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Purification and Characterization of Lipase from *Shewanella* SP. CMST GISA-MSU through Solid State Fermentation and Its Positional Specificity.

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ABSTRACT

Extracellular lipase was produced by *Shewanella* sp CMST GISA-MSU isolate from the gut of a marine fish *Sardinella longiceps* has been investigated in various cheapest agricultural wastes were selected for solid state fermentation of lipase by *Shewanella* sp CMST GISA-MSU. Among the tested substrates, *Brassica oleracea* shreads were favoured more on the production of lipase by *Shewanella* sp CMST GISA-MSU. On optimization, 15% moisture contents, 0.2% (0.03g) $MgSO_4$, 1.2% (180µl) of gingelly oil, 1.6% (0.25g) mannitol (carbon source) and 1% (0.15g) tryptone (nitrogen sources), different concentrations of NaCl 10% (1.5g). Furthermore, 60 kDa molecular weight of purified lipase was determined by SDS and Native PAGE analysis. Lipase relative activity was clarified to be stable within the temperature range of 10–70°C and pH range of 5-9, with maximal relative activity at 60°C and pH (7). Finally, determined the positional specificity of purified *Shewanella* sp CMST GISA-MSU lipase by TLC analysis.

Keywords *Brassica oleracea* shreads. Lipase. *Sardinella longiceps gut bacterium.* Chromatography. Positional specificity.

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INTRODUCTION

Lipase (Triacylglycerol acylhydrolases, EC 3.1.1.3) are a class of enzymes that catalyze the hydrolysis of long chain triglycerides to intermediate and short chain di and monoglycerides, free fatty acids and glycerol [1]. Apart from hydrolyses, lipases are also involved in a wide range of conversion reactions that include esterification, interesterification, transesterification, alcoholysis, acidolysis and aminolysis in non aqueous media [2]. In particular different species of fish and crustaceans have a specific resident gut microbiota, which is capable of synthesising enzymes in nature [3]. Many authors reported to, the bacterial and fungal strains were identified as a strong lipase producer when streaked on spirit blue agar medium. These mediums are individually supplemented with Tween 80, olive oil and glycerol-tributyrin [4].

Recently some reports proved that solid state fermentation (SSF) is highly effective in the production of lipase. The solid state fermentation (SSF) is an interesting alternative for microbial enzyme production due to the possibility of using residues and by-products of agro industries as nutrient sources and support for microorganism development. The use of by-products as substrates for lipase production, adds high value, and low-cost substrates may reduce the final cost of the enzyme [5]. Bacterial lipases are mostly extracellular and are greatly influenced by nutritional and physico-chemical factors, such as temperature, pH, nitrogen and carbon sources, inorganic salts and agitation [6]. Downstream processing is fundamental for any fermentation process and it involves isolation and purification sequences to obtain a pure and homogenous product like enzymes [7]. In view of the present was undertaken to investigate the optimization of lipase production, purification and characterization by *Shewanella* sp. CMST GISA-MSU through solid state fermentation using *Brassica oleracea* shreads.

MATERIALS AND METHOD

Selection of bacterium and lipase activity

The bacterium used in this study was isolated from the gut of a marine fish *Sardinella longiceps* collected from the Colachal coast of Kanyakumari District, Tamil Nadu, India. Based on the colour morphology and appearance totally 15 different bacterial strains (A1 to A15) were individually subjected to screening to determine their lipase production capability using Sprit blue agar (SBA) medium. Among the tested 15 bacterial strains, only 8 strains showed lipase positive result. Along with the eight lipase strains (A1 to A8), simply one strain, i.e. A6 performed the highest lipase production, it was confirmed through the maximum zone formation (Table 1). The 16S rRNA sequence of *Shewanella* sp CMST GISA-MSU was compared with other similar bacterial groups by NCBI- BLAST data base program and then it was deposited in NCBI data bank (Accession no: JN093116). Founded on the screening and identification results, the lipase positive strain (*Shewanella* sp CMST GISA-MSU.) 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 [8].

Screening of various cheapest agricultural waste products on lipase production by the candidate bacterium through SSF

In favor of lipase production underneath solid state fermentation, 2 g each of various cheapest agricultural wastes such as *Carica papaya* peel (Papaya peel), *Solanum tuberosum* peel (Potato peel), *Brassica oleracea* shreads (Cabbage shreads), *Beta vulgaris* waste (Beetroot waste) and *Allium cepa* peel (Onion membrane) were added individually in 250 ml flask containing 15 ml nutrient solution (tryptone : 0.09g ; yeast extract : 0.03g ; olive oil : 0.225 ml ; $CaCl_2.2H_2O$: 0.0003g ; $MgSO_4.7H_2O$: 0.001g and $FeCl_3.6H_2O$: 0.15g) were sterilized at 121^0C for 15 min. After that, 4 ml of seed culture of *Shewanella* sp. CMST GISA-MSU was inoculated individually and allowed for incubation at 37^0C for 48h. The effect of the individual supplemented substrates on lipase production was determined through lipase assay. Since this, the maximum lipase yielding substrate (*Brassica oleracea* shreads) was selected for additional studies.

Solid-state fermentation

The selected lipase positive organism *Shewanella* sp. CMST GISA-MSU was enriched using nutrient solution with 2 g of *Brassica oleracea* shreads. Before inoculating the organism, the medium mixture was

sterilized at 121^{0}C for 15 min. Then 4 ml of enriched seed culture (*Shewanella* sp. CMST GISA-MSU) was inoculated and incubated at 37^{0}C for 48h. Afterward, the moldy substrate with the culture was homogenized with 50 ml of distilled water with the help of shaker at 150 rpm for 1 h. After 1 h, the filtrate was collected by ordinary filter paper [9]. The filtrate was centrifuged at 10,000 rpm for 15 min and the supernatant was used for lipase estimation. Lipase activity was quantified according to the modified method of [10]. The lipase activity in the culture supernatant was determined by UV-Vis Spectrophotometer (Techomp - 8500), with the help of p-nitro phenol standard graph. One unit of lipase activity is equivalent to one microgram of p-nitro phenol released under standard assay condition.

Media Optimization for Lipase Production

The lipase production by the selected bacterium was optimized through supplying different parameters such as moisture contents, metal ions, triglycerides, carbon sources, nitrogen sources, and sodium chloride.

Optimization of different concentration of moisture on lipase production

The effect of moisture on lipase production during SSF was determined . For this , 2 g of dry *B. oleraceae* shreads substrate was moistened with different concentrations of nutrient solution (5, 10, 15, 20, 25 and 30%). Then 4 ml each of seed culture was inoculated into the individual sterilized flasks and incubated for 48h. After incubation, the lipase productions was estimated.

Optimization of metal ions on lipase production combined with substrate

To select suitable trace elements for enzyme production by *Shewanella* sp. 0.1% of metal ions such as $FeCl_3$, $MgSO_4$, $CaCl_2$ and $CuSO_4$ were individually added into the basal medium and inoculated with 4 ml seed culture of *Shewanella* sp. The lipase production was measured at after 48h of incubation at 37°C. Among these, maximum lipase producing metal ion was further optimized by varying its concentration (0.06%, 0.1%, 0.2%, 0.26% and 0.33%). After 48 h of incubation, the lipase productions was determined.

Optimization of triglycerides on lipase production combined with substrate

To study the triglycerides -induced production of lipase, six different triglycerides were tested (olive oil, sunflower oil, coconut oil, neem oil, palm oil and gingelly oil). The selected triglycerides were incorporated individually into the nutrient medium at a concentration of 1% (150 µl) and the medium without triglycerides was taken as control. Then, triglycerides showing the highest production on lipase production was further optimized by varying concentrations such as 0.6%, 0.8%, 0.9%, 1%, 1.2% and 1.3%.

Optimization of carbon sources on lipase production combined with substrate

For the identification of suitable carbon sources such as glucose, lactose, maltose, fructose and mannitol were screened individually at the level of 0.6%. Subsequently, the maximum lipase-producing carbon source was further optimized by varying its concentrations such as 0.6%, 1.0%, 1.3%, 1.6% and 2% in the production medium. They were tested individually, and the effect of lipase production after 48 h of incubation at 37^{0}C.

Optimization of nitrogen sources on lipase production combined with substrate

For the selection of nitrogen sources on lipase production by the *Shewanella* sp. under SSF, five different nitrogen sources such as tryptone, yeast extract, peptone, NH_4HCO_3 and $NaNO_3$ were used. They were supplied individually at the concentration of 0.6% in the basal medium. The effects of these nutrients were determined after 48 h of incubation. After screening, the maximum lipase yielding nitrogen source was taken for further optimization through varying its concentration They were supplied individually (0.3%, 0.6%, 1%, 1.3% and 1.6%) along with the basal medium, and the lipase production was determined after 48 h of incubation.

Optimization of sodium chloride on lipase production combined with substrate

The effect of NaCl on lipase production was tested by adding different concentrations of NaCl in the production medium. 2g each of dry substrate was taken in individual basal medium with different concentrations of NaCl (3.3%, 6.6%, 10%, 13.3% and 16.6%) and 4 ml of seed culture of *Shewanella* sp. was inoculated in sterilized flasks. The media were incubated for 48h at 37^0C. After incubation, the lipase production was determined.

Purification of *Shewanella* sp CMST GISA-MSU lipase

After 48 h one- liter fermentation of optimized *Shewanella* sp. culture broth was filtered on Whatman paper No. 1 to eliminate all biomass. The filtrate was centrifuged at 10,000 rpm for 30 min at 4ºC. After centrifugation collected the supernatant allowed for precipitation with 60% ammonium sulphate fractionation. After that, the precipitated sample was dissolved in 10 ml Tris-HCl buffer and dialyzed (10 KDa dialysis membrane) overnight against 4 L of the 0.05M Tris-HCl buffer. Then the fraction was preactivated DEAE cellulose column (column length: 25 cm and packing length:10 cm). The enzyme was eluted with linear gradient of 50ml of 100 mM Tris- HCl buffer (pH 7.2), and also equilibrated with different concentrations of NaCl solution with the flow rate of 5ml/min. 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 molecules. Sephadex G -75 column and equilibrated with 75ml of 0.05M sodium phosphate buffer (pH 7). At 2 minutes intervals, 5 ml each of individual fractions were collected. Then each fraction was tested for lipase and protein contents through Spectrophotometric assay method [11]. The protein content at each stage of enzyme purification was determined according to the method of [12] with Bovine serum albumin as the standard. The relative molecular mass and activity of the purified lipase were estimated by 10% SDS and Native PAGE according to the method of Laemmli [13] and Hiol *et al.* [14] and the proteins were stained with Coomassie brilliant blue R-250.

Characterization of purified lipase

Effect of different pH stability on purified lipase

pH stability profiles of purified lipase were studied at pH range of 5, 6, 7, 8 and 9 using Tris– HCl buffer. For stability studies, 0.1ml of purified lipase was mixed with 1ml of individual each pH range of buffer and incubated for 1h at 37ºC. After incubation, 200 µl of sample was used for measuring the relative activity (stability) under normal assay conditions.

Effect of different temperature stability on purified lipase

The optimum temperature for purified lipase stability was determined by assay method. The enzyme activity at different temperatures (10, 20, 30, 40, 50, 60 and 70ºC) was monitored by incubating the enzyme extract (0.1ml) with 1ml Tris- HCl buffer (pH – 7.2) at respective temperatures for 1 h. After that, 200 µl of sample was taken from the mixture and measured the enzyme stability under standard normal assay conditions.

Determination of kinetics parameters

Purified lipase was incubated with various concentrations of *P*- nitrophenol palmitate in Tris-HCl buffer (pH 7.2). The lipase activity was measured by Spectrophotometric assay method. Kinetic constants were calculated from the double reciprocal Line weaver-Burk plot equation [15].

Positional specificity of purified lipase by thin layer chromatography

Positional specificity was determined by TLC method described by Deepa *et al.* [16]. 10mg of pure tripalmitin (Sigma) and 5 ml of 0.1 M Tris- HCl (pH 7.2), containing 1 mM $CaCl_2$ were emulsified by sonication and separated into two equal portions in two individual tubes. Then, one portion was treated with 0.5 ml of purified lipase and another portion was not treated with purified lipase as it served as a control. The mixture was incubated at 37ºC in a shaking water bath for 3 h. After incubation, the reaction products were extracted

by the addition of 5 ml n-hexane. The extract was concentrated by evaporation and applied onto a silica gel plate (Merck). A standard mixture (Sigma) were used as a reference glycerides. Plates were developed with a 96:4:1 mixture (by volume) of hexane/ diethyl ether/acetic acid. The spots were visualized by placing the plate in an iodine chamber. Lipid spots were identified with respect to the Rf values of lipid standards.

Statistical Analysis

The data obtained in the present study were subjected to the following statistical analysis using computer software SPSS-16.0 (SPSS Inc, USA) and were analyzed using One-way ANOVA test at 5% significant level.

RESULTS AND DISCUSSION

The biological diversity of marine and estuarine species provides a wide array of enzymes with unique properties [17]. Esakkiraj *et al.,* [18] studied the lipase producing ability of *Staphylococcus epidermis* CMST-Pi1 was isolated from the gut of shrimp *Penaeus indicus*. Lipases are glycerol ester hydrolases that catalyze the hydrolysis of triacylglycerols into fatty acids, partial acylglycerols and glycerol [19]. In the present study, lipase producing bacterial strain was isolated from the gut of a marine fish *S. longiceps* and then it was screened for its lipase producing ability on spirit blue agar. From the result, it was found that the bacterial strain produced a clear zone of lipase production (17 mm) after 48h of incubation and it was due to the hydrolysis of tributyrin (Figure.1). Among the 8 lipase positive strains, only one strain (A6- Shewanella sp. CMST GISA-MSU) showed maximum zone formation of more than 17 mm by hydrolyzing 1% glycerol tributyrin in sprit blue agar plate (Table. 1).

Further, the *Shewanella* sp. CMST GISA-MSU was established by amplifying the 16S rRNA region and the sequence of the strain (1447 bases) was examined by BLAST analysis. The 16S rRNA genome sequence of the strain showed 100% similar identity with that of JQ824139; *Shewanella* sp. SMK1-12. This 16S rRNA sequence was submitted to the NCBI GenBank, its accession number is JNO93116 (Fig. 2). Dutta and Ray, [20] identified *Bacillus cereus* (AB 24464) an alkaline thermostable lipase producing bacterial strain from spoiled coconut sample through 16S rRNA sequencing analysis. The fluorescence dye Rhodamine B agar plate assay is described as a true lipase assay, which indicates the zone of lipolysis as an orange fluorescent under UV light at 350 nm. In the present study, the conformation of lipase positive bacterial strain (*Shewanella* sp CMST GISA – MSU) in Rhodamine B agar showed orange halos around the colony after 3 days of incubation. Lipase production was monitored by irradiating plates with UV light at 350nm (Figure. 3).

In recent years, considerable research has been carried out using agricultural wastes, which are renewable and abundantly available to produce value added products. Ul-Haq *et al.* [21] were reported that various agro products including rice, corn flour, wheat flour, barley, oat, wheat bran, rice bran, wheat husk and soy bean powder were identified as the best supporter for whole cell synthesis of lipase (WCSL) by *Chinensis* CCTCCM201021. This study revealed that, among the tested substrates, *Brassica oleracea* shreads waste (Cabbage leaf) was identified as the best supporter as it gave the highest (466.07 U/g) synthetic activity of lipase (Figure. 4). The one-way ANOVA test conducted on lipase production as a function of variation between different substrates was statistically more significant ($F = 285876.3$; $P < 0.001$).

. The moisture content of substrate plays a vital role in microbial growth and for effecting biochemical activities in SSF [22]. The influence of initial moisture content of 60% v/w had shown maximum lipase production by *Yarrowia lipolytica*. In accordance to these, in the present study, initial moisture content of 15% v/w had shown maximum (208.02 U/g) lipase production (Table. 2). Further increasing or decreasing level of moisture content beyond the optimum level lead to a reduction in production of lipase. The statistical one-way ANOVA test revealed that the variation between different moisture content on lipase production was highly significant ($F = 31163.04$; $P < 0.001$).

Metalic ions are believed to be influencing enzyme production, according to Tan *et al.,* [23], magnesium (Mg^{2+}), sodium (Na^{+}) and potassium (K^{+}) were beneficial for the biosynthesis of lipases. In their study, they suggested that maximum (1240 U/ml) lipase production was recorded in magnesium sulphate supplemented medium. Similarly in the present study, maximum (150.23 U/g) lipase production was observed in magnesium sulphate supplemented medium by *Shewanella* sp CMST GISA-MSU through solid state

fermentation (Table. 2 and Figure. 5). The statistical one-way ANOVA test revealed that the variation between different metal ions on lipase production was more significant (F =36914.38; P < 0.001). Among the tested concentrations, maximum (304.25 U/g) lipase production was recorded at 3% (0.03g) concentration. The one-way ANOVA test conducted on lipase production as a function of variation between different concentrations of $MgSO_4$ was statistically more significant (F = 131923.2; P < 0.001).

Triglycerides induced lipase production by *Shewanella* sp. was confirmed by the addition of lipids to the culture medium. Among the tested triglycerides, gingelly oil was found to be suitable for maximizing (378.01 U/g) the lipase production (Table. 2). The one-way ANOVA test conducted on lipase production as a function of variation between different triglycerides was statistically more significant (F = 736353.8; P < 0.001). In the same way, Dayanandan *et al.*, [24] investigated that, the maximum (978 U/g) lipase was produced in 5% of gingilly oil added medium by *Aspergillus tamarii* MTCC 5152 under solid state fermentation for 48h of incubation. The result on the effect of different volume of gingelly oil ranged between 0.6 to 1.3% (100 and 200 µl) on lipase production is given in Figure. 6. After 48 h of incubation, maximum lipase production of 139.93 U/g was recorded in the medium containing 1.3% (180 µl) of gingelly oil. The one-way ANOVA test conducted on lipase production as a function of variation between the different volume of gingelly oil was statistically more significant (F = 16351.16; P < 0.001).

Several studies have shown that lipase production was influenced by different carbon sources present in media [25]. In the present study, the effect of various supplementary carbon sources on lipase production by *Shewanella* sp CMST GISA-MSU. revealed that, the mannitol was influenced more effect than that of the other tested carbon sources and it yielded 181.09 U/g of lipase (Table. 3 and Figure.7). The one-way ANOVA test conducted on lipase production as a function of variation between carbon sources was statistically more significant (F = 65355.39; P < 0.001). Similarly, Sekhon *et al.,* [26] emphasized that *Bacillus megaterium* AKG-1 was found to be produced maximum lipase (848 units/ml) with the help of basal medium contains 0.2% of mannitol through submerged fermentation. But in the present study, the lipase production by *Shewanella* sp CMST GISA-MSU. had given positive effect when the culture medium was supplied with 1.6% (0.25g) mannitol. Among the tested different concentrations of mannitol, 1.6% (0.25g) was optimum concentration to produce maximum (167.99 U/g). The one-way ANOVA test conducted on lipase production as a function of variation between different concentrations of mannitol was statistically more significant (F = 12278.9; P < 0.001).

Various nitrogen sources were used for producing maximum extracellular lipase from *Acinetobacter calcoaceticus* [27]. Among these nitrogen sources, tryptone produced maximum (122.26 U/g) lipase when compared to others Table. 3 and Figure. 8. The statistical one-way ANOVA test revealed that the variation between different nitrogen sources on lipase production was more significant (F = 11003.22; P < 0.001). Similarly, Mohamed *et al.,* [28] inferred that the highest lipase production of 0.12 U/ml (1.8 fold) was obtained in tryptone added medium when compared to the basal medium by *Bacillus* sp. strain 42 through submerged fermentation. The effect of different concentrations of yeast extract (1, 2, 3, 4, 5 and 6 %) on lipase production by *Saccharomyces cerevisiae* was studied by Shirazi *et al.*, [29] and they pointed out that the highest lipase activity with 5% yeast extract. In the present study, the influence of different concentrations of tryptone on lipase production showed that 1% (0.15g) was the suitable concentration for enhancing the lipase production. The one-way ANOVA test conducted on lipase production as a function of variation between different concentrations of tryptone was statistically more significant (F = 194351.6; P < 0.001).

In the present study, the origin of the candidate strain was in marine forms, therefore the effect of NaCl on lipase production was studied and the result showed that at 10% (1.5g) NaCl concentration, maximum (142.97 U/g) lipase production was obtained by *Shewanella* sp CMST GISA-MSU. through solid state fermentation (Table. 3). This study supports the findings of Khunt *et al.,* [30], who investigated that, the maximum (19.4 U/ml) lipase production was observed in 10% NaCl supplemented medium by *Halomonas salina* Ku-19 under submerged fermentation. The one-way ANOVA test conducted on lipase production as a function of variation between different concentrations NaCl was statistically more significant (F =9044.967; P < 0.001).

A representative purification profile is summarized in Table 4. In the present study, precipitation of enzyme was carried out by ammonium sulphate, since it was highly soluble in water, cheap and had no deleterious effect on the structure of protein. The result revealed that, among the different tested saturations, 60% saturation was effective concentration with 18 fold specific activity. This 60% ammonium sulphate

saturated and precipitated protein was dialyzed against 0.05M Tris-HCl buffer for 3 h. During this dialysis 10 - 20 kDa impurities were removed from the sample and it was proved to be very effective raising specific activity to 323 U/ml with 34 U/mg specific activity and 1.7 purification fold. This result matches with the purification strategies followed by Kumar *et al.,* [31] and it revealed that 60% saturation was proved to be effective for maximum *Bacillus pumilus* RK31 lipase specific activity of 123 U/mg with purification fold of 1.53. The 60% ammonium sulphate saturated and precipitated protein was dialyzed against distilled water for 24 h. Dialysis removed 10 -20 kDa impurities from the sample and it proved very effective raising specific activity to 211.40 U/ml with 1.74 purification fold. In the present study, the DEAE – Cellulose lipase positive fraction showed 7.5 purification fold and 149 U/mg of specific activity. Likewise, Islam *et al.,* [32] documented that, the DEAE Cellulose purified fraction of *Cirrhinus reba* lipase was obtained with 6.21 U/mg^{-1} of specific activity and 34.5 purification fold. The 7.5% purification fold lipase of *Shewanella* sp CMST GISA-MSU. was further filtered with the help of size exclusion chromatography of Sephadex G-75 and it resulted in 11% fold purified lipase and yield 240%. Zheng –Yu *et al.,* [33] documented that the lipase produced by *Aspergillus niger* F044 was allowed for purification by Sephadex G-75, it resulted with 73.71% fold purification and 33.99% yield. In the present study, the molecular weight of purified lipase protein was estimated to be 60 kDa using purified Sephadex G-75 fraction sample by 10% SDS-PAGE (Figure. 9). Its lipase activity was observed by using 10% Native polyacrylamide gel electrophoresis analysis (Figure. 10). Gaur *et al.,* [34] recorded the molecular weight of purified lipase from *Pseudomonas aeruginosa* PseA was 60kDa through SDS –PAGE analysis by using sodium dodecyl sulfate and native polyacrylamide gel electrophoresis.

pH is a critical parameter that influences the change in the activity of lipase. In the present study, the lipase produced by *Shewanella* sp. showed the maximum relative (stability) activity of 90% at pH 7 (Figure. 11). Rathi *et al.,* [35] evidenced that, the *Burkholderia cepacia* RGP-10 lipase retained its activity at 100% in the treatment of pH 7 at 50°C through submerged fermentation. Park *et al.,* [36] reported an extracellular lipase from *Burkholderia* sp. which exhibited maximum activity (90%) at 60ºC. In the present study the effect of temperature on the relative activity of lipase produced by *Shewanella* sp CMST GISA-MSU., revealed that it was stable in the temperature range from 10 to 70°C and it was specifically high 90% at 60ºC (Figure. 12).

The effect of substrate concentration on the initial velocity of the reaction was studied and a typical Michaelis-Menton relationship was obtained. The kinetic parameters - maximum reaction rate (*Vmax*) and Michaelis - Menton constant (*Km*) for the purified lipase was estimated from the Lineweaver –Burk equation plot [37]. They reported that the kinetic studies of the purified *Aspergillus fumigatus* MTCC 9657 lipase showed a *Km* value of 0.54 mmol/L and a *Vmax* value of 271.2 U for the maximum substrate concentration. Similarly, In the present study, the *Vmax* and *Km* value of lipase were derived from the Double reciprocal Lineweaver -Burk plot and found to be *Vmax* = 80 U/ml/min and *Km* = 0.9 mM/ml (Figure. 13).

The positional specificity of purified lipase was determined through TLC analysis. The result indicated that the lipid sources like monoglyceride (Rf = 0.07), 2, 3 diglyceride (Rf = 0.20), free fatty acids (Rf = 0.49) and triglyceride (Rf = 0.56) were observed in the tripalmitin (control) sample (Figure. 14). Similarly, Akhila Rajan *et al.,* [37] evidenced that, the *Aspergillus fumigatus* MTCC 9657 lipase hydrolysed 10 mg of pure tripalmitin to 1, 3 or 1, 2 diglycerides, monoglycerides and free fatty acids were analyzed by TLC.

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Table 1: Screening of gut bacterial isolates for lipase producing ability in spirit blue agar plates

Symbol for test organisms	Clear zone formation (mm)
A1	++
A2	+
A3	+
A4	+
A5	++
A6	+++
A7	+
A8	++
A9	-
A10	-
A11	-
A12	-
A13	-
A14	-
A15	-

High zone formation : +++ (15 -20mm)
Moderate zone formation : ++ (11- 15mm)
Low zone formation : + (5- 10mm)

Table 2: Optimization of different moisture content, metal ions and triglycerides on lipase production by *Shewanella* sp. CMST GISA-MSU

Different moisture content (%)	Lipase Production (U/g)	Different metal ions (0.1%)	Lipase production (U/g)	Different triglycerides (1%)	Lipase production (U/g)
5	176.78 ± 0.19	$FeCl_3$	126.3 ± 0.16	Gingelly oil	378.01 ± 0.008
10	165.04 ± 0.016	$MgSO_4$	150.23 ± 0.09	Sunflower oil	360.06 ±0.016
15	208.02 ± 0.008	$CaCl_2$	149.45 ± 0.016	Coconut oil	348.28 ± 0.12
20	203.73 ± 0.25	$CuSO_4$	106.61 ± 0.24	Neem oil	344.68± 0.34
25	194.60 ± 0.24	Control	121.02 ± 0.008	Palm oil	151.41 ± 0.24
30	189.18 ± 0.03	-	-	Control	124.06 ± 0.024
Control	154.87 ± 0.11	-	-	-	-

Each value is the Mean ± SD of triplicate analysis

Table 3: Optimization of different carbon sources, nitrogen sources and different concentrations of NaCl on lipase production by *Shewanella* sp. CMST GISA-MSU

Different carbon source (0.6%)	Lipase production (U/g)	Different nitrogen source (0.6%)	Lipase production (U/g)	Different concentrations of NaCl (g)	Lipase production (U/g)
Glucose	137.13 ± 0.01	Yeast extract	112.28 ± 0.10	3.3	114.93 ± 0.26
Fructose	114.31 ± 0.17	Tryptone	122.26 ± 0.11	6.6	116.10 ± 0.08
Maltose	128.16 ± 0.097	Peptone	105.61 ± 0.08	10	142.97 ± 0.26
Lactose	120.74 ± 0.33	NH_4HCO_3	115.80 ± 0.16	13.3	124.71 ± 0.16
Mannitol	181.09 ± 0.008	$NaNO_3$	102.68 ± 0.27	16.6	121.27 ± 0.09
Control	96.18 ± 0.002	Control	90.625 ± 0.009	Control	104.25 ± 0.19

Each value is the Mean ± SD of triplicate analysis

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Table 4: Summary of purification of lipase produced by *Shewanella* sp. CMST GISA-MSU

Purification steps	Lipase activity (U/ml)	Protein content (mg/ml)	Total lipase (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold (%)	Yield (%)
Crude lipase	227 ± 1.63	101 ± 1.63	104693 ± 4.08	5009 ± 7.34	20 ± 4.13	-	100 ± 00
Ammonium sulphate precipitation	263 ± 2.44	93 ± 2.44	121297 ± 4.89	4613 ± 6.53	26 ± 4.25	1.3 ± 0.2	115 ± 2.4
Dialysis	323 ± 3.26	87 ± 3.26	148969 ± 5.71	4315 ± 5.74	34 ± 5.76	1.7 ± 0.1	142 ± 3.3
DEAE - Cellulose	434 ± 4.08	27 ± 4.08	200163 ± 6.53	1339 ± 4.89	149 ± 6.54	7.5 ± 5.7	191 ± 4.1
Sephadex G-75	545 ± 4.89	24 ± 4.89	251357 ± 7.34	1190 ± 5.72	211 ± 7.36	11 ± 6.5	240 ± 4.9

Each value is the Mean ± SD of triplicate analysis

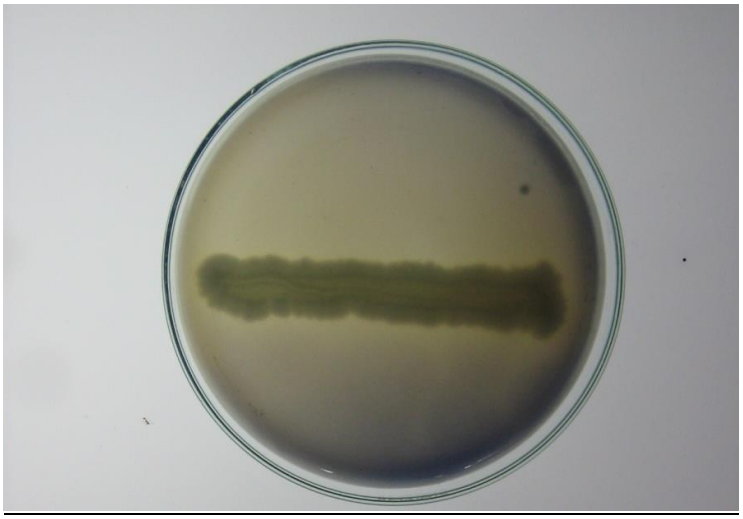

Figure 1. Lipolytic activity of *Shewanella* sp. in spirit blue agar

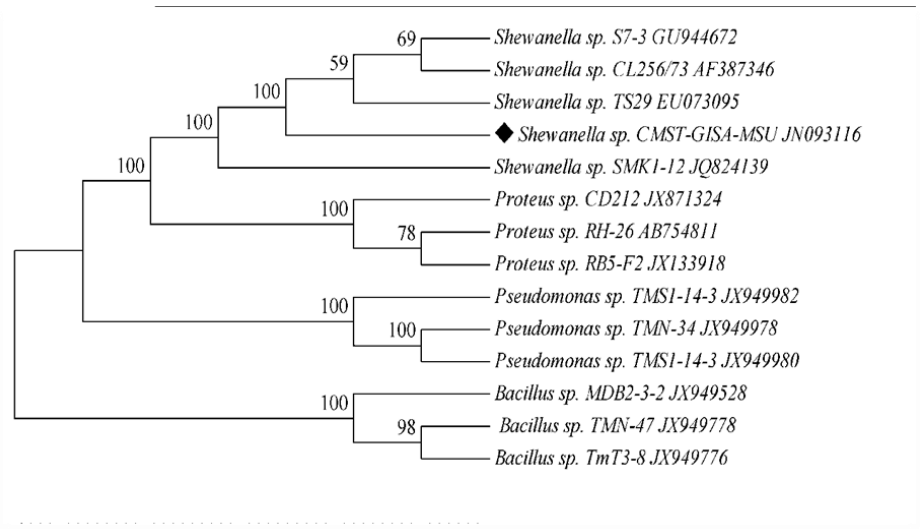

Figure 2. Phylogenetic relationship of *Shewanella* sp. CMST-GISA-MSU with closely related sequences

♦- Candidate bacterium (*Shewanella* sp. CMST-GISA-MUS JN093116)

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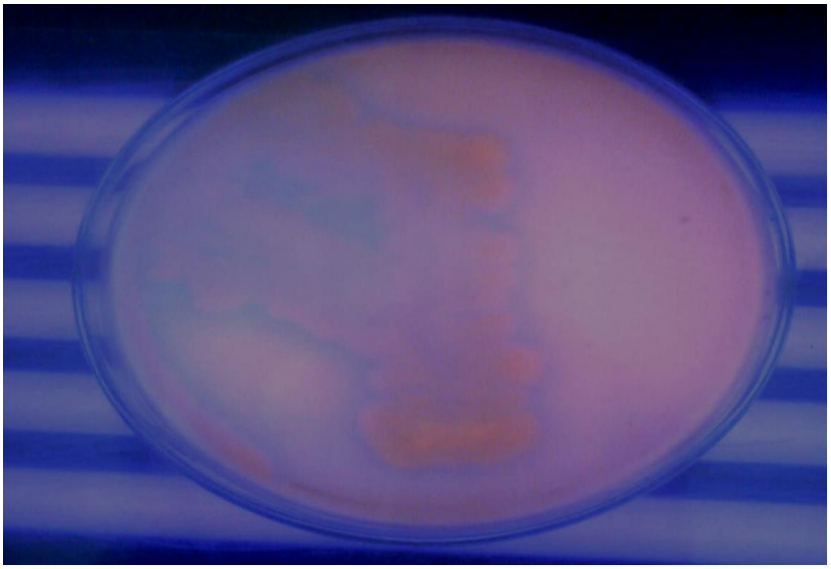

Figure 3. Lipolytic activity of *Shewanella* sp. CMST-GISA-MUS in Rhodamine B agar

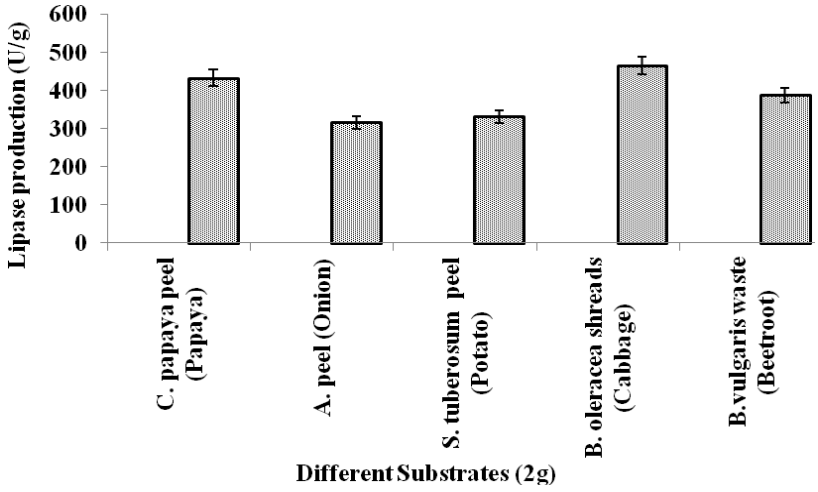

Figure 4. Screening of different substrates on lipase production by *Shewanella* sp. CMST-GISA-MUS

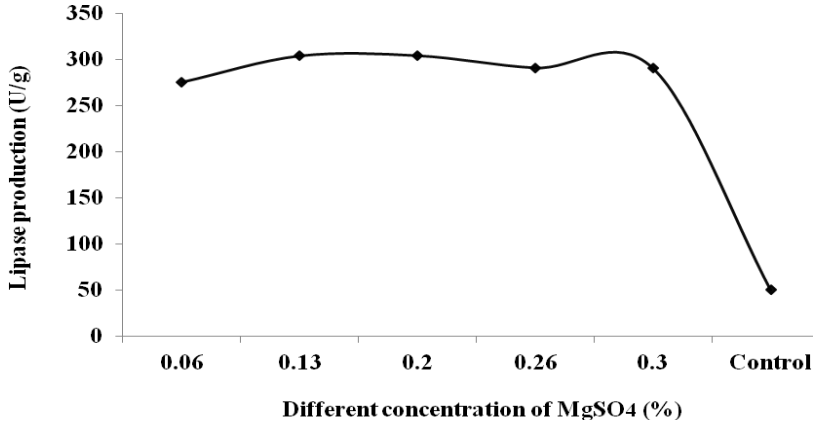

Figure 5. Optimization of different concentrations of $MgSO_4$ on lipase production by *Shewanella* sp. CMST-GISA-MUS

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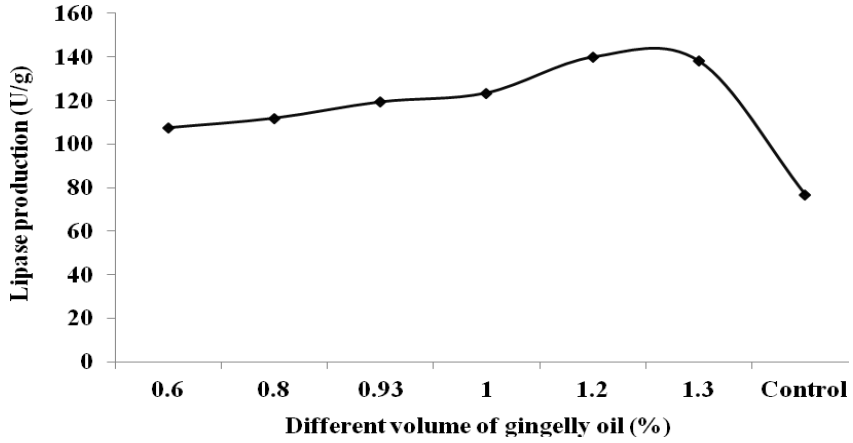

Figure 6. Optimization of different volume of gingelly oil on lipase production by *Shewanella* sp. CMST-GISA-MUS

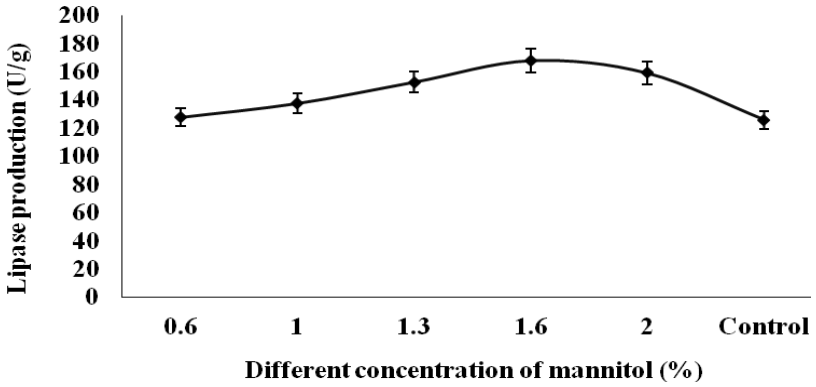

Figure 7. Optimization of different concentrations of mannitol on lipase production by *Shewanella* sp. CMST-GISA-MUS

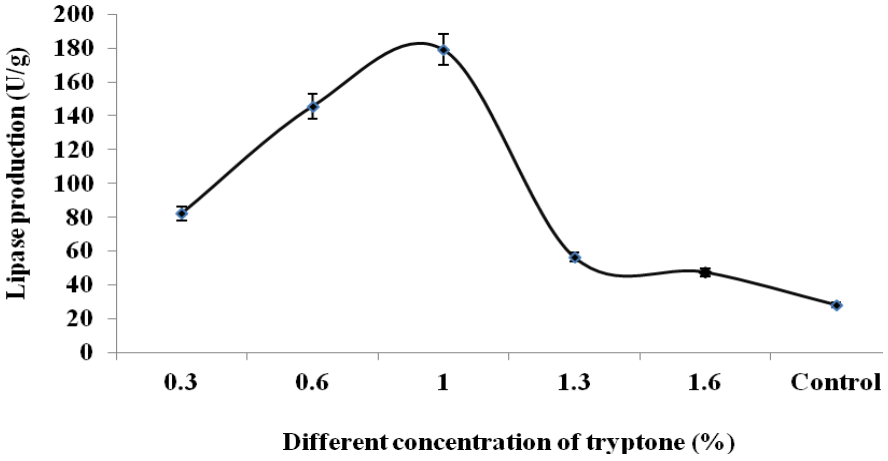

Figure 8. Optimization of different concentrations of tryptone on lipase production by *Shewanella* sp. CMST-GISA-MUS

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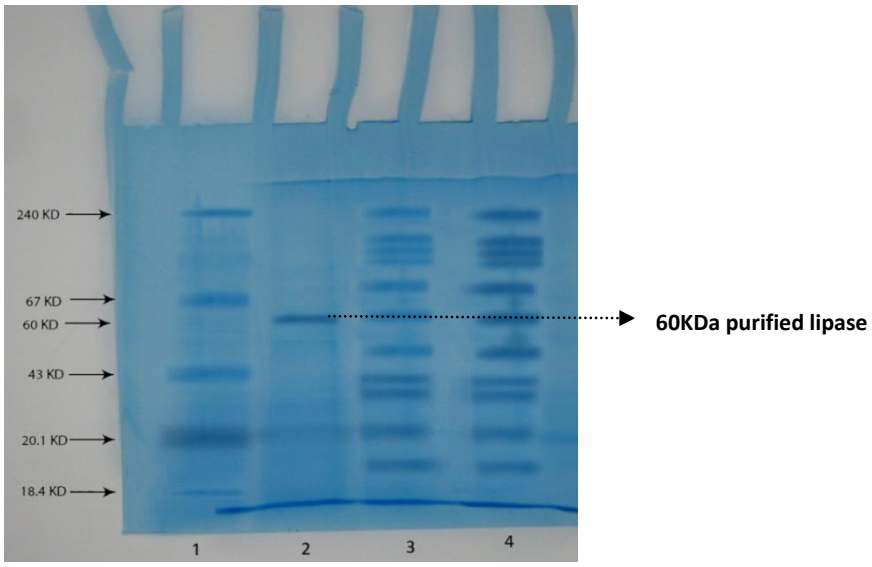

Figure 9. SDS-PAGE analysis of purified lipase from *Shewanella* sp.
Lane: 1 Molecular markers, Lane 2: Pooled fractions eluted from the Sephadex G-75, Lane 3: Pooled fraction of DEAE-Cellulose, Lane 4: Dialyzed enzyme.

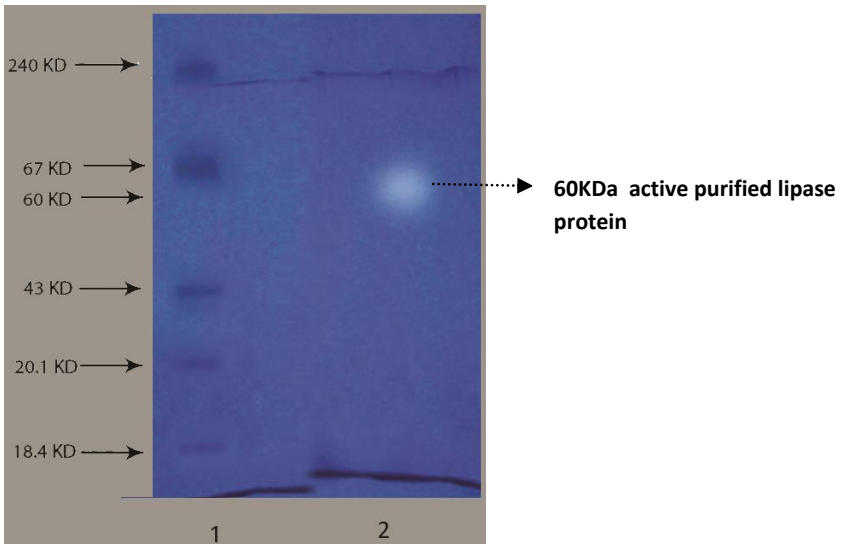

Figure 10. Detection of purified lipase activity by Native PAGE
Lane:1 Molecular markers, Lane 2: Lipase activity zone.

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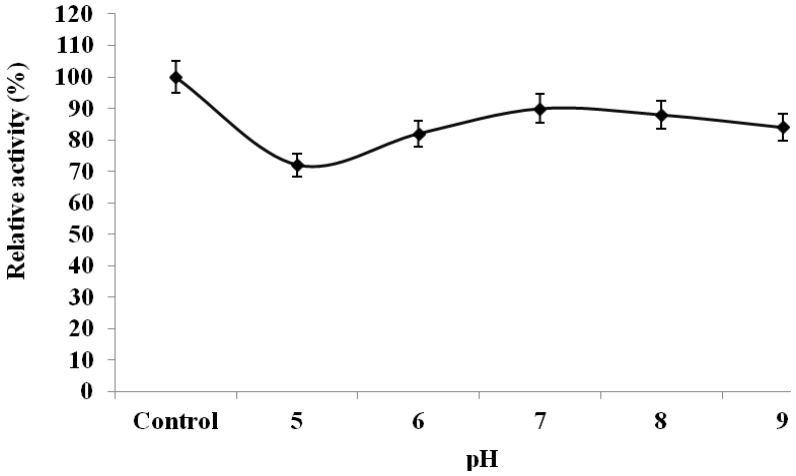

Figure 11. Effect of different pH stability on purified lipase

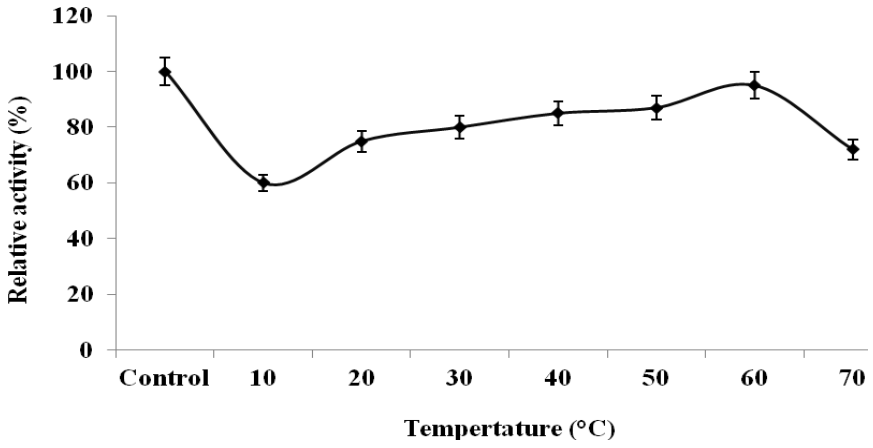

Figure 12. Effect of different temperature stability on purified lipase

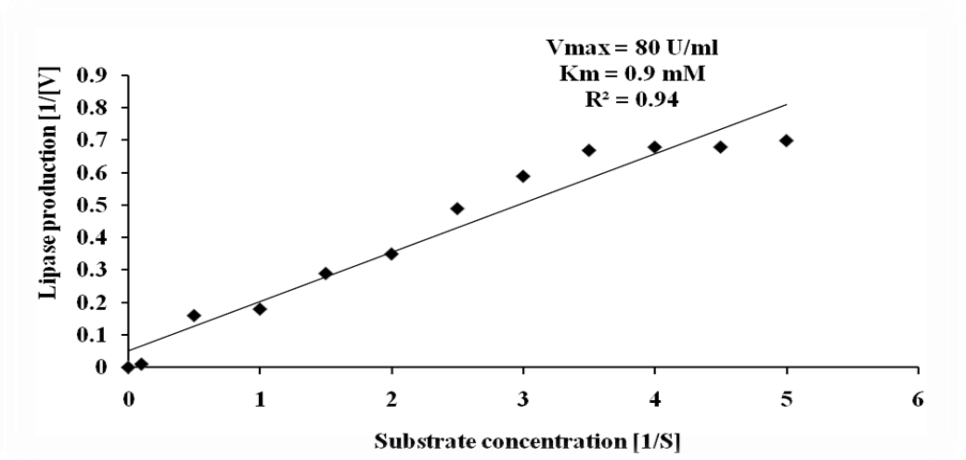

Figure 13. Double reciprocal Lineweaver – Burk Plot

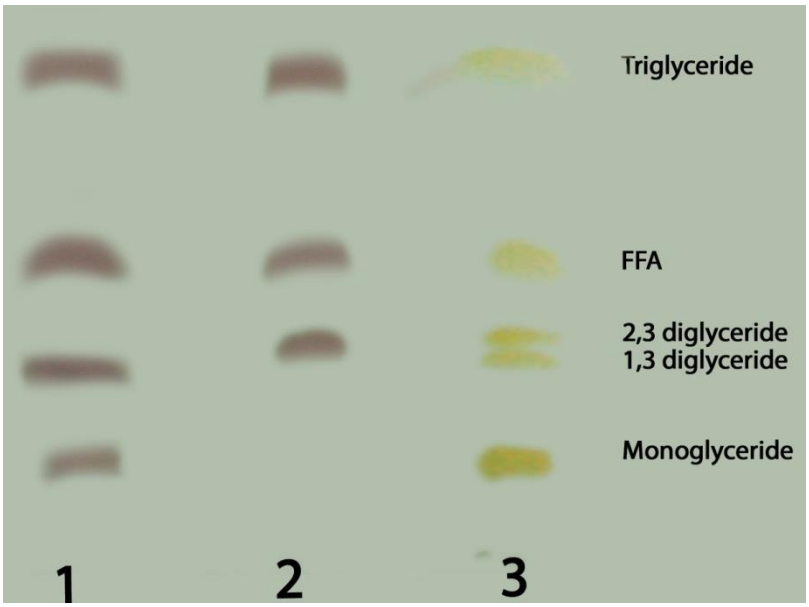

Figure 14. Determination of positional specificity of purified lipase by TLC
Lane 1: Tripalmitin, Lane 2: Tripalmitin +Enzyme, Lane 3: Lipid standards

CONCLUSION

In the present study, an attempt was made to isolate and identify a novel lipase producing bacterium *Shewanella* sp CMST GISA-MSU. from the gut of a marine fish *S. longiceps*. The lipase production by the candidate bacterial strain through SSF was found to be accelerated at optimized culture conditions such as moisture and various substrates concentrations. From the result it could be concluded that, the optimum substrates required for enhancing production of lipase were 15% moisture contents, 0.2% (0.03g) $MgSO_4$, 1.2% (180µl) of gingelly oil, 1.6% (0.25g) mannitol and 1% (0.15g) tryptone and (1.5g(10%) NaCl. Further, the lipase produced by *Shewanella* sp. was purified and the molecular weight of this purified lipase was determined as 60 kDa. Through this study, it could be confirmed that *Shewanella* sp. is a potential strain for lipase production by utilizing the cheapest substrate *Brassica oleracea* shreads (Cabbage shreads) waste through SSF.

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