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Partial purification and biochemical characterisation of a novel thermos table extracellular lipase from Serratia marcescens scl1 isolated from medicinal waste

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Abstract

Lipases find wide application in modern industrialization. Thermostable lipases are a challenge for various applications in different industrial segments. Microbial enzymes are a cheap source of lipase. In the present study isolation, partial purification and characterization of a novel thermostable lipase produced from Serratia marcescens scl1 was carried out. The lipase producing bacteria was isolated from medicinal waste and identified as Serratia marcescens using 16S rDNA sequencing method. Biochemical characterization performed showed the optimum growth conditions for the bacteria at 37°C temperature, pH7 in production media using 1% (w/v) olive oil as a carbon source, after 48 hours of incubation. Extracellular lipase produced by the bacteria was partially purified by ammonium sulphate precipitation and dialysis. Biochemical characterization revealed that the enzyme showed optimum activity at temperature 75°C and pH 8. The substrate saturation kinetics showed maximum at 1.3mM [S] and activity 5.43±0.05 X10-2 unit/ml. The protein concentration determined is 240µg/ml and specific activity of the enzyme is 22.58 unit/mg. The quantitative assay of lipase activity was carried out using 2.0 mM pNPP as substrate in 50.0mM Tris-HCl measuring absorbance at 410nm. The study revealed that isolated bacterium is able to produce a thermostable lipase which can satisfy the requirements of modern industrialization.

Keywords: *Serratia marcescens, phylogenetic analysis, biochemical characterisation, thermostable lipase, purification*

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INTRODUCTION

The versatility of extracellular microbial enzymes makes them a potential need to the mankind. Lipase is the widely used industrially important enzyme that catalyses hydrolytic conversion of triacylglycerols to acylglycerols and fatty acids (Litthauer et al., 2002), and is also able to carry out diverted trans-esterification (Macrae, 1983), aminolysis, inter-esterification (Liu et al., 2008) and alcoholysis (Jaeger, 1994 reactions). It's immense application can be observed in fat hydrolysis (detergents), garbage of petroleum

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clearance (Abhijit, 2012), fine chemicals manufacturing (synthesis of esters), coal industry (Hasan et al., 2006), food flavour modification, oily substance removal from waste water, removal of lipids from animal skins in leather industry and blood triglyceride analytical assays (Kamini et al., 2000). The demand of microbial lipase is increasing worldwide, especially in developing countries such as China, Brazil and India. The microbial lipase market was of USD 400.2 million and estimated to be USD 459.9 million in 2019. It is projected to reach USD 590.2 million by 2023, growing at a CAGR of 6.8% from 2018, in terms of value (Microbial Lipase Market Report Code: FB 6158).

Lipases can be isolated from fungi (Goswami et al., 2012), plants (Francis et al., 2002), animals (Jaeger et al., 2002) and also from bacteria (Hasan et al., 2006; Singh et al.,2011). Inspite of various sources of this enzyme, very small number of species are capable of producing thermostable, biosynthetically active, manipulatable product (Cardenas et al., 2001). In our finding we partially purified and characterized a novel thermostable extracellular lipase from *Serratia marcescens* scl1 isolated from medicinal effluent of pharmaceutical industry.

MATERIALS AND METHODS

Sample collection and bacteria enumeration.

The Lipase producing bacterial strain was isolated from medicinal effluent of a pharmaceutical industry located in West Bengal, India. 1ml of collected sample dissolved in 100ml of 0.8% saline water and the debris precipitated by centrifugation at 100rpm for 30 minutes under normal laboratory environment. The soup obtained was diluted up to 10^{-8} in 0.8 % saline water. 0.1 ml of 10^{-6}, 10^{-7}, 10^{-8} dilutions were taken and plated in nutrient agar medium. After 48 hours of incubation at 37^0C numbers of bacteria were counted by using the formula of Niemela S, 1983 with minor modifications.

$$N = \frac{\Sigma C}{(1xa) + (0.1xb) + (0.01xd)}$$

Here, N is total number (in terms of CFU) of bacteria present per ml of isolated sample; ΣC is summation of total colonies appeared on all plates (10^{-6}, 10^{-7}, 10^{-8}); a is the number of colonies appeared on 10^{-8} diluted plate; b is the number of colonies present in 10^{-7} diluted plate; d is the number of colonies appeared on 10^{-6} diluted plate.

Isolation and screening for lipase producing microorganism.

In order to isolate and screen lipase producing microbes, replica plating was done by taking colonies from 10^{-7}diluted plate on to TBA (Tributirin Agar) media having composition [0.3% yeast extract (w/v), 0.5% peptone (w/v), 2% agar, 1% tributyrin (v/v) at pH 7.0]. Lipase production was analysed by observing a zone of clearance after 48 hours of incubation at 37^0C. Further screening was done by re-plating the positive colonies on Tributirin agar media and confirmed by zone of clearance. Lipolytic activity of the bacterial strain was reconfirmed by zone of clearance on Tween 20 and Tween 80 agar plates analogous to Kirby-Bauer test (1% Tween 20/ Tween 80 with 0.02% methyl red in 2% agar) (Brenner et al., 2015; Lapage et al., 1976) with supernatant of bacteria growing in nutrient broth under 150 rpm at 37^0C for 24 hours.

Bacterial strain identification.

Isolated strain was identified in a multistep process starting from Bergey's Manual of Determinative Bacteriology (Holt et al., 1994) a) to BLAST searching in NCBI database. 1% seeding culture was allowed to grow on nutrient broth at 37^0C for 48 hours under 150 rpm and young culture was used for different morphological and biochemical identification. Gram staining was done for morphological identification, SEM (Scanning Electron Microscopy) study was done at CRNN (Centre for Research in Nanoscience and Nanotechnology), Kolkata, West Bengal, India for size and any special morphology determination and Catalase, Oxidase, Urease, H_2S, Nitrate reduction, Indole test, Methyl Red reduction test, Voges-Proskauer test and Citrate tests were done for biochemical identification. The fermentative

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properties of the strain were tested on different sugar viz. Sucrose, Lactose, Glucose, Maltose, Mannitol, Inositol (Gurtler et al., 1996; Clarridge et al., 2004). The data were compared with Bergey's Manual of Determinative Bacteriology (Holt et al., 1994). The strain was also examined for different enzyme production capabilities viz. Caseine hydrolysis, Starch hydrolysis, Gelatin hydrolysis, Lipid hydrolysis. Further characterization was done by growing on different growth media viz. TSI (Triple-Sugar-Iron Agar), SSA (Shigella-Salmonella Agar), HEA (Hektoen Entric Agar) media. To find out evolutionary relationship, the phylogenetic construction was done by 16S rDNA amplification using universal primer based PCR. BLAST was done on NCBI server to find sequence similarities and identities with other organisms. Then Multiple Sequence Alignment (MSA) was done using Clustal W programme and RDP database based distance matrix. Data of MSA of our organism used to construct a phylogenetic tree using MEGA 4 software (Prasad et al., 2013; Lee et al., 1993).

Medium formulation for optimum growth of bacteria.

The isolated strain was grown by seeding 1% inoculum under shaking condition at 150 rpm at 37^0C for different time intervals (viz. 24, 48, 72 hours) on to three different medium viz. Standard medium [Peptone- 5gm, Yeast extract- 5gm, Glucose- 5gm, NaCl- 0.25gm, $MgSO_4.7H_2O$- 0.5gm, Olive oil- 5%, Distilled water- 1000ml (pH- 7.0)], Nutrient Broth [Peptone- 5gm, Beef extract- 3gm, NaCl- 5gm, Distilled water- 1000 ml (pH-7.0)], Production Medium [Peptone- 5gm, Yeast extract- 10gm, NaCl- 5gm, Olive oil- 1%, Distilled water- 1000ml (pH- 7.0)] (Dandavate et al., 2009). Optimal growth was observed in Production Medium (PM) at 24 hrs and was used for further continuation.

The bacteria are grown in Production Medium at different pH (4.0, 5.0, 6.0, 7.0, 8.0, 9.0) and at different temperatures (25^0C, 35^0C, 45^0C, 55^0C) for 24 hours under shaking condition. The growth of bacteria was analysed using UV-Visible spectrophotometer (Systronic- 105) at 600 nm.

Medium formulation for optimum lipase production.

The parameters for optimal lipase production were determined for isolated organism onto different medium, pH, and temperatures. Three different growth medium were used viz. SM (Standard Media), NB (Nutrient Broth), PM (Production Media). The bacteria were allowed to grow at different time intervals (24, 48, 72 hours) under shaking condition at 150rpm at 37^0C. Lipase production capabilities were analysed on the basis of zone of clearance on Tween-agar plates and the analysis was done through extraction of supernatant of the growth culture. The effect of pH on lipase production was also evaluated with 1% inoculum in optimal medium (production medium) under shaking condition (150 rpm) at 37^0C for 24 hours at different pH (4.0, 5.0, 6.0, 7.0, 8.0, 9.0) adjusted by 0.1(N) HCl or 0.1 (N) NaOH. The lipase production was assayed spectrophotometrically at 410nm using pNPP (p-Nitrophenylpalmitate) as substrate. Evaluation of optimal temperature for lipase production was done in same medium with pH 7.0 at different temperatures (25^0C, 35^0C, 45^0C, 55^0C).

Crude and Partially purified Lipase production.

The isolated strain was grown under optimal condition for production of the enzyme lipase. The cultured broth was centrifuged at 8000 rpm for 15 minutes at room temperature for crude lipase. The protein concentration in supernatant of crude lipase was determined by Bradford assay.

For partial purification, the crude extract was saturated with 60% ammonium sulphate under continuous stirring at 4^oC. The fraction was then dialysed at 4^oC against 50 mM Tris-HCl buffer for 24 hours at pH 8.0. During the dialysis procedure the buffer was changed three times.

Amount of protein in partially purified lipase was estimated by Bradford assay (1976). In this case 200 µl of dialyzed protein was mixed with 800µl of Tris- HCl buffer at pH 8.0. 2000 µl

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of commercial Bradford reagent was added to the mixture. Absorbance was measured spectrophotometrically at 595 nm. Concentration of protein was determined using standard curve prepared against known BSA (bovine serum albumin) concentration.

Assay of lipase activity.

Spectrophotometric and titrimetric analysis of the partially purified protein were done (Debadrita et al., 2015; Boonmahome et al., 2013) to analyse the lipase activity. The titration was carried out using 1% olive oil (Reetz et al., 2005) as substrate. The composition of titration mixture was 0.1 ml of partially purified extract, 1.8 ml 50 mM Tris-HCl buffer at pH 8.0, 1% olive oil and the mixture was incubated for 20 minutes at 75^0C. Ethanol-acetone (1:1) mixture was used to terminate the reaction and amount of free fatty acid production was analysed by titrating with 0.05(N) NaOH and phenolphthalein as indicator. The calculation of lipase activity of crude enzyme extract was done using the equation described by Manickam et al., 1996.

$$Activity = \frac{(V_S - V_B) \times N \times 1000}{S \times T}$$

Here, V_s is volume of NaOH solution (ml) added during titration; V_B is amount of NaOH (ml) required to titrate control (enzyme void); N is the NaOH strength (0.05N in our experiment); S is the final volume of the reaction mixture (here 2 ml); T is the incubation period (20 minutes).

In spectrophotometric analysis pNPP (p-Nitrophenylpalmitate) was used as substrate (Janssen et al., 1996). Substrate stock solution was prepared in HPLC grade isopropanol where the concentration of the substrate was pNPP 20Mm. From the stock 0.1 ml substrate was taken and mixed with 1.8 ml of 50mM Tris-HCl buffer at pH 8.0. 0.1ml of crude enzyme was added to it to make a cocktail mixture. This mixture was incubated at 75^0C for 20 minutes in a water bath. After incubation (1:1) ethanol- acetone mixture was added to terminate the reaction. The enzymatic activity of lipase produces pNP from pNPP was measured spectrophotometrically at 410 nm. Lipase activity was assayed using standard curve of pNP (2-20 mg/ml in 50 mM Tris-HCl buffer at pH 8.0). One unit (IU) of lipase is the amount of enzyme that catalytically produces 1µmol of pNP under optimal condition in 1 minute.

Characterization of lipase.

Enzyme activity is affected by few but strict parameters like the substrate concentration, temperature and pH. Substrate concentration is considered as the most important parameter in enzyme catalysed reaction. As the substrate concentration increases the velocity of the reaction also increases by following first order kinetics (for single substrate) but when maximal velocity is achieved it reaches a saturation point and follows zero order kinetics. Different concentrations of pNPP (0.1 mM to 2.0 mM) was added to crude enzyme extract of fixed volume (0.1 ml). The reaction was catalysed in 1.8 ml of Tris- HCl buffer of pH 8.0. The experiment was carried out under optimal temperature (75^0C) and absorbance was measured spectrophotometrically at 410 nm after termination of the reaction (Sirisha et al., 2010; Li et al., 2005).

Most lipase producing bacteria are mesophilic and their extracellular enzymes show optimal activity at 30^0C- 50^0C. To determine the optimal temperature of isolated lipase, its activity was checked at different temperatures viz. 15^0C, 25^0C, 35^0C, 45^0C, 55^0C, 65^0C, 75^0C, 85^0C keeping the reaction medium pH at 8.0. Interpreted data was compared with standard curve (Nevel et al., 1996; Freinkel et al., 1969).

Optimal pH of the enzyme lipase was determined by analysing its activity in different buffer solution of different range of pH viz. pH 5.0- 6.0 (Citrate Buffer), pH 7.0 (Phosphate Buffer), pH 8.0- 9.0 (Tris- HCl Buffer), pH 10.0- 11.0 (Glycine- NaOH Buffer). In each case 50 mM of buffer solution was taken. All the analysis was performed thrice at 75^0C for 20 minutes using different buffer solution and the assay mixture (Borel et al., 1994; Charoenpanich et al., 2011).

Enzyme kinetics was analysed under optimal condition. The activity of enzyme lipase

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was assayed on the basis of different concentration of the substrate p- Nitrophenyl palmitate (pNPP). The optical density for each pNPP concentration was used to calculate the lipase activity. Km and Vmax of the extracted enzyme was determined by plotting the experimental data in LB plot (Lineweaver-Burk Plot). Km is indirectly proportional to the substrate affinity of an enzyme and in general remains in between 10^{-1}M to 10^{-5}M for industrially important enzymes. Activity of the extracted enzyme and corresponding substrate (pNPP) concentration both were plotted reciprocally to get a linear plot (Lotrakul et al., 1997; Sugihara et al., 1991)

RESULT AND DISCUSSION

Isolation of lipase producing organism.

The lipase producing bacteria were isolated from medicinal waste of a pharmaceutical industry in West Bengal. At the beginning qualitative and quantitative analysis was done by plating on Nutrient agar plates. From there screening for lipase production was done on Tributyrin agar (TBA) plates. Following the formula of Niemela S, 1983 calculated microbial load was 3.5 X 10^8/ml of medicinal waste sample. 10^{-7}dilution was capable of producing 33 colonies onto nutrient agar plate. Among them only 3 colonies were susceptible to produce zone of clearance on TBA plate (Fig1a). The most proficient colony (giving highest zone of clearance) was used for further continuation of our work. Cup-plate assay using methyl red indicator on Tween 20/80 agar plates were able to assert the lipolytic activity of the strain (Fig1b,d). In this case 48 hours incubated culture was first centrifuged at 8000 rpm for 5 minutes. 100 µl of supernatant were added to each of the cup made on the plates and incubated at 37^0C for 24 hours. Tween 20/80 which are made up of fatty acid esters polyoxyethylenesorbitan, on lipolysis they produce simpler fatty acid chains thus the pH of the medium also changes, this change is detected by indicator (methyl red), the break down also produces halozone which is observable under UV light (Fig1c,e).

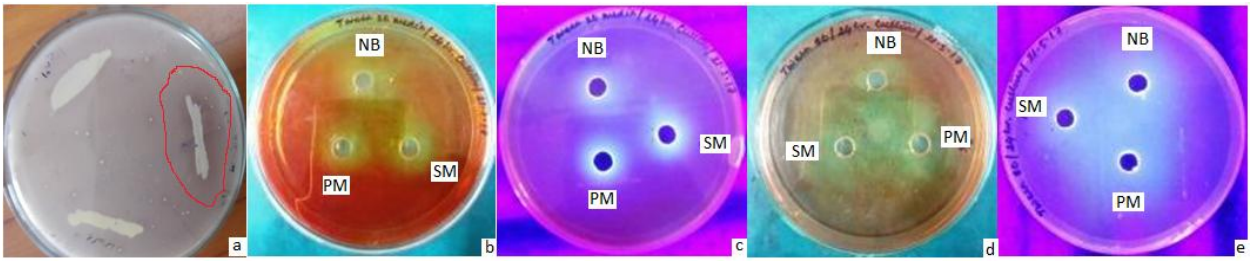

Figure 1. (a) Lipolytic activity on TBA plate (b) Tween 20 plate (c) Tween 20 plate in UV (d) Tween 80 plate (e) Tween 80 plate in UV.

Bacterial strain identification.

The outlook of the strain`s colony was glossy, smooth, circular, non- pigmented on nutrient agar plates. The aerobic microorganisms appear as small reddish-pink rod under microscope on Gram staining thus are Gram negative (Fig2a) and are able to fermenting different types of sugars viz. glucose, sucrose, maltose, lactose, mannitol, inositol with or without producing of gas. IMViC test of the strain interprets negative in indole and methyl red (MR) but positive Voges- Proskauer (VP) and citrate utilization test. They are capable of catalase production but not oxidase and urease. They do not produce H_2S but are able to reduce nitrate. Apart from nutrient agar and TBA, the isolated bacteria show white marginated circular colony on Hektoen Entric Agar and show noticeable growth on Mac Conkey agar plate, SS agar plate (Table1). ABIS online software confirmed us that our organism of interest belong to the genera *Serratia and* confirmed by matching with Bergey's Manual of Determinative Bacteriology (Holt et al., 1994)

Clear rod shaped bacteria were observed on micrograph under 30,000 X magnification on

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Scanning electron microscope. According to calculation of the device, size of the bacterium was 1.8773 µm and diameter was 0.846 µm (Fig2b).

To find out evolutionary relationship with other bacteria, phylogenetic analysis was done of our organism of interest. To construct such tree, amplification of 16S rDNA was done by the use of universal primer based PCR. BLAST was done on NCBI server that shows quiet interesting result. The 16S rDNA sequence of the organism shows 98% sequence identity with many of the culturable and unculturable *Serratia* strains available in NCBI database. Then Multiple Sequence Alignment (MSA) was done using Clustal W programme and RDP database based distance matrix. Result of this MSA shows that our organism of interest is a novel one of whose phylogenetic tree was constructed using MEGA 4 software (Fig3). The ribotyping data of *Serratia marcescens* scl1 has been deposited in GeneBank database under Accession No: KT877002.

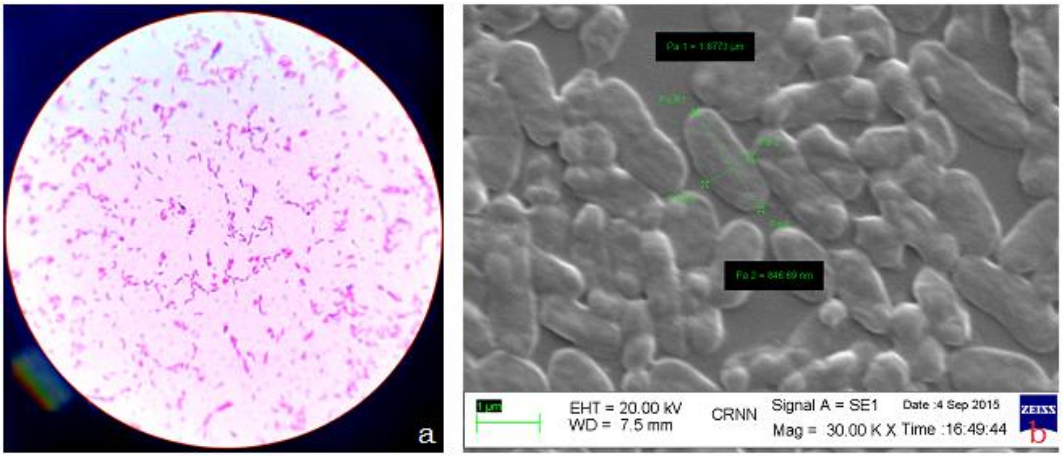

Figure 2. Bacterial morphology at 400X after Gram staining (a); SEM micrograph on 30,000X magnification (b)

Table1- Characterization parameter for bacteria (Morphological, Fermentative, Biochemical, Enzyme production, Growth in different media, Antibiotic sensitivity)

Characteristics	***Serratia marcescens* scl1**
Morphology	
Appearance	Circular, glossy, smooth
Pigmentation	Non pigmented
Shape	Rod shaped
Gram character	Gram negative
Size	Small
Metabolism	Aerobic
Growth in carbohydrates	
Sucrose	+ve, gas production
Lactose	+ve, no gas production
Glucose	+ve, gas production
Maltose	+ve, gas production
Mannitol	+ve, gas production
Inositol	+ve, no gas production
Biochemical tests	
Catalase	+ve

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Oxidase	-ve
Urease	-ve
H_2S production	-ve
Nitrate reduction	+ve
Indole	-ve
Methyl red	-ve
Voges- Proskauer	+ve
Citrate	+ve
Enzyme production	
Protease	+ve/-ve
Amylase	-ve
Gelatinase	-ve
Lipase	+ +ve
Growth on different media	
Triple Sugar Iron Agar	+ve
S.S Agar	+ve
Hektonicentric agar	+ve
Mac Conkey	+ve
Tetrathionate broth	+ve
Antibiotic sensitivity	
Amoxicillin, Ampicillin, Penicillin, Vancomycin, Methicillin	No clear zone of inhibition, resistance
Chloramphenicol, Ciprofloxacin, Ceftazimidine, Cefotaxime, Cloxacillin, Streptomycin, Lincomycin, Oxytetracycline, Ofloxacin, Gentamycin, Novobiocin	Clear zone of inhibition, sensitive

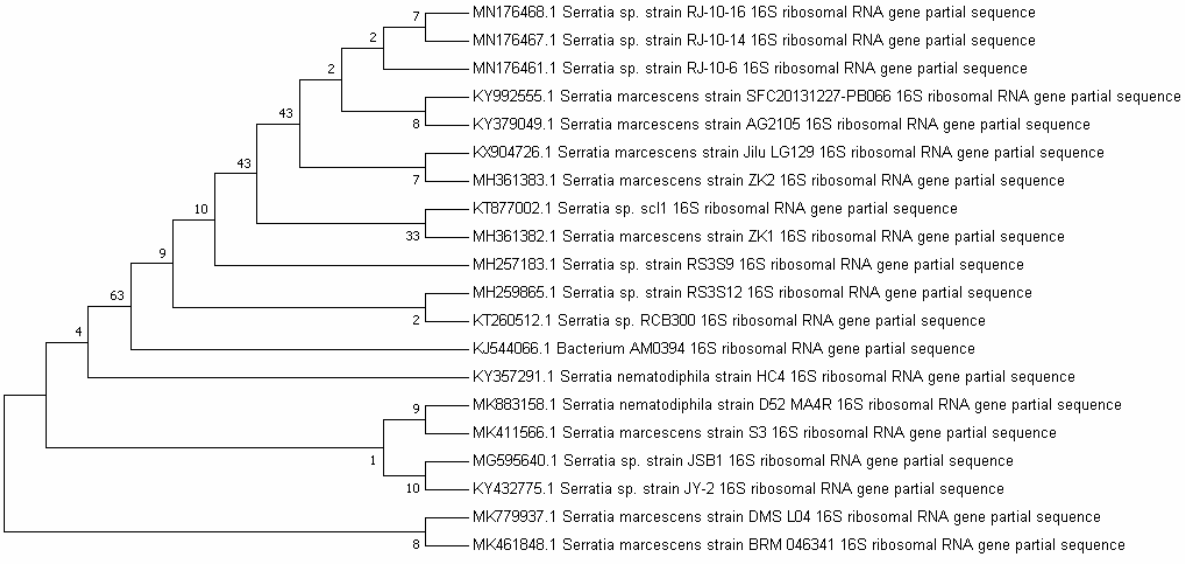

Figure 3. -Phylogenetic analysis based on 16S rDNA

Medium formulation for optimum growth and growth kinetics.

Growth medium formulation.

Proper medium is necessary for optimal growth of microorganism. We formulate three different medium viz. Standard medium (SM), Nutrient broth (NB) and Production medium (PM) for different time (viz 24 hr, 48 hr and 72 hr.) to analyse the optimum one. Spectrophotometric measurement at 600 nm after 48 hours of

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incubation interpreted that the production media was the most efficient growth medium for our organism where nearly 7.34 x10^8 bacterial cells were present per ml of broth (Fig4).

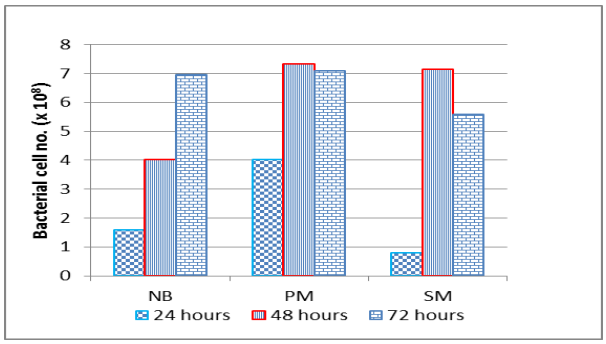

Figure 4. Growth of *Serratia marcescens* scl1 on different growth medium

Optimum temperature and pH.

Cellular metabolism of bacteria is much more dependent on environmental factors. Among several factors pH and temperature are pivotal. Most of the *Serratia* strains are mesophilic.

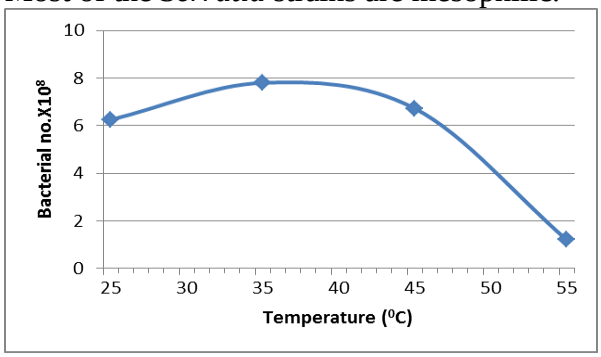

Figure 5. *Serratia marcescens* scl1 growth at different temperatures

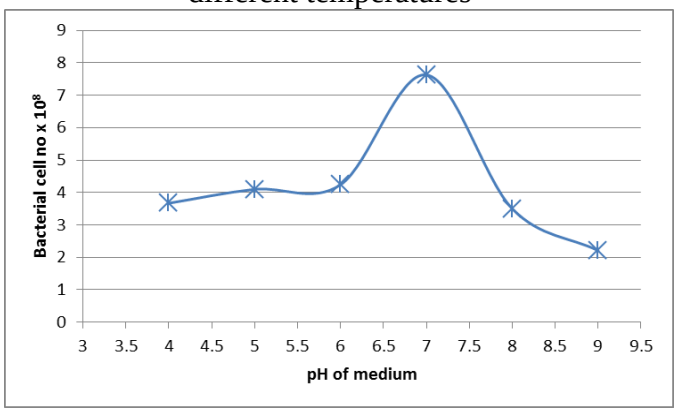

Figure 6. *Serratia marcescens* scl1 growth at different pH

We evaluate growth of our novel organism at different temperature (Fig5) and pH (Fig6) range to determine optimal one. Spectrophotometric study revealed that temperature 35^{0}C and pH 7.0 was the ideal for *Serratia marcescens* scl1 growth.

Growth kinetics of Bacteria

We headed towards growth kinetics of bacteria after determining optimal growth condition taking initial bacterial load 3.2 X 10^7 cells/ml. The first phase of the bacterial growth, the lag phase was about 8 hours for *Serratia marcescens* scl1. Then the bacteria enter into log/ exponential phase and spent about 20 hours at this phase. Spectrophotometric measurement reveals about 4.5 X 10^9 cells/ml at end of this phase. In optimal growth conditions, growth rate and generation time for *Serratia marcescens* scl1 were calculated and found to be generation time is 50 minutes i.e. the bacteria were able to complete 1.15 generation/ hour (growth rate) (Fig7).

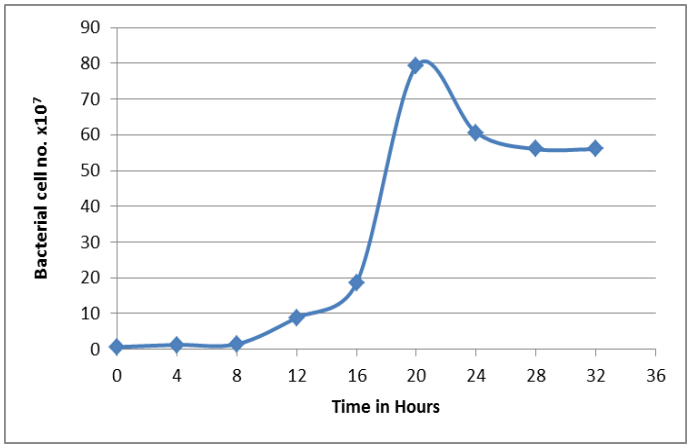

Figure 7. *Serratia marcescens* scl1 growth curve

Medium optimization for lipase production.

Medium and time course of lipase production.

Most microbes produce several types of extracellular enzymes and sometime induction is necessary. The enzyme production rate is dependent on growth medium component and the environmental parameter at which the bacterium is growing. The isolated *Serratia marcescens* scl1 shows optimal growth and maximal extracellular lipase on production medium. In order to identify time course for lipase production we used culture-

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soup of our isolated organisms on Tween- agar plates. The bacteria produced maximum lipase on 24 hours but extended time (viz; 48 hr, 72 hr) of growth decrease its production (Fig8).

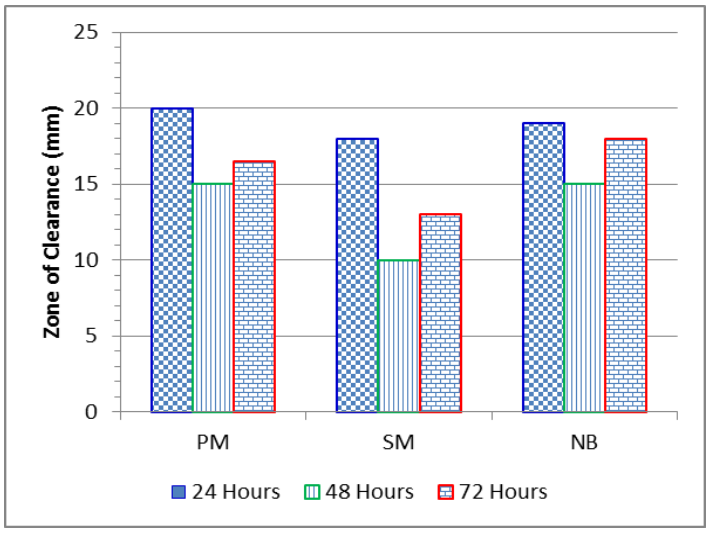

Figure 8. Lipase activity analysis on different growth medium

Incubation temperature for lipase production.

Temperature is an important factor in microbial metabolism. This parameter plays crucial role on production rate of extracellular enzymes by bacterial cell. We set different range of temperatures to find out the particular temperature at which lipase production is maximum. In our findings the temperature was 35^0C is shows optimal one and increase or decrease of it lowers the lipase production (Fig9). Spectrophotometric analysis using pNPP was done to analyse lipase activity.

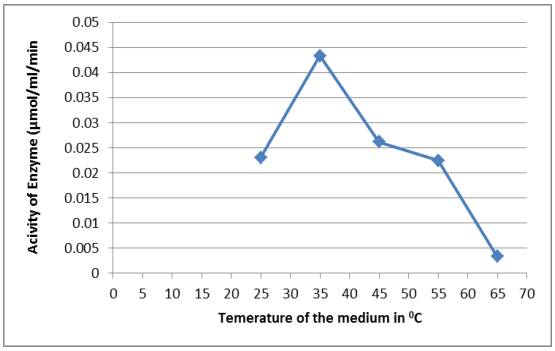

Figure 9. Lipase production analysis on different temp. of the medium

Incubation pH for Lipase production

Among several important factors for microbial growth, pH is a major one. This factor also play pivotal role in bacterial metabolism thus extracellular enzyme production. To find out the particular pH at which *Serratia marcescens* scl1 produces maximum amount of lipase, different range of pH was set. Analysis conclude that lipase production by the bacteria is increasing with pH and is highest at pH 7.0, further elevation in pH drastically reduces the production (Fig10).

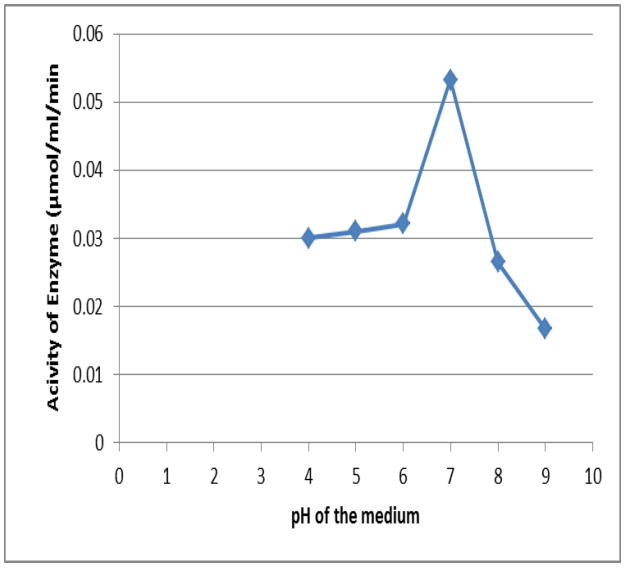

Figure 10. Lipase production analysis on different pH of the medium

Partial purification of lipase and analysis of lipase activity

Lipase Purification

The crude lipase of *Serratia marcescens* scl1 was extracted by growing the bacteria in production medium for 24 hours in ideal condition. By centrifugation of culture broth at 8000 rpm for 15 minute at room temperature, the supernatant is collected. Partially purification of lipase in order to carry out further experiment was done by 60% ammonium sulphate precipitation followed by dialysis at 4^0C under continuous shaking. The extracted enzyme was subjected to 10% SDS-PAGE to analyse the probable molecular weight of isolated lipase (Fig11).

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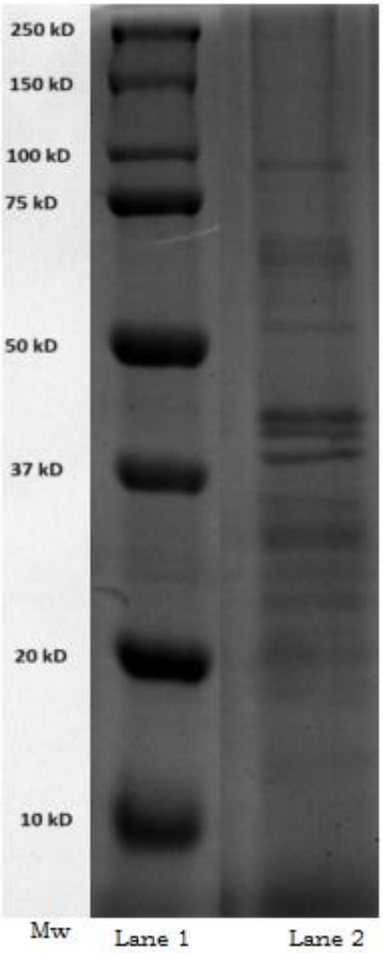

Figure 11. SDS-PAGE of partially purified lipase from *Serratia marcescens* scl1; Lane1- Protein marker, Lane2- dialyzed lipase

Assay of lipase

Current researchers take different approaches like Thin Layer Chromatography (TLC), spectrophotometric analysis, titrimetric analysis, Tween-agar diffusion analysis and many more to determine extracellular lipase activity. In order to determine the lipase activity, two types of assay method was followe; one is titrimetric analysis and the other one is spectrophotometric analysis. The activity assay was done by the procedure of Manickam et al. and found to be 3.75 X 10^{-2} unit/ ml in titrimetric and pNPP based spectrophotometric activity was found to be 5.4 X 10^{-2} unit/ml.

Total protein assay

The supernatant was collected after centrifugation at 8000 rpm for 15 minutes, and the total protein content was determined by Bradford's method. The total protein content was found to be 240 µg/ml. Using this data specific activity was determine for crude extract was 22.58 unit/ mg of protein.

Characterization of lipase.

Enzyme lipase has wide range of applications nowadays. Several parametric analyses was carried out to find out whether on not the lipase produced by *Serratia marcescens* scl1 is fit for large scale production.

Substrate saturation kinetics.

Scientific research papers on lipase activity reveals use of several types of substrates to analyse extracellular lipase activity. Among such frequently used substrates p-Nitrophenyl Palmitate (pNPP) is a major one. Absolute enzyme activity is measurable only when all the active site of an enzyme is completely filled i.e. in saturated state. Different pNPP concentration ranging from 0.1mM up to 2.0 mM was set to determine enzyme activity. The 1.3 mM of pNPP was the concentration where the enzyme gets saturated (Fig12).

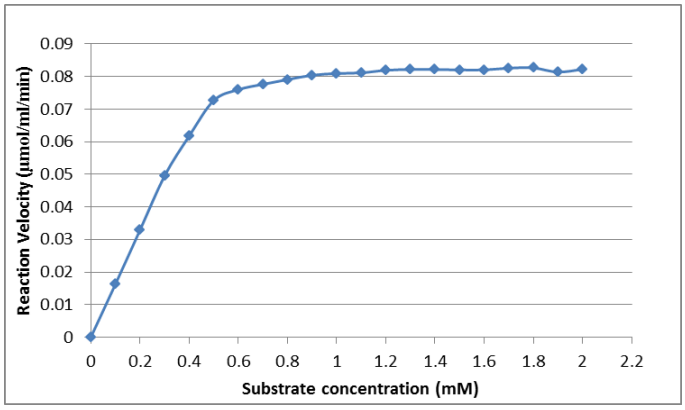

Figure 12. Substrate saturation kinetics of lipase under optimal condition

Effect of temperature and temperature optimum.

Thermostable extracellular lipases have a gigantic demand in today's industry as most of the lipases produced by microbes are stable up to 50^0C temperature. Not only that they can show only

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about 75% of their activity at this temperature and further elevation can diminished their activity. Very few culturable organisms are capable of thermostable lipase production. Among such species *Pseudomonas sp.* (Dharmsthiti et al., 1999; Wang et al., 1995) Aeromonas sp. (Kim et al.,2000; Wang et al., 1995) and Bacillus sp. (Iizumi et al.,1990; Sugihara et al., 1992;Dharmsthiti et al., 1999) are most frequent. The lipase produced by our *Serratia marcescens* scl1 has a unique property i.e. the enzyme show optimal activity at a temperature ranging from 55^0C to 85^0C, with highest activity at 75^0C. This phenomenon of the novel enzyme from our bacterium ensures its thermostability. During the assessment the activity of the enzyme at 75^0C was 5.5 X 10^{-2} unit/ml (Fig13).

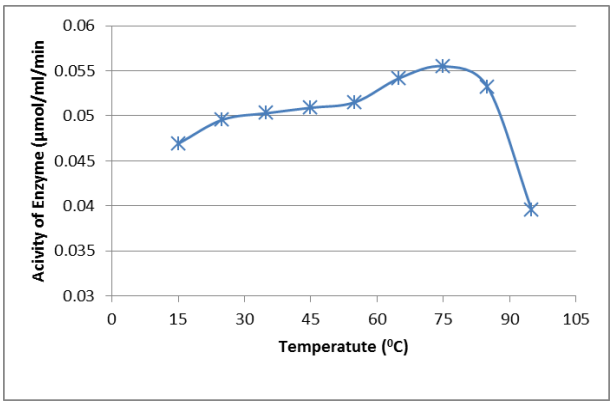

Figure 13. Optimum temperature determination

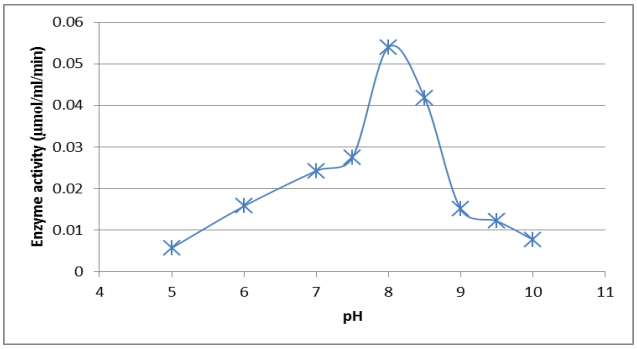

Figure 14. Optimum pH determination

Effect of pH and pH optimum.

Current research on lipases reveals that these enzymes show their optimal activity at a less alkaline solution (Ramani et al., 2010; Gutarra et al., 2009) and most of them at nearly neutral (Kim et al., 2008; Ramani et al., 2010). Very few, with some exception show their activity at acidic condition (Ramani et al., 2010). Activity of our lipase produced by *Serratia marcescens* scl1 was assessed over a wide pH ranging from 5.0 to 10.0. The resultant data interpret that the enzyme show optimum activity at pH 8.0 and calculated activity at that pH was 5.4 X 10^{-2} unit/ml (Fig14).

Determination of Km and Vmax

Vmax is the maximal velocity of an enzyme and Km is the substrate concentration at which the velocity is half of the maximal velocities are the two important internal parameter of enzyme. I.L Michaelis and M. Menten, two pioneer of enzymology had formulated several equations and postulated their valuable thoughts about enzyme kinetics. Thus our next moto was to follow Michaelis-Menten framework but the hyperbolic plot does not give exact value of Vmax (Maximum Velocity). This issue enforces to switch our work to follow Lineweaver-Burk (LB) equation and plot. In kinetics study we used different concentration of pNPP as the substrate for the enzyme lipase in LB framework, the calculation shows that the Km of the enzyme was 3.349 X 10^{-3} M and the Maximum velocity (Vmax) was 5.68 X 10^{-1} unit/ml (Fig15).

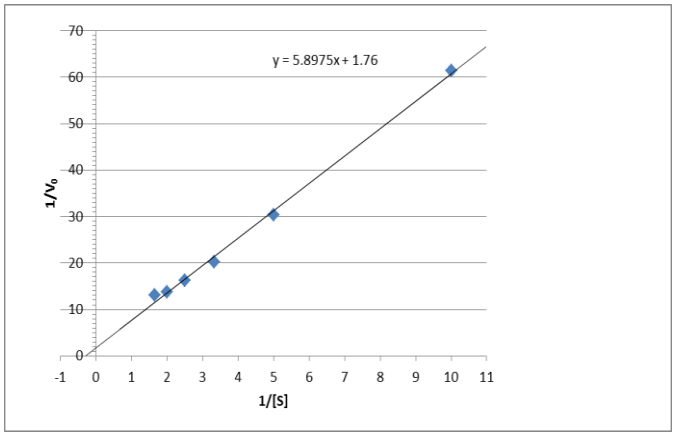

Figure 15. Km and Vmax determination through LB plot

CONCLUSION

The research suggests that the isolated microorganism is a novel bacterial (*Serratia marcescens* scl1) strain from medicinal waste. Several morphological and biochemical experiments were performed to find out the

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uniqueness of the organism through several tests. Ribotyping i.e. 16S rDNA sequencing was done and submitted to NCBI database with an Accession No: KT877002. It was observed that the novel organism (*Serratia marcescens* scl1) shows optimal growth at 35^0C at neutral pH and takes 50 minutes to complete a generation. *Serratia marcescens* scl1 produces extracellular lipase that show optimal activity at pH 8.0, 75^0C with a Km value of 3.3 X 10^{-3} and Vmax 5.68 X 10^{-1} unit/ml. The organism is capable of thermostable lipase production which can have wide range of use in modern industries.

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