ammonium sulphate	72.824 ± 0.1731	Control	8.3017 ± 0.0826	3.0	166.58 ± 0.1143
7	Control	172.53± 0.0653	Control	48.934 ± 0.2547	-	-	3.5	154.22 ± 0.0898
8	-	-	-	-	-	-	4.0	150.90 ± 0.2449
9	-	-	-	-	-	-	Control	92.794 ± 0.1813

Each value is the Mean ±SD of triplicate analysis

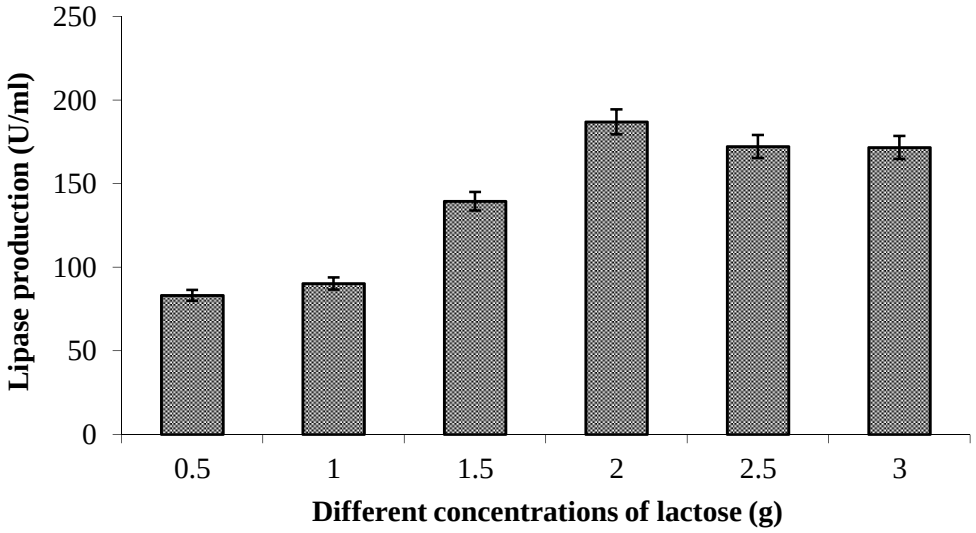

Figure. 3: Effect of different concentration of lactose on lipase production by *Alcanivorax* sp. GI-CMST 1

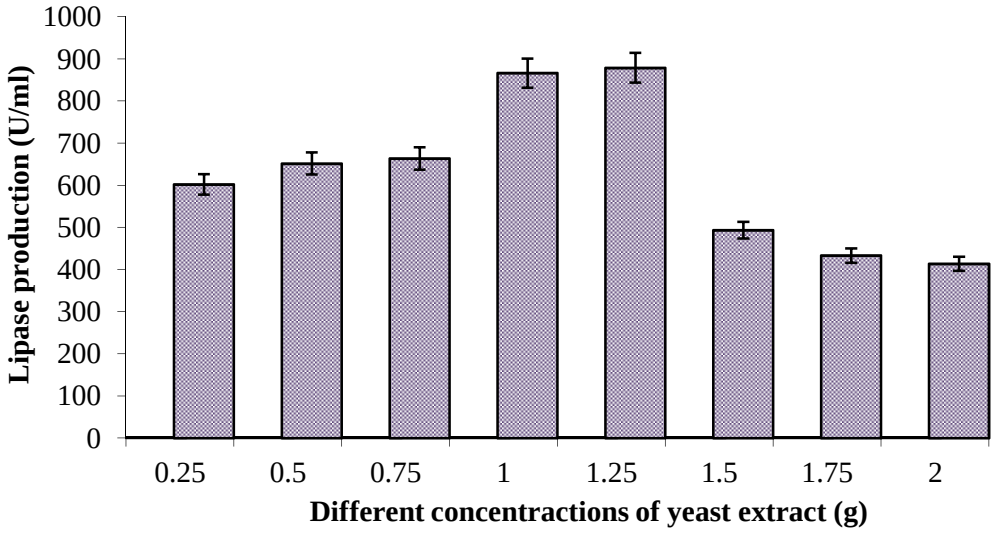

Figure.4: Effect of different concentration of yeast extract on lipase production by *Alcanivorax* sp. GI-CMST 1

Table 1 provides the effect of different nitrogen sources on lipase production by *Alcanivorax* sp. GI-CMST 1. Among these sources, high lipase production was observed in organic nitrogen source yeast extract (625.30 U/ml). The one- way ANOVA for the data on lipase production as a function of variation due to different nitrogen sources was statistically highly significant ($F = 2793286$; $P < 0.0001$). Among the different concentrations of yeast extract tested, 1.25 g (2.5%) was found to be an optimum to produce the highest amount of lipase (1193.14 U/ml) (Figure 4). The one-way ANOVA for the data on lipase production as a function of variation due to different concentration of yeast extract was statistically very much significant ($F = 1766064$; $P < 0.0001$). This finding was supported by the studies of [19] documented that, the maximum lipase production (186.9 U/L) was obtained in 0.1% yeast extract supplement medium by *Pseudomonas aeruginosa* BTS-2 through submerged fermentation.

Lipid- induced lipase production by *Alcanivorax* sp. GI-CMST 1 was investigated by the addition of lipids to the culture medium. The lipase production in lipid supplemented medium gave better production than the control medium. In general, *Alcanivorax* sp. GI-CMST 1 lipase preferred natural triglycerides, compared to synthetic triglycerides. It hydrolyzed all tested triglycerides with the highest degree of affinity to olive oil (461.71 U/ml) (Table 1). The one-way ANOVA for the data on lipase production as a function of variation due to different triglycerides was statistically very significant ($F = 3154678$; $P < 0.0001$). Among the different volume of olive oil tested, 0.5µl was found to be an optimum to produce the highest amount of lipase (331.51 U/ml) (Figure 5). The one -way ANOVA for the data on lipase production as a function of variation due to a different volume of olive oil was statistically highly significant ($F = 56109$; $P < 0.0001$). This study gives significant results and it was correlated with the studies by [20] reported that the olive oil has significantly influenced lipase production by thermophilic *Bacillus* sp.LBN4.

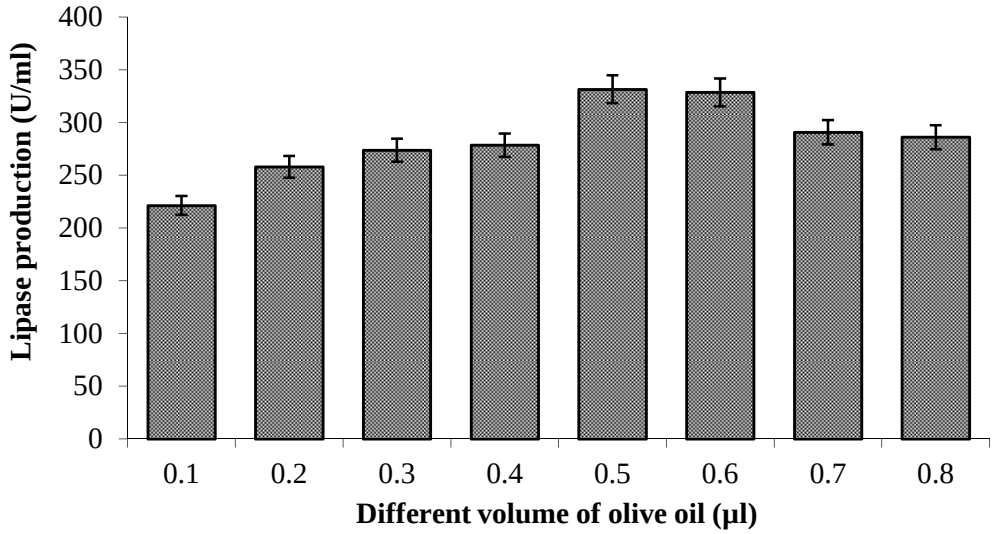

Figure.5: Effect of different volume of olive oil on lipase production by *Alcanivorax* sp.

GI-CMST1

NaCl is an important nutrient factor in fermentation studies with marine organisms. The effect of NaCl concentration on lipase production by *Alcanivorax* sp. GI-CMST 1 revealed that 2.5g (5%) NaCl was the suitable concentration to produce more amount of lipase (259.61 U/ml) (Table 1). The one- way ANOVA for the data on lipase production as a function of variation due to different concentration of NaCl was statistically grand significant ($F = 118701$; $P < 0.0001$). This study supports the previous findings of [21] reported that, the lipase production by halophilic bacterium *Staphylococcus epidermidis*. They observed the lipase production by this particular bacterium was high at the optimized culture concentration of 0.3 - 4 M NaCl (14-16%).

The effect of media pH on lipase production by *Alcanivorax* sp GI-CMST1 is given in Table 2. The lipase production was maximum (260.07 ± 0.0245 U/ml) at alkaline pH 8. The one - way ANOVA for the data on lipase production as a function of variation due to different pH was statistically very significant ($F = 559553.0225$; $P < 0.0001$). Similarly,[22] were documented that the maximum (12.15 Eu/ml) lipase production was obtained at required pH 8 in basal medium for 48h incubation by *Staphylococcus* sp. Lp12 through submerged fermentation.

Table 2: Effect of different pH, temperature and incubation time on lipase production by *Alcanivorax* sp. GI-CMST1

S.NO	Different pH	Lipase production (U/ml)	Different temperature (ºC)	Lipase production (U/ml)	Different incubation (h)	Lipase production (U/ml)
1	3	7.425 ± 0.1733	10	100.54± 0.0327	24	66.459 ± 0.1886
2	4	34.129 ± 0.1739	20	112.94± 0.0082	48	186.32 ± 0.1714
3	5	40.171 ± 0.0245	30	122.95± 0.0163	72	244.16 ± 0.0244
4	6	40.909± 0.2474	40	123.717± 0.0287	96	152.84 ± 0.3429
5	7	130.19± 0.0327	50	129.09± 0.1203	-	-
6	8	260.07± 0.0245	60	67.059± 0.0041	-	-
7	9	115.67± 0.2613	70	60.325± 0.0057	-	-

Each value is the Mean ±SD of triplicate analysis

The effect of different temperature on lipase production by *Alcanivorax* sp GI-CMST1 is given in Table 2. The lipase production was maximum (129.09 ± 0.1203 U/ml) 50ºC. The one way ANOVA for the data on lipase production as a function of variation due to different temperature was statistically very significant (F = 658096.1464; P < 0.0001). Similarly, [23] were reported that the maximum lipase production (12.8 EUm^{-1}) was observed at 50°C by *Acinetobacter baylyi* through response surface methodology (RSM).

The incubation time is an important factor for the production of extracellular lipase by *Alcanivorax* sp GI-CMST1. The maximum lipase production was observed on the second and third day (72h) depends upon strains as shown in Table 2. In the present study, maximum (186.32 ± 0.1714 U/ml) lipase production was achieved during 72 h of incubation by *Alcanivorax* sp. GI-CMST1. The one - way ANOVA for the data on lipase production as a function of variation due to different incubation time was statistically very significant (F = 320662.61; P < 0.0001). Similarly, [24] accepted that the maximum (925 U/g) production of lipase in coconut cake supplemented medium after 72h (3days) of incubation through solid sate fermentation by *Candida rugosa*.

The purification of lipases, independent of the enzyme source, has a restricted capacity to improve the specific activity, reaching a maximum of 5 times the initial value[25]. Enzyme activity was found in supernatant up to 75% saturation. Among the result of lipase activity and protein content was 24576 U and 109 mg/ml, its specific activity was 22.26 U/mg and purification fold 15 respectively (Table 3). Similarly, [14] evidenced that the crude *Mucor hiemalis f. hiemalis* lipase was obtained 17 fold purification and 47 U/mg specific activity by the treatment of 75% ammonium sulfate precipitation.

Table 3: Purification of *Alcanivorax* sp. GI-CMST 1 lipase

S.NO	Purification step	Lipase activity (U/ml)	Protein content (mg/ml)	Total lipase (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Yield (%)
1	Crude lipase	254± 17.96	104± 4.08	14016 ± 36.7	9984± 57.1	1.40± 0.20	-	100 ± 0.00
2	Ammonium sulphate precipitation	256± 28.58	109± 16.33	24576 ± 40.8	1104± 16.3	22± 4.90	15 ± 4.08	150 ± 4.90
3	DEAE-Cellulose column	295± 32.66	114± 20.41	28320 ± 44.9	1094± 8.16	25± 6.53	18 ± 8.16	180 ± 6.53
4	Sephadex G-75 chromatography	386± 45.72	143± 24.49	37056 ± 48.9	1372± 40.8	27± 8.16	19 ± 12.2	190 ± 8.16

Each value is the Mean ±SD of triplicate analysis

Alcanivorax sp. GI-CMST1 DEAE – Cellulose elution fractions 4 (0.05M NaCl) which showed maximum enzyme activity (28320 U) was pooled and lyophilized to concentrate the sample. Protein was concentrated to 114 mg/ml and its purification fold was 18 after IEC (Fig. 6 and Table. 3). It was stored at 4ºC until loading to GPC. Likewise, the strategy was adopted by [26] with 42.99 fold purification by ion exchange chromatography using DEAE A-50 for lipase from *P. aeruginosa* PseA. And the Sephadex G-75 column nine elution fractions which showed maximum lipase activity 37056U, protein 143 mg/ml and purification fold 19 was collective by *Alcanivorax* sp. GI – CMST 1 (Figure 7 and Table 3). In the same way, *Fusarium oxysporum* lipase was occurred 302.2 U/mg specific activity and 41.4 fold purity was observed after filtration of Sephadex G-75 [27]. The purified enzyme was represented by a single band corresponding to a molecular weight and lipase active compound (Clear zone) of about 42KDa, as revealed by SDS and native PAGE analysis (Fig.9 and 10). Likewise,[28] reported that the molecular weight and activity of lipase (Clear zone formation) from *Mucor* sp. strain isolated from palm fruit was 42KDa by SDS and native PAGE.

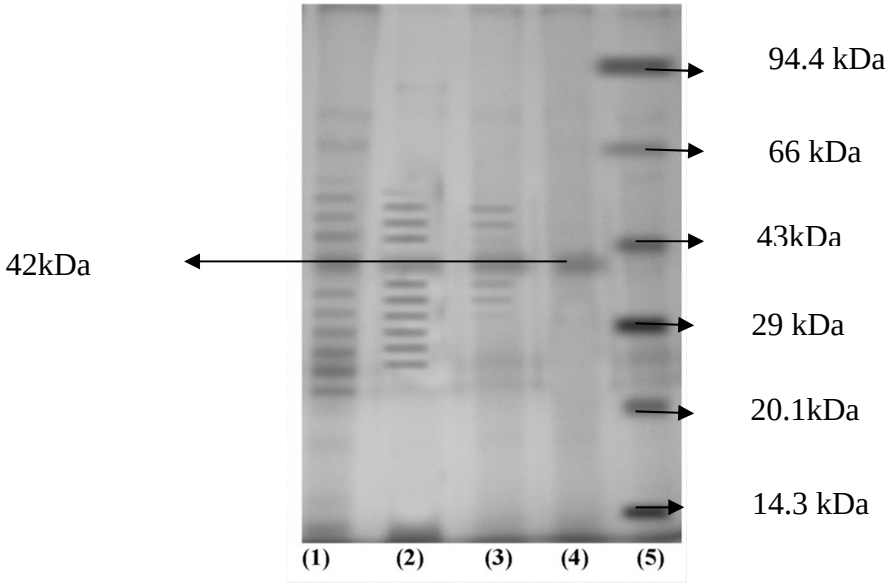

Figure. 6: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis pattern of purified lipase

SDS-PAGE was conducted in 10% gel. Lane 1: Crude sample, Lane 2: 75% ammonium sulphate precipitated sample, Lane 3: DEAE –Cellulose fraction sample; Lane 4: 42 kDa Purified lipase from Sephadex G- 75 column fraction; Lane 5: Protein molecular weight markers

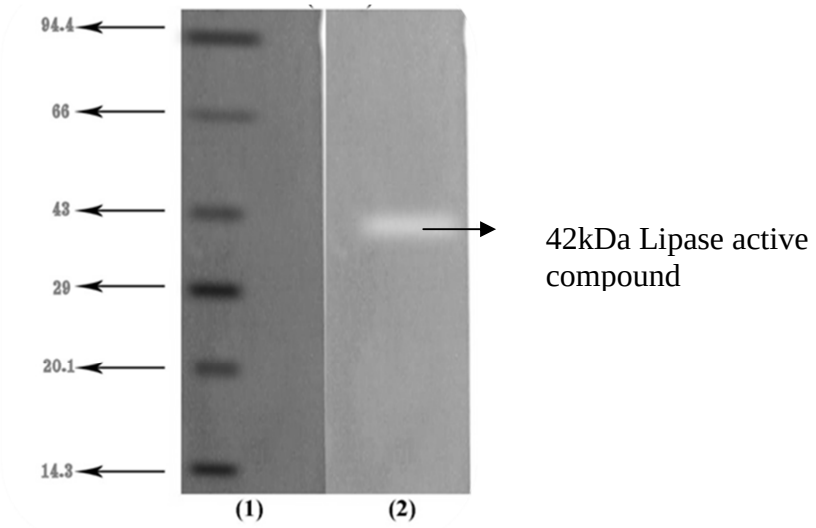

Figure. 7: Native-polyacrylamide gel electrophoresis pattern of purified lipase.

Native -PAGE was conducted in 10 % gel. Well 3, 4, 5, 6 showing clear zone was indicated for 42 kDa lipase activity from crude, 75 % ammonium sulphate, DEAE-Cellulose and Sephadex G- 75 column fraction sample

CONCLUSION

The lipase producing strain was isolated from the gut of marine fish *Sardinella longiceps.* The obtained results showed that the medium composition for lipase production was lactose 2g, yeast extract 1.25g, olive oil 0.5µl, NaCl 2.5g, pH 8.0, temperature 50ºC and incubation time 72h. The purification of lipase by using ammonium sulphate at 75% saturation showed the high total activity (24576U), DEAE - Cellulose (28320U) and Sephadex G-75 (37056U). The molecular weight and activity of purified lipase protein were 42KDa. This alkaline and thermo stable lipase were mainly used in many fields especially detergent, textiles, food and pharmaceutical industries.

Acknowledgements
The authors wish to thank all the scientists and scholars who helped to complete this project.

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