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ISOLATION, PARTIAL PURIFICATION AND CHARACTERIZATION OF A NOVEL THERMOSTABLE LIPASE FROM SERRATIA MARCESCENS SCL1

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ABSTRACT
Thermostable lipases have become challenge for wide application in modern industrialization. The present study aims in the isolation, partial purification and characterization of a thermostable lipase producing bacterial strain, *Serratia marcescens* scl1 from medicinal waste. The bacteria isolated on Tributyrin agar plates was characterized by different biochemical tests and confirmed by 16s-rDNA sequencing. The growth parameters of bacteria showed best growth at 35^0C and pH 7.0 with 1% olive oil as carbon source after 48 hours of incubation. The bacterial growth curve was analyzed and lipase partially purified taking 24 hour old cultures. The lipase production analyzed with Tween 20 and Tween 80 agar plates and quantitative assay of lipase activity carried out using 2.0 mM pNPP as substrate in 50.0mM Tris-HCl, measuring absorbance at 410nm. The optimum temperature and pH of extracellular lipase produced by the bacteria occurred at 75^0C and pH 8.0. 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 240 µg/ml and specific activity of the enzyme is 22.58 unit/mg. The results indicate the isolate are able to produce low cost high profile enzyme which can satisfy the requirements of future goal to modern industrialization.

KEYWORD: Thermostable lipase; 16s rDNA; KT877002; para Nitrophenyl palmitate.

1. INTRODUCTION
Extracellular microbial enzyme is now a potential need marker for different industry due to its ability to improve the products. These enzymes have recently found wide industrial application globally. The hydrolytic enzymes with their potentiality include amylase[1], protease[2], cellulose[3], pectinase[4], xylanase[5], esterase[6], lipase[7,8,9] etc. have become an emerging field in applied and industrial microbiological as well as enzyme engineering sector. Lipases (Triacylglycerol lipase, EC3.1.1.3) are the water soluble versatile hydrolytic enzymes responsible for hydrolysis of triacylglycerol to diaceyl glycerol, monoacyl glycerol, fatty acid and glycerol in both aqueous and non-aqueous media including their interface, and are also involved various important reactions such as trans-esterification[10], inter-esterification, aminolysis[11], alcoholysis.[12] Since discovery of lipase by Claude Berbard in 1856, scientists focused on the isolation and characterization of lipase producing microorganisms due to immense importance of the extracellular enzyme in industrial field.[13,14] Lipases can be extracted from bacteria[15,16], fungi[17], plant[18] and animals.[19] Among them the bacterial lipases have received more interest due to its consistency in supply by easy cultivation in inexpensive media without seasonal fluctuation. As also improvement in product yield by genetic manipulation is possible more over they are non-toxic, eco-friendly and green synthesis is possible.[20,21,22] Bacterial lipase is a multi-faceted enzyme with lipolytic as well as esterolytic activity and show versatility with respect to wide range of substrates, regio-specificity[23], enantio-selectivity[24], chiral selectivity[25] and stability over a wide range of pH[26] and temperature[27,28] than the corresponding animal and plant lipases. The bacteria of genus *Bacillus*[29], *Pseudomonas*[30], *Staphylococcus*[31], *Chromobacterium*[32], *Achromobacter*[33], *Burkholderia*[34], *Alacaligens*[35], *Arthobacter*[36] etc. are widely used for production of industrially important lipases are found to be ubiquitous and are isolated from diverse habitats especially from industrial wastes of oil industry[37], food and vegetable industry[38], oilseed[39], oil contaminated soil[40,41], garbage of petroleum[42] and coal industry.[43] The worldwide demand of microbial lipases is increasing especially in developing countries like India, China and Brazil.[44] Microbial lipases have a vast application in industrial field especially in waste water treatment (detoxification and degradation of contaminant)[45], food (flavour modifying enzyme)[46], pharmaceutical (digestive enzyme)[47], cosmetic (for removal of lipids)[48], leather (animal skin fat elimination), pulp & paper industry (to remove the pitch

from the pulp)[49] detergent industry (hydrolysis of oil and fats)[50], production of fine chemicals[51], fermentation of vegetable and meat, synthesis of surfactant and polymer, production of biodiesel[52] etc. The microbial lipase market is estimated to be USD 425.0 million in 2018. It is projected to reach USD 590.2 million by 2023, growing at a CAGR of 6.8% from 2018, in terms of value (cicion prNewswire). Approximately 13 billion tons of detergent is produced each year using 1000 tons of lipases. With modernization of society, the need for thermostable and alkali resistant lipase is in high demand for detergent production used in machine wash in laundry.

The bacterial lipase production is greatly influenced by medium composition and physiological factors such as temperature, pH, inducer etc. During bacterial lipase production different lipidic carbon and organic nitrogen sources are used as major controller. Considering the ever increasing demand for extracellular industrially important bacterial lipase with novel character, the present study focuses on the isolation and characterization of a thermostable lipase producing bacterial strain*Serratia marcescens* scl1 andPartial purification followed by activity analysis of lipase under different physiological condition.

2. MATERIALS AND METHODS

2.1. Isolation and characterization of lipase producing Strain

The lipase producing bacterial strain was isolated from medicinal waste of a pharmaceutical industry in West Bengal, India. Serially diluting up to 10^{-8} with 1 gm of soil sample dissolved in 100 ml of saline water (0.8%) and incubated for 30 minute at 100 rpm in laboratory condition. 0.1ml sample of last three dilutions were plated in nutrient agar plates and incubated for 48 hours at 37^0C. The average no. of bacteria are counted using formula as demonstrated by Niemela, S. 1983 with some modification.[53]

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

Where, N is the total no. of bacteria (in terms of CFU) per ml of isolated sample; ∑C is sum of total colonies on all plates counted (last three plates); a is the number of bacteria from third countable plate (10^{-8} dilution); b is the number of bacteria from second countable plate (10^{-7}dilution); d is the number of bacteria from first countable plate (10^{-6}dilution).

A replica was prepared taking 33 colonies from 10^{-7} diluted plate on Tributirin agar (TBA) media (containing 0.5% (w/v) peptone, 0.3% (w/v) yeastextract, 1% (v/v) Tributyrin and 2% agar, pH 7.0) to analyze lipase production by appearance of a clear zone after incubation at 37^0C for 48 hours. The suspected colonies are further screened by replating in TBA media and confirmed by the appearance of clear zone on incubation. The bacterial strain with maximum lipolytic activity was reconfirmed by collecting the supernatant of bacteria growing in nutrient broth under same conditions with shaking at 150 rpm and tested for zone of clearance in Tween 20 and Tween 80 agar plates (1% Tween 20/Tween 80 with 0.02% methyl red in 2% agar).[54,55]

2.2. Identification of lipolytic strain

2.2.1. Morphological and biochemical identification

Taxonomic status of isolated bacteria was identified according to Bergey's Manual of Determinative Bacteriology (Holt et al.,1994) through morphological (Gram staining), biochemical (H_2S production, Nitrate Reduction, Catalase, Oxidase, Urease, Indole, MR reduction, VP Test and Citrate test), fermentative (Glucose, Sucrose, Maltose, Lactose, Mannitol, Inositol)[56,57] and enzyme production (Starch hydrolysis, Lipid hydrolysis, Casine hydrolysis, Gelatin hydrolysis) tests. The strain was further characterized by growing in Mac Conkey agar plate, Shigella-Salmonella agar (SSA), Hekteon entric agar (HEA) and Triple-Sugar-Iron Agar (TSI) media. The strain was tested for different antibiotic susceptibility.

2.2.2. Scanning Electron microscopy (SEM) study

The Scanning Electron microscopy (SEM) of isolated strain grown in Nutrient broth for 48 hours at 37^0C under 150 rpm with 1% seeding culture was performed at Centre for Research in Nanoscience and Nanotechnology (CRNN), Kolkata, India.

2.2.3. Identification and phylogenetic tree by 16s rDNA sequencing

Identification of the isolated strain was confirmed by extracting the genomic DNA of bacteria using DNA extraction kit (Eurofinscat. no. 5224700305). The fragment of 16S rDNA was selectively amplified from genomic DNA by PCR using universal oligonucleotide primers and amplicon was confirmed by single band of 1500 bp on 1% agarose gel. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 16SF and 16SR primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer (16SF primer-CGGACGGGTGAGTAATGTCT and 16SR primer-CTCAGACCAGCTAGGGATCG). Consensus sequence of 16S rDNA gene was generated from forward and reverse sequence data using aligner software. The 16S rDNA gene sequence was used to carry out BLAST with the nrdatabase of NCBI genbank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 4.[58, 59]

2.3. Medium formulation for optimum growth and growth kinetics

2.3.1. Growth medium formulation

The isolated strains were grown in different media and physical environment to optimize and formulate the

growth kinetics. Three types of media with varying composition were used for this purpose viz. nutrient broth (Peptone 5gm, Beef extract 3 gram, NaCl 5gm, Distilled Water 1000ml. pH-7.0), standard medium (Peptone 5gm, Yeast extract 5gm, Glucose 5gm, NaCl 0.25 gm and $MgSO_4$. $7H_2O$ 0.5gm, Olive oil 5%, Distilled Water 1000ml. pH-7.0) and production medium (Peptone 5gm, Yeast extract 10 gram, NaCl 5gm, Oilve oil- 1%, Distilled Water 1000ml. pH-7.0).[60] All tests were performed in triplicate in 250ml conical flask with 100ml medium containing 1% inoculum at 37^0C under 150 rpm shaking condition for different time duration (viz. 24 hours, 48 hours and 72 hours). The production medium (PM) showed best result and was used for further experiment.

2.3.2. Optimum temperature and pH for bacteria

The optimum physical environment viz. temperature and pH for isolate was analyzed in production medium (PM) at different temperature (25^0C, 35^0C, 45^0C and 55^0C) and at different pH (4, 5, 6, 7, 8, and 9) under 150rpm shaking condition for 24hours. All growth measurements were done by recording absorbance values at 600nm by UV-Visible spectrophotometer (Systronic-105).

2.3.3. Growth kinetics of bacteria

The growth kinetics of bacteria was performed at optimum temperature and pH in production medium containing 1% inoculum at 37^0C under 150 rpm shaking condition for about 32 hours recording absorbance values at an interval of 4 hours. The generation time and growth rate of the bacteria for the formulated medium is calculated from exponential stage of growth.

2.4 Partial purification of lipase

2.4.1 Preparation of crude lipase enzyme

Production of lipase enzyme was carried out by growing the bacteria *Serratia marcescens* scl1 in the desired medium under optimum conditions of growth. The culture supernatant containing crude enzyme lipase was obtained by centrifugation of the culture broth of *Serratia marcescens* scl1 at 8,000rpm for 15min at 37 ^{0}C. The protein estimation and lipase assay was performed as described previously.

2.4.2 Ammonium sulphate fractionation

The cell free supernatant obtained in the previous step was saturated with 30% ammonium sulphate at 4^0C with continuous stirring. The ammonium sulphate fraction was the dialysed against 50mM Tris-HCL buffer, pH 8.0 for 24hrs at 4^0C in a dialysis bag. The buffer used during dialysis was changed three times during dialysis. The concentrated partially purified enzyme obtained after dialysis was checked for protein estimation and lipases assay as described previously.

2.5. Medium optimization for lipase production

2.5.1. Medium and time course for lipase production

The lipase production ability of the bacteria was analyzed in three different media viz. nutrient broth (NB), standard media (SM) and production medium (PM) with respect to different time course to standardize the time required for optimum lipase production. The best medium and suitable time for optimum lipase production was analyzed by culturing the isolate in three different media at optimum physical environment under shaking condition for different time course of 24 hours, 48 hours and 72 hours. The lipase production ability was analyzed by extracting supernatant of grown culture and production of clear zone in Tween-agar plates.

2.5.2. Incubation temperature for lipase production

To evaluate the effect of incubation temperature on lipase production, 1% isolate was inoculated in production medium and incubated for 24 hours at different temperatures viz. 25^0C, 35^0C, 45^0C and 55^0C with shaking condition and then assayed for lipase production.

2.5.3. Incubation pH for lipase production

Investigations on effect of medium pH on lipase production were carried out by growing the bacteria in production medium for 24 hours in different pH. The pH of the medium was adjusted to 4, 5, 6, 7, 8 and 9 with 0.1N HCl or 0.1N NaOH and then assayed for lipase production by spectroscopic method.

2.6. Partial purification of lipase and analysis of lipase activity

2.6.1. Titrimetric assay of lipase

Extracellular lipase, an inducible enzyme is partially extracted from 1% olive oil supplemented production medium by centrifugation at 8000rpm for 5 min at room temperature. The supernatant collected and lipolytic activity analyzed by titrimetric method and spectroscopic method.[61, 62]

In titration 1% olive oil was used as substrate.[63] The reaction mixture contained 1% olive oil, 0.1 ml supernatant, 1.8 ml 50mM Tris-HCl buffer, pH8.0 and was incubated at 75^0C for 20 min. The reaction was terminated with ethanol- acetone (1:1) mixture and liberated free fatty acid analysed by titration using 0.05N NaOH with phenophathelin as indicator. The lipase activity is calculated by free fatty acid liberated (in micromole) per ml of supernatant (crude lipase) using equation described by Manickam etal.

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

Where, Vs is amount of NaOH solution needed for titration (ml); V_B is volume of NaOH (ml) needed to titrate control (without enzyme); N is the strength of NaOH used (0.05N); S is the volume of total reaction mixture (2 ml); T is the time of incubation (20 minute).

One unit of enzyme is the amount of enzyme required to liberate 1 µmol of free fatty acid from the substrate (olive oil) under optimal condition in one minute.

2.6.2. Spectroscopic assay of lipase

In spectroscopic method pNPP (para Nitrophenyl palmitate) [Sigma-Aldrich/CAS Number 1492-30-4] was used as substrate.[64] 20mM substrate stock of pNPP was prepared in HPLC grade isopropanol. In this assay a cocktail was prepared using 1.8ml of Tris-HCl buffer, pH8.0; 0.1ml pNPP stock and 0.1 ml crude enzyme mix. The resultant mixtures are incubated in water bath for 20 minute at 75^0C. The reaction was terminated withethanol-acetone (1:1) mixture and analyzed amount of para Nitrophenol (pNP) released due to enzymatic activity analysed using spectrophotometer at 410nm. The activity of lipase is calculated using standard curve of pNP (2-20 mg/ml in 50mM Tris-HCl buffer, pH 8.0). One unit (IU) of lipase activity is amount of enzyme able to liberate 1 µmol of pNP in one minute under standard condition.

2.6.3. Total protein assay

The total protein content of the supernatant was determined using Bradford method (1976). 100µl of extracted supernatant was mixed with 700µl of buffer (Tris-HCl buffer, pH8.0) and 2000µl of commercial Bradford reagent was added. Absorbance values were recorded at 595nm. The standard curve is prepared using known concentration of bovine serum albumin (BSA).

2.7. Characterization of lipase

To investigate extracellular lipase, cell was separated by centrifugation and supernatant stored at 4^0C for further use. Kinetics of enzyme was analyzed under optimal condition. Each experiment was performed in triplicate and mean taken for interpretation of the result.

2.7.1. Substrate saturation kinetics

The concentration of substrate is one of most important parameter affecting rate of enzymatic reaction. During enzymatic reaction the velocity of reaction increase as the substrate concentration increases until saturation is reached. To measure optimum substrate utilization, enzyme concentration (0.1ml crude extract) and buffer volume (1.8ml Tris-HCl, pH 8.0) was retained constant while varying substrate concentration (pNPP in isopropanol) from 0.1mM to 2.0mM. The reaction was performed in ideal condition (temp. 75^0C) and absorption taken at 410nm followed by termination of reaction.[65, 66]

2.7.2. Effect of temperature and temperature optimum

The extracellular lipase activities of most bacteria are in between 30^0- 50^0C. For determination of optimum temperature, the effect of temperature on enzymatic activity are determined at pH 8.0 at various temperatures, i.e. 15^0C, 25^0C, 35^0C, 45^0C, 55^0C, 65^0C, 75^0C and 85^0C under optimal assay condition and data compared with standard curve.[67, 68]

2.7.3. Effect of pH on activity

The lipolytic activity of crude enzyme was determined at pH range of 5.0-10.0 using 50mM of various buffer solutions i.e. citrate buffer (pH 5.0-6.0); phosphate buffer (pH 7.0); Tris-HCl buffer (8.0-9.0), Glycine-NaOH buffer (10.0-11.0). The assays were performed in triplicate manner at 75^0C for 20 minute using assay mixture with different buffer.[69,70]

2.7.4. Determination of km and Vmax

The effect of para-nitrophenyl-palmitate (pNPP) concentration on enzymetic activity was measured at different concentration. The absorption data for each pNPP concentration was used to calculate enzymatic activity. The Lineweaver-Burk plot of above data was used in determination of the Km and Vmax of isolated enzyme. Km of the enzyme interprets affinity for the substrate and a low value indicates high affinity. It is reported that most of the industrial enzymes have Km ranges 10^{-1} to 10^{-5}M. To determine Km and Vmax of isolated enzyme each value of pNPP concentration and corresponding activity is plotted in reciprocal manner to generate to linear plot.[71, 72]

3. RESULTS AND DISCUSSION

3.1. Isolation and characterization of lipase producing strain

The bacteria from medicinal waste of pharmaceutical industry of West Bengal was screened quantitatively and qualitatively by plating in nutrient agar followed by tributyrin agar plates and selected colonies screened for lipase production by replica plating in TBA plates. The microbial load was 3.5 x10^8/gm in the collecting soil sample as calculated using the formula demonstrated by Niemela, S. 1983. Among total 33 colonies of 10^{-7} diluted nutrient agar plate, 3 colonies were able to produce clear zone on TBA media as analysed by replating. The colonies which produced maximum zone of clearance was screened and used for further study. The confirmation of lipolytic/esterolytic activity of isolated strain was performed by cup-plate method in Tween 20 and Tween 80 agar plates using methyl red as indicator. The supernatant collected from 48 hours agitated culture in nutrient broth by centrifugation at 8000rpm was added to the well (100 µl) of Twee-agar plates and incubated for 24hrs at 37^0C. Lipase, a hydrolytic enzyme is responsible for the breakdown of Tween 20/80 (fatty acid esters of polyoxyethylene sorbitan) and results in a change in pH of the medium as detected by change in colour of methyl red and appearance of a halo zone under UV light (Fig. 1a,b,c,d,e).

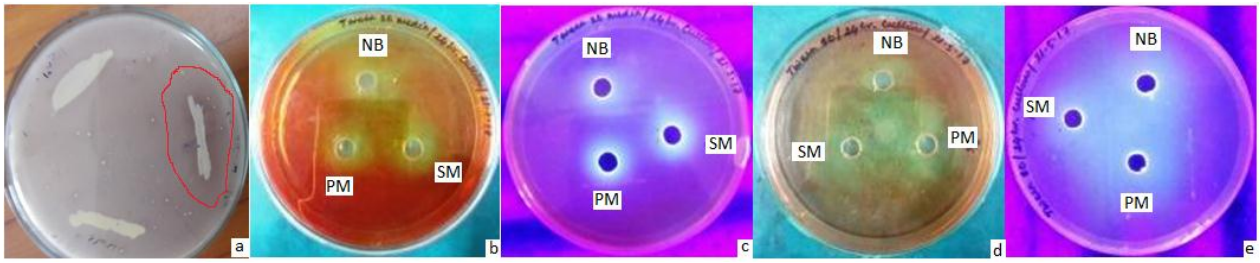

Fig. 1: Lipolytic activity of bacteria in TBA plate (a), Tween 20 plate (b); Tween 20 plate in UV light (c); Tween 80 plate (d); Tween 80 plate in UV light (e).

3.2. Identification of lipolytic strain

3.2.1. Morphological and biochemical identification

To identify the isolated strain different morphological and biochemical experiments were carried out. The bacteria are Gram negative, small rod shaped, aerobic and are non-pigmented circular colony producer (Fig. 2a).

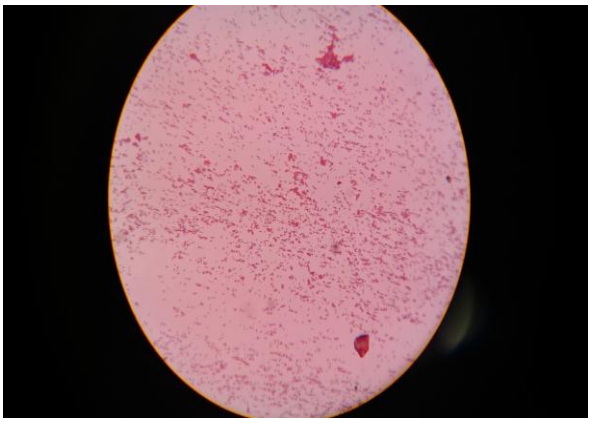

Fig. 2a. Morphological of bacteria at 400x after Gram staining.

The bacteria indicates a positive VP and Citrate but negative Indole and MR test as characterized by biochemical experiments (Table 1). The bacteria is able to ferment different type of sugar with or without production of gas (Table 1). The bacteria produces excess lipolytic enzyme but was unable to hydrolysis casine or gelatin although starch was slightly degraded. The bacteria grow in Mac Conkey, S.S. agar and Hektonen agar plate with white marginated circular colony. Based on the data, the isolated strain is characterized as belonging to the genus of *Serratia* from comparing with Bergey's Manual of Determinative Bacteriology (Holt et al., 1994) with the help of software ABIS online.

Table 1: Different biochemical test for *Serratia marcescens* scl1.

Morphological/Biochemical test		**Result**
Morphological test	Colony morphology	Circular, glossy, smooth
	Pigmentation	Non- pigmented
	Bacteria Shape	Rod Shaped
	Gram character	Gran negative
	Size	Small
	Metabolism	Aerobic
Carbohydrates fermentation	Glucose	+; gas production
	Sucrose	+; gas production
	Maltose	+; gas production
	Lactose	+; no gas production
	Mannitol	+; gas production
	Inositol	+; no gas production
Biochemical test	H_2S production	-
	Nitrate Reduction	+
	Catalase	+
	Urease	+
	Indole	-
	Methyl Red	-
	VP	+
	Citrate	+
	Oxidase	-

Extracellular enzyme production	Amylase	-
	Lipase	++
	Protease	-/+
	Gelatinase	-
Growth in different media	Mac Conkey	+
	Triple Sugar Iron Agar	+
	Tetrathionate broth	+
	S.S. Agar	+
	Hektonic entric agar	+
Antibiotic sensitivity test	Penicillin, Amoxicillin, Methicillin, Ampicillin, Vancomycin	No zone of inhibition; resistance
	Ciprofloxacin, Lincomycin, Cefotaxime, Ofloxacin, Ceftazimidine, Cloxacillin, Novobiocin, Chloramphenicol, Gentamycin, Oxytetracycline, Streptomycin	Clear zone of inhibition; sensitive

3.2.2. Scanning Electron microscopy (SEM) study

The Scanning electron microscopy (SEM) show that the bacteria are rod shaped and the size is 1.8773 µm in length and 0.846µm in diameter (Fig. 2b). The data was taken under 30,000x magnifications.

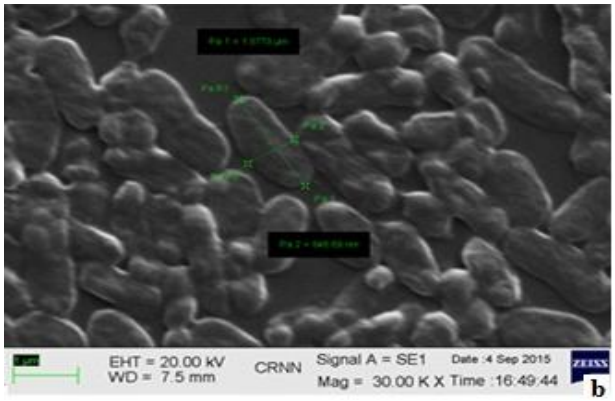

Fig. 2b. Scanning Electron micrograph 30,000x magnification.

3.2.3. Identification and phylogenetic tree by 16s rDNA sequencing

To confirm that the isolated bacteria is of novel strain and to construct phylogenetic analysis, the DNA was extracted and 16s rDNA amplified by PCR using universal primers. The sequence alignment by BLAST with NCBI gene bank database (www.ncbi, and nlm.gov/Blast/blast_help.html) show that the rDNA sequence is 99% similar with *Serratia marcescens NvL01, CH-B4, InBio 40520 and SW2-9-3* (Fig. 3).

Sequences producing significant alignments:

Description	Max score	Total score	Query coverage	E value	Max ident	Accession
Serratia marcescens strain NvL01 16S ribosomal RNA gene, partial sequence	1772	1772	100%	0	99%	KJ397961.1
Serratia sp. CH-B4 16S ribosomal RNA gene, partial sequence	1772	1772	100%	0	99%	KP325087.1
Serratia sp. INBio_4052O 16S ribosomal RNA gene, partial sequence	1772	1772	100%	0	99%	KM242496.1
Serratia marcescens strain SW2-9-3 16S ribosomal RNA gene, partial sequence	1772	1772	100%	0	99%	KM252937.1
Uncultured bacterium clone nck236c06c1 16S ribosomal RNA gene, partial sequence	1772	1772	100%	0	99%	KF097300.1
Uncultured bacterium clone nck235f10c1 16S ribosomal RNA gene, partial sequence	1772	1772	100%	0	99%	KF097243.1
Uncultured bacterium clone nck235b01c1 16S ribosomal RNA gene, partial sequence	1772	1772	100%	0	99%	KF097189.1
Uncultured bacterium clone nck233h12c1 16S ribosomal RNA gene, partial sequence	1772	1772	100%	0	99%	KF097136.1
Uncultured bacterium clone nck233a04c1 16S ribosomal RNA gene, partial sequence	1772	1772	100%	0	99%	KF097104.1
Uncultured bacterium clone nck208g05c1 16S ribosomal RNA gene, partial sequence	1772	1772	100%	0	99%	KF095457.1

Fig. 3: Sequence alignment by BLAST with NCBI gene bank database.

The isolated strain is novel strain and is *Serratia marcescens* scl1was confirmed using multiple alignment programs of Clustal W and distance matrix using RDP database. The phylogenetic tree is constructed based on alignment data using software MEGA4 (Fig. 4).

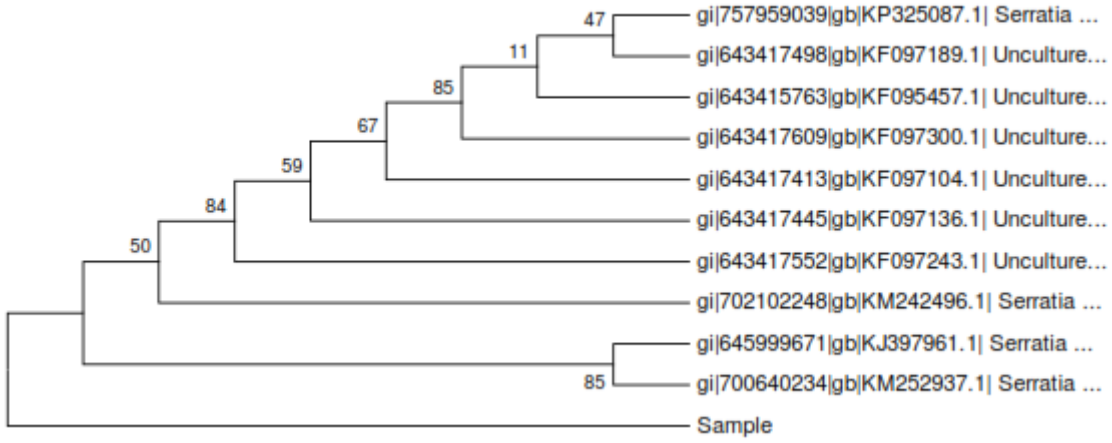

Fig. 4: Phylogenetic tree of isolate *Serratia mercescens scl1* constructed from its 16s rDNA sequence using MEGA 4.

The 16S rDNA sequence of *Serratia marcescens* scl1 has been deposited in NCBI/GeneBank database under accession no. KT877002.

3.3. Medium formulation for optimum growth and growth kinetics

3.3.1. Growth medium formulation

Formulation of medium is one of the most important parameter for optimum growth of bacteria. To determine optimal medium for cultivation of *Serratia marcescens* scl1three types of media of different composition was used viz. nutrient broth, Standard medium and production medium and growth analyzed by absorption measurement at 600nm. The production medium shows maximum growth with respect to time interval of 48 hours (cell no. $7.34x10^8$cell/ml) (Fig. 5).

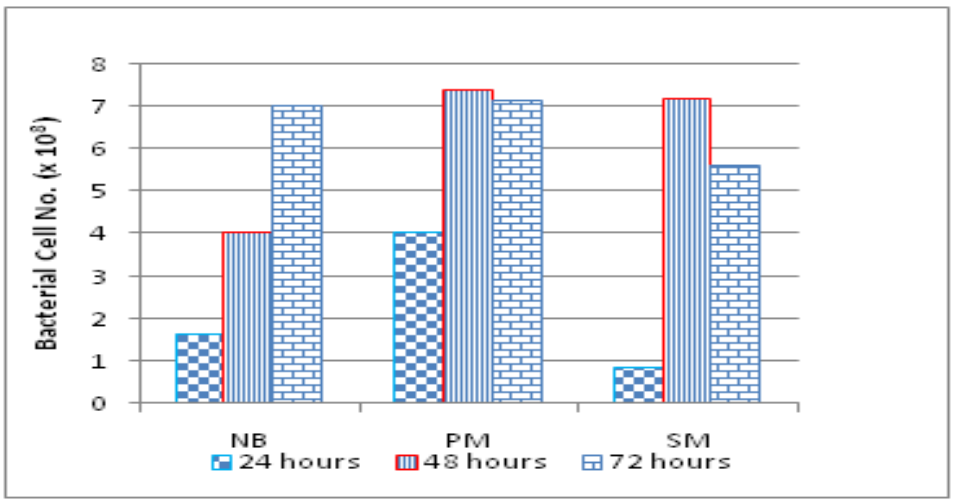

Fig. 5: Growth of *Serratia mercescens scl1* in different media and at different time interval; NB indicate nutrient broth; PM indicate production medium and SM indicate standard medium.

3.3.2. Optimum temperature and pH for bacteria

Temperature affects the enzymatic/metabolic activity of the bacteria, thus affecting growth and is an important growth determinant. Different bacteria grow at different temperature. Most of the *Serratia sp.* are mesophilic and are able to grow at a temperature range between 25^0-45^0C. In our study we used different temperature to analyze the optimum one for our isolate *Serratia marcescens* scl1 and found it to be 35^0C (Fig. 6). pH is the second most physical requirement for ideal growth of bacteria. It also affects the metabolic process of bacteria as well as availability of nutrient form. The isolate *Serratia marcescens* scl1 show maximum growth at pH 7.0 (Fig. 7).

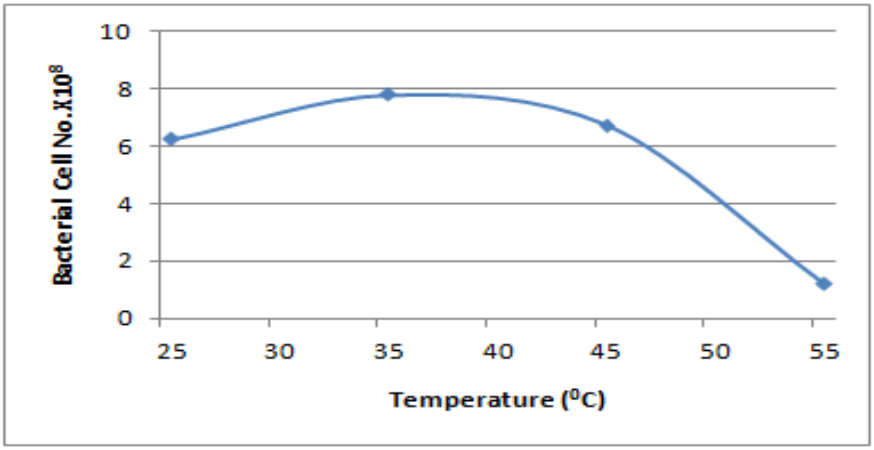

Fig. 6: Bacterial growth with respect to temperature.

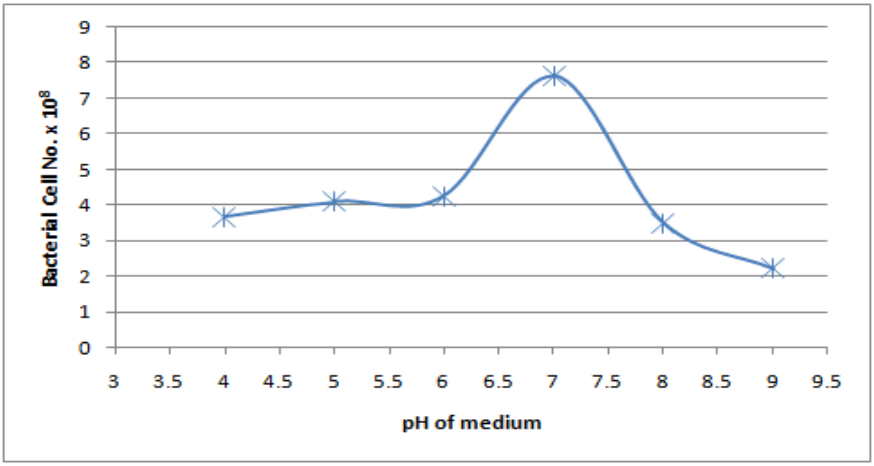

Fig. 7: Bacterial growth with respect to pH of the medium.

3.3.3. Growth kinetics of bacteria

The growth curve of bacteria was performed using $3.2x10^7$ cell/ml inoculum and shows bacteria remain in lag phase for about first 8 hours and then enter in exponential phase up to 20 hours of inoculation. Finally it enters the stationary phase. The number of bacteria at the end of the exponential phase calculated using standard method is $4.5x10^9$ cell/ml. The generation time and growth rate of *Serratia mercescens* scl1is 50min/generation and 1.15 generation/hour respectively in production medium under optimum condition (pH 7.0; temp. 35^0C) as calculated from exponential phase (Fig. 8).

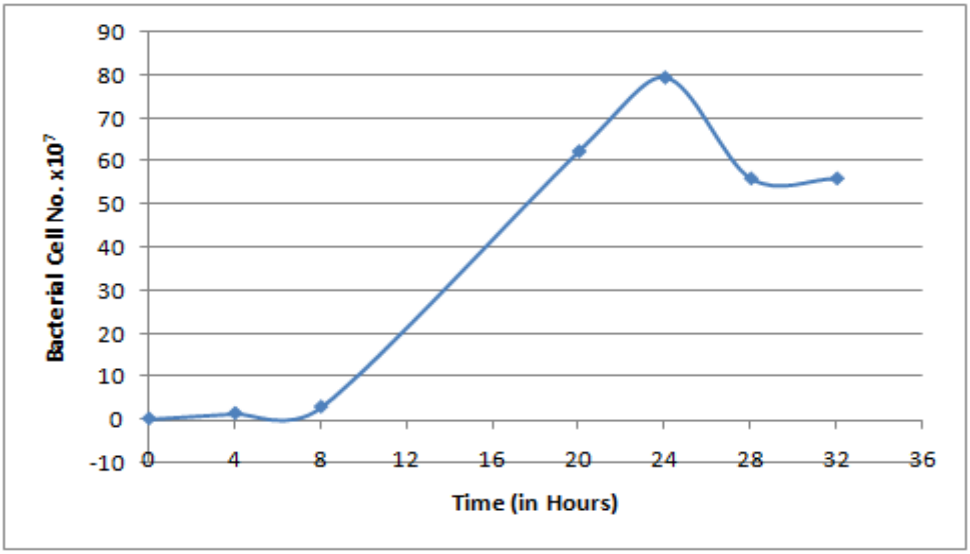

Fig. 8: Bacterial growth curve in respect to optimum temperature and optimum pH of the medium.

3.4. Lipase Purification

The partial purification of lipase enzyme from *Serratia marcescens* scl1 involved ammonium sulphate precipitation of crude enzyme followed by dialysis. This partially purified lipase was subjected to SDS-PAGE (Fig. 9).

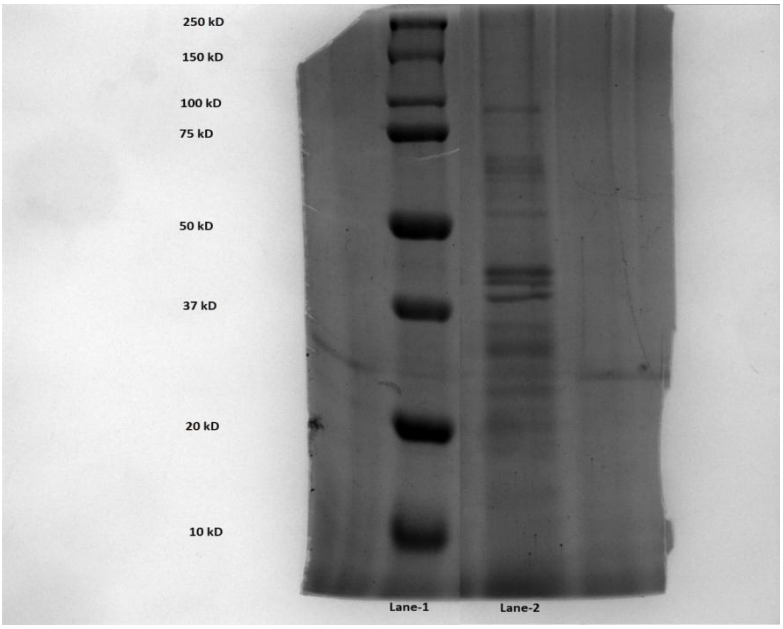

Fig. 9: SDS PAGE electrophoresis of partial purification of lipase enzyme, lane 1. Molecular marker and lane 2. Partial purified enzyme.

3.5. Medium optimization for lipase production

3.5.1. Medium and time course for lipase production

Most bacteria produce extracellular enzymes depending on external force such as composition of medium supplied and also time of growth. The medium and time course of lipase production reveals that the isolated *Serratia marcescens* scl1 is able to produce maximum lipase on production medium. The Tween-agar plate experiment showed that extracellular lipase is mostly produced at time interval of 24 hours and subsequent incubation decreases lipase production ability of the bacteria. The result indicates that the bacteria produce growth linked extracellular lipase under optimum condition (Fig. 10).

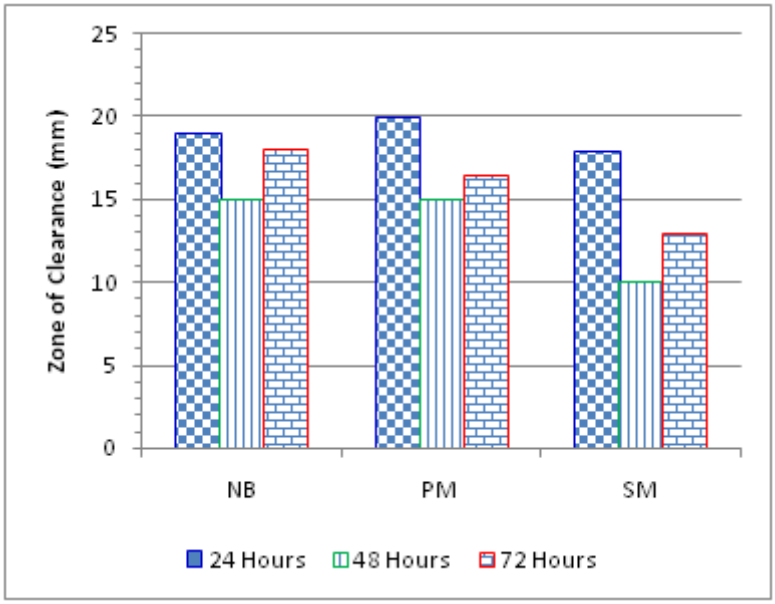

Fig. 10: Lipase activity analysis by zone of clearance in tween-agar plates.

3.5.2. Incubation temperature for lipase production

Experiment on effect of incubation temperature on lipase production in production medium after 24 hour incubation showed that *Serratia mercescens* scl1 wasable to produce maximum lipase at 35^0C and incubation at 55^0C no lipase activity was found as growth are drastically diminished. The lipase activities were analyzed by spectroscopically using pNPP as substrate.

3.5.3. Incubation pH for lipase production

The optimum pH for lipase production determined in production medium at experimental condition showed bacteria was able to produce maximum lipase at pH 7.0. The lipase production increased from pH 6.0 and showed a maximum at pH 7.0. Subsequent increase in medium pH revealed that the lipase production ability of bacteria is decrease. Thus pH 7.0 is suitable for stabilization and accumulation of extracellular lipase of isolate *Serratia marcescens* scl1.

The experiments hereafter are conducted in production medium at 37^0C, pH 7.0 and 24 hours of incubation time.

3.6. Partial purification of lipase and analysis of lipase activity

3.6.1. Assay of lipase

In literature there are several ways to determine the extracellular lipase produced from bacteria such as titrimetric, thin layer chromatography, Tween agar diffusion, spectroscopic method and others. In our study we use the titrimetric method and spectroscopic method mostly to determine activity of extracellular lipase of supernatant from our isolate *Serratia mercescens* scll grown in production medium under experimental condition. In titrimetric method the activity determined according to Manickam etal. is $3.75x10^{-2}$unit/ml whereas in spectroscopic method using pNPP as substrate the calculated activity is $5.4x10^{-2}$ unit/ml.

3.6.2. Total protein assay

The protein content of supernatant was determine by standard protocol using Bradford reagent and found to be 240µg/ml. Using the amount of protein content the specific activity was calculated and found to be 22.58unit/mg of crude protein.

3.7. Characterization of lipase

Characterization of enzyme is one of the most important parameter for further study to be used for industrial application if possible. In our study we performed different parametric study with our isolated enzyme to satisfy the requirements of future goal in modern industrialization.

3.7.1. Substrate saturation kinetics

Different types of substrates are used in literature to study activity of extracellular lipase. Para Nitrophenyl palmitate (pNPP) is one of the most widely used substrate for determination of lipase activity. Enzyme kinetics depends on the substrate concentration because true activity of enzyme is not possible if the enzyme is not fully saturated. To determine saturation kinetics we used 0.1mM to 2.0mM pNPP and found that the enzyme gets saturated at 1.3mM of substrate concentration (Fig. 11).

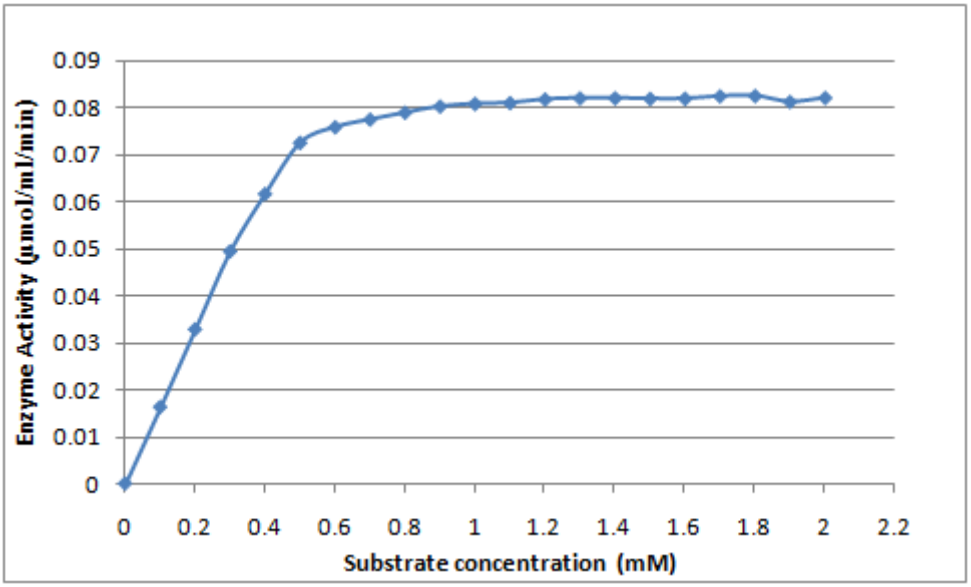

Fig. 11: Saturation kinetic of enzyme under optimal condition.

3.7.2. Effect of temperature and temperature optimum

Most bacterial lipases are stable up to 50^0C with to 75% maximum activity if incubated for 30 minute at said temperature. The demand of thermostable lipase for industry is on growing. Few bacterial species able to produce thermostable lipase are *Aeromonas sp*.[73,74], *Bacillus sp*.[75,76,77], *Pseudomonas sp*.[78,79] The activity of lipase from *Serratia marcescens* scl1was determined at various temperatures. The lipase showed activity over a

wide temperature range from 55^0C to 85^0C with optimum 75^0C (Fig. 12.).

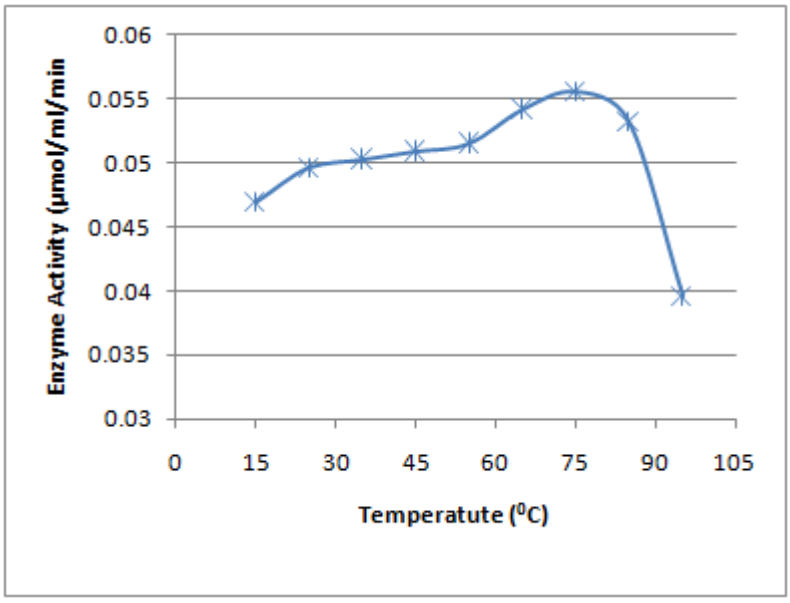

Fig 12: Determination optimum temperature of enzyme.

The result indicates that the bacteria were able to produce highly thermostable lipase. The activity of this thermostable lipase at optimum temp. is $5.5x10^{-2}$unit/ml.

3.7.3. Effect of pH and pH optimum

Generally lipases are optimally active at neutral[80, 81] or slightly alkaline pH.[81,82], with exception for a few at acidic pH.[83,84] The activity of lipase is carried over a pH range 5.0 to 10.0 (Fig. 13).

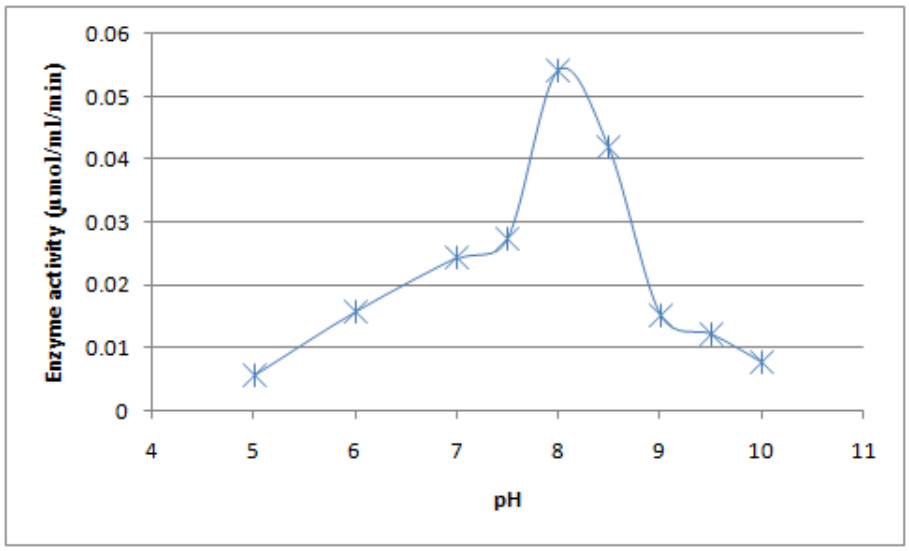

Fig. 13: Determination optimum pH of enzyme.

The data represent that the lipase of *Serratia marcescens* scl1has optimal pH 8.0 i.e. maximum activity under alkaline condition with a value of $5.4x10^{-2}$ unit/ml.

3.7.4. Determination of km and Vmax

It is good approach to describe the enzyme kinetics with respect to Michaelis and Menten framework which give rectangular hyperbolic reaction velocity (v) vs. substrate concentration ([S]) curve through origin. Since the curve is hyperbolic it is difficult to accurately measure Vmax and the [S] that yield ½ Vmax i.e. Km (Fig.14).

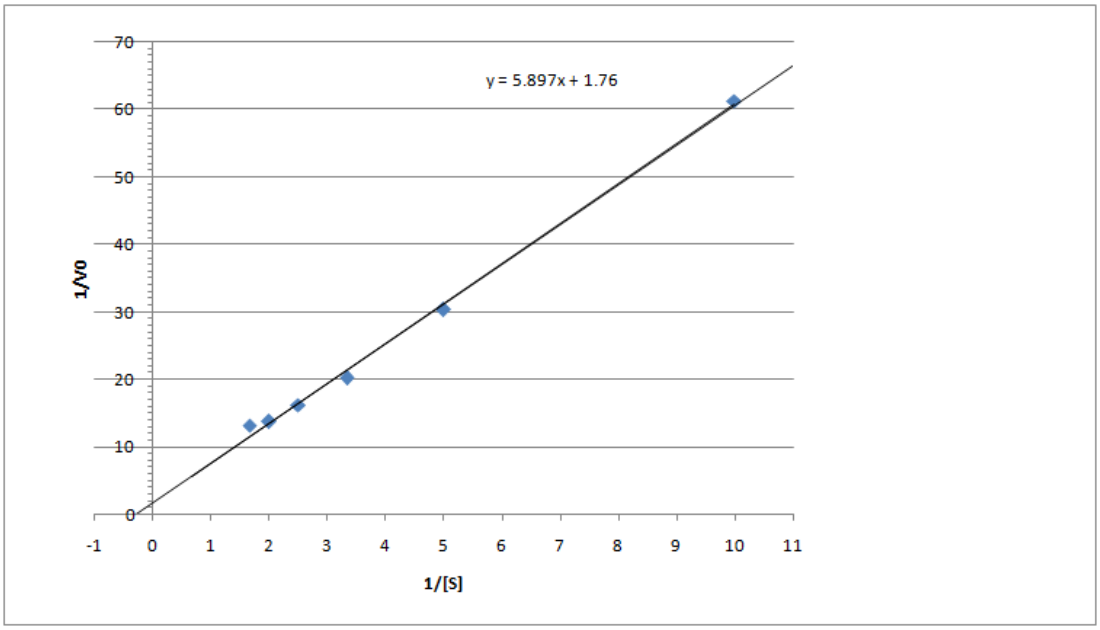

Fig. 14: Lineweaver-Bruk plot for determination Km and Vmax of enzyme.

Therefore to determination the kinetic value of enzyme the Lineweaver-Burk plot is a good approach for the exact determination of Km and Vmax. To determine the Km and Vmax of the enzyme of *Serratia marcescens* scl1the enzyme was treated with different pNPP concentrations ([S]) and the activity plotted in respect to liner equation. The result show the kinetic constant Km is $3.349x10^{-3}$M and optimum velocity (Vmax) under experimental condition is $5.68x10^{-1}$ unit/ml.

4. CONCLUSION

In this study, a novel bacterial strain *Serratia marcescens* scl1 have been isolated from medicinal waste and characterized by different biochemical tests and 16S rDNA technology. The sequence has been submitted to NCBI database (Accesstion no: KT877002). The qualitative and quantitative study to identify the optimum condition for bacterial growth was carried out. The bacteria showed 50 minute generation time. The isolate was able to produce inducible thermostable (optimum temp. 75^0C) extracellular lipase with optimum pH 8.00. The lipase showed a Km value of $3.3x10^{-3}$M indicating a high affinity for substrate (lipid) and hence satisfy the desired property to be used in industrial sector with its excellent thermotolerant activity ($5.68x10^{-1}$ unit/ml). Thus the strain can be used as potential source of such a lipase which can be applied in different industrial aspect.

Disclosure of potential conflicts of interest: The authors declare that they have no conflict of interest.

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