

# Production and Purification of a New Inhibitor of Pancreatic Lipase and Hormone Sensitive Lipase from Soil Actinomycetes

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**ABSTRACT:** A novel lipase inhibitor Shamstatin was isolated from soil isolate, identified as *Streptomyces sp.* The lipase inhibitor was produced by the organism at optimized fermentation conditions. The intracellular Shamstatin was extracted using ethanol as a solvent and subjected to chromatographic purification. Shamstatin was elucidated to be: 6-ethyl-5-methyldihydro-2H-pyran-2,4(3H)-dione, a cyclic diketone having molecular mass of 156.17908. Shamstatin with an  $IC_{50}$  of 23 ng/ $\mu$ l showed dose dependent mixed type inhibition towards porcine pancreatic lipase. It also exhibited *in vitro* lipase inhibitory activity against hormone sensitive lipase.

**KEYWORDS:** lipase inhibitor, Shamstatin, *Streptomyces*, pancreatic lipase inhibitor, hormone sensitive lipase inhibitor

## I. INTRODUCTION

Obesity is defined as weight that is higher than what is considered as a healthy weight for a given height (CDC), which culminates into serious health problems like cardiovascular diseases, heart disease, hypertension, atherosclerosis, diabetes, certain types of cancer and osteoarthritis, if not controlled [1]. There are many theories and methods to reduce obesity, though in recent years treatment with lipase inhibitors has proven to be a very effective method for managing obesity [2]. Pancreatic Lipase inhibition is one of the most widely studied mechanisms to assess the anti obesity capacity of a selected compound. Actinomycetes have been considered a source of lipase inhibitors.

Tetrahydrolipstatin (THL), a hydrogenated analogue of lipstatin from actinomycetes (*Streptomyces toxytricini*), is a potent inhibitor of gastrointestinal lipases which is commercially sold as Orlistat [3]. However the treatment has a few side effects, such as, it causes hyperuricemia, diarrhea, nausea, gastric irritation, oily spotting, flatulence, flatus with discharge, fecal incontinence and dry skin [4]. A second lipase inhibitor of 176 Daltons belonging to the class of enol acetate of p-amino phenyl acetaldehyde was isolated from *Streptomyces sp.* against gastrointestinal lipase [5]. The success of these microbial compounds for treatment of obesity has expedited the research for the identification of newer, safer pancreatic lipase inhibitors. Many other microbial and plant based compounds have been isolated and identified as pancreatic lipase inhibitors, some of which are protamines,  $\epsilon$ -polylysine, polysaccharides like chitosan [6]. These lipase inhibitors act only on a particular type of lipase. Hence, it is imperative to isolate better and safer lipase inhibitors preferably with more diverse properties.

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In this study, we discuss a lipase inhibitor having both pancreatic and hormone sensitive lipase inhibitory activity with the intention of targeting obesity at two different levels.

## II. MATERIALS AND METHODS

### 2.1 Culture

Actinomycetes were isolated from various soil samples by serial dilution method and were spread plated on Actinomycetes Isolation Agar (AIA). Pure isolates were obtained by subculturing onto fresh media.

### 2.2 Actinomycetes isolation Media

The isolation media as formulated by Olsen[7], had the following composition: Sodium caseinate 2.0g, L-Asparagine 0.1g, Sodium propionate 4.0g, Dipotassium phosphate 0.5g, Magnesium sulphate 0.1g, Ferrous sulphate 0.001g Agar 15.0g Final pH (at 25°C) 8.1±0.2.

### 2.3 Screening of actinomycetes for lipase inhibitory activity

These isolated pure cultures were grown individually in actinomycetes isolation broth for the production of lipase inhibitor. A loop full of well grown 7 day old culture was inoculated and the culture flasks were incubated in an incubator shaker rotating at 220 rpm at 30°C. After 7-10 days of growth the mycelial mat was separated by centrifugation at 10,000rpm, 4°C, 20 min. The mycelium was collected and solvent extracted to obtain the lipase inhibitor.

### 2.4 Characterization of the actinomycetes

The screened and isolated lipase inhibitor producing actinomycetes was further characterized by Gram staining, microscopy and biochemical tests[8]. The confirmation of identity of the culture was done by 16S rRNA sequencing (Triyat Scientific).

### 2.5 Optimization of growth parameters

#### 2.5.1 Optimization of the growth media

Culture was grown in twelve different media such as Actinomycetes Isolation broth, Kusters broth[9], Kenknight broth[10], Potato dextrose broth, YMB broth, M3 broth, Starch casein broth, Botcher conn's broth[11], CzapekDox broth, Vegetable high media broth, Soya meal broth, Oatmeal broth. 50ml of specified type of media were inoculated with culture (10% inoculum) individually. The flasks were incubated at room temperature in a shaker rotating at 220 rpm at 30°C for a period of 7 days. The mycelial mat was separated and used for the extraction of lipase inhibitor.

#### 2.5.2 Growth Temperature optimization

Culture was subjected to growth in liquid medium at different temperatures viz 20°C, 28°C, 37°C, 45°C for a period of 7 days under shaking conditions.

#### 2.5.3 Time course of inhibitor production

This study is carried out to understand the relationship between actinomycetes growth rate and inhibitor production. Seven 250 ml flasks containing 50 ml actinomycetes isolation broth were inoculated with the isolated culture. Flasks were harvested every 24 hours and used for further studies [12].

#### 2.5.4 Optimization of Fermentation conditions for inhibitor production

The optimized conditions obtained in the earlier experiments were used to obtain biomass on a large scale. The 10L fermentor was setup with 7200ml working media and inoculated with pregrown seed inoculum at 10% level. A 50L fermenter with 31.5L working media and 3.5L inoculum was also set up. The fermentation was maintained at 30°C, 180rpm, and 0.625vvm with a backpressure of 0.5bars for 5 days.

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## 2.6 Isolation of lipase inhibitor

The bioactive was isolated by treatment of mycelial mat with different solvents. Four types of extractions, aqueous, methanolic, ethanolic and ethyl acetate were carried out. The mycelial mass was macerated in different solvents using sterile sand. This was filtered using Whatmann 1 filter paper. The solvent fraction was separated and the extraction of the inhibitor from the residual biomass using solvent was repeated twice. The solvent fractions were pooled and dried under vacuum at 37 °C. The dried residue was collected and re-suspended in DMSO and inhibitory activity was checked.

## 2.7 Purification and characterization of the isolated lipase inhibitor

### 2.7.1 Purification of the lipase inhibitor

The isolated inhibitor was analyzed using isocratic analytical HPLC, RP-C18 column [5µm, 250 mm x 4.6 mm, Shimpack CLC-ODS (L1), LC-10A (Shimadzu, Japan). A flow rate of 1 ml/minute was maintained and scanned over a UV-Vis spectrum of 210-320nm, methanol and acetonitrile were used as mobile phase (82:18).

The peaks observed in the analytical column were subjected to purification by preparative HPLC following the same analytical conditions. The individual peaks collected were dried and resuspended in DMSO and lipase inhibitory assay was carried out. The selected peak was further purified by preparative TLC using methanol and chloroform (10:90) as mobile phase on Silicagel 60 TLC plates (MERCK). The lipase inhibitor eluted as a single band with  $R_f$  0.85 was scrapped off and extracted using ethanol. Ethanol was evaporated to dryness and the inhibitor was used to assess the inhibitory activity.

### 2.7.2 Characterization of the lipase inhibitor

The purified sample giving highest lipase inhibitory action was subjected to LC-MS/MS analysis for molecular weight determination in ESI +ve mode. The structure of the inhibitor of microbial origin was elucidated using NMR ( $^1\text{H}$ ,  $^{13}\text{C}$ , HSQC, DEPT), FT-IR, GC and GC MS/MS.

### 2.7.3 Chemical structure elucidation

NMR spectra were recorded on a Bruker Avance instrument with 400 MHz for  $^1\text{H}$  and 100 MHz for  $^{13}\text{C}\{^1\text{H}\}$  experiments. Proton and carbon chemical shifts are given relative to either residual solvent peaks or standard tetramethylsilane. Assignments of  $^1\text{H}$  and  $^{13}\text{C}$  signals were based on  $^1\text{H}$ ,  $^{13}\text{C}$ , HSQC (heteronuclear single quantum coherence) and DEPT (distortionless enhancement by polarization transfer) experiments.

## 2.8 Lipase Inhibitory Activity *in Vitro*

Pancreatic lipase (PL) inhibitory activity was determined using the substrate *p*-nitrophenyl butyrate (pNPB) [13]. The substrate, *p*-nitrophenyl butyrate (pNPB) was dissolved in acetonitrile to give a stock solution with a concentration of 50 mM/L. The purified microbial extract was dissolved in DMSO (1mg/ml). Porcine pancreatic lipase (type II, Sigma) was dissolved in sodium phosphate buffer with Triton X-100 to get 60-70 units/mL of the lipase. One mL reaction mixture contained 99mM sodium phosphate, 149 mM sodium chloride, 0.5%(v/v) Triton X-100, 0.50 mM *p*-nitrophenyl butyrate (pNPB), 6-7 units porcine pancreatic lipase and 0.20 mg/mL of crude extracts. The reaction mixture was incubated at 37°C for 25 min. Ethanol was added to stop the reaction. The absorbance of released *p*-nitrophenol was measured at 405 nm using a microplate reader (Varioskan Flash). The assay was performed in triplicates and the results were expressed as average value. Controls without the inhibitor were also run parallel. Percentage inhibition of lipase activity was calculated using the formula:

Lipase inhibition =  $(A-B/A)100$ , where A is lipase activity, B is activity of lipase when incubated with the extract.

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## 2.9 Kinetics of Enzyme Inhibition

Single phase kinetics was determined to study the inhibition kinetics. Initial rate of enzymatic reaction was found for increasing enzyme concentrations (0- 0.1mg/mL). Inhibitory kinetics was performed by maintaining fixed enzyme concentration 0.01mg/ mL and varying substrate concentration (0-0.2mM pNPB concentration). The kinetic graph was drawn using standard Micheles-Menten curve for porcine pancreatic lipase. The reciprocal plot was used to obtain the Lineweaver-Burk (LB) plot. Using the LB kinetic plot as reference, inhibitory assay was done with two different concentrations of the lipase inhibitor (50µg/mL, 100µg/mL).

## 2.10 Determination of IC<sub>50</sub>

IC<sub>50</sub> of the isolated lipase inhibitor was calculated using varying concentrations of the lipase inhibitor ranging from 250pg/µL to 1000µg/µL.

## 2.11 Specificity of the 6-ethyl-5methyl-dihydro-2H-pyran-2,4(3H)-dione

The lipase inhibitory activity was assessed using different lipases: Pancreatic lipase (PL), Hormone sensitive lipase (HSL) [13], Monoacylglycerol lipase (MAGL), 4-methylumbelliferyl butyrate (MUB) was used as the fluorimetric substrate. 6.0 mg of MUB was dissolved in 1000 µL of DMSO and further diluted fivefold. 10 µL of the microbial lipase inhibitor and 200 µL of 50 mM phosphate buffer solution at pH 7.4, 50 µL of substrate solution were added in each well were added. The fluorescence emitted at 445 nm after excitation at 365 nm was immediately measured with the fluorescence spectrometer. Each lipase was analysed separately with and without the inhibitor. The speed of fluorescence development is directly proportional to the product formation and therefore to the lipase activity [14]. Pancreatic lipase, Monoacylglycerol lipase, Hormone sensitive lipase were used at a concentration of 0.20µg/mL.

## III. RESULTS AND DISCUSSIONS

### 3.1 Characterization of the actinomycetes

Forty two purified soil isolates were screened to assess the lipase inhibitor producing potential. The isolate CFTR 31, showed maximum lipase inhibitory activity, (Table 1) in all the solvent fractions. Further the 16S rRNA identification revealed 85% similarity to *Streptomyces griseus* which was in confirmation to the biochemical tests. The 16S rRNA has been deposited and the GenBank accession number of the sequence is KT784805.

### 3.2 Optimazation of inhibitor production

CFTR 31 grew well on Actinomycetes isolation broth followed by Potato dextrose broth and Botcher Conn's broth (Fig 1). Further studies were carried out using Actinomycetes isolation broth and Potato dextrose broth. The CFTR 31 isolate was grown at different temperatures (20 °C, ambient temperature (28-30°C), 37 °C and 45 °C. Growth and inhibitor production increased with temperature, reached maximum at ambient temperature and decreased thereafter (Fig. 2). The growth in both the media used showed the same pattern. Among the two media used, Actinomycetes isolation broth was observed to be the best with 95% lipase inhibitory activity. This media was used in further experiments. The production of lipase inhibitor also increased with time (Fig 3), reached optimum on day 5 and decreased thereafter. These optimized conditions were used for maximum inhibitor production. Extractions with different solvents, ethanol, methanol, ethyl acetate were compared with aqueous extract. Ethanol was found to be the best solvent for the extraction of the inhibitor as compared to methanol, ethyl acetate and water.

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Table 1.Screening of different isolates for lipase inhibitory activity

Culture no.	% Percentage inhibition by aq.extract	% Percentage inhibition by ethanol extracts	% Percentage inhibition by methanol extracts	% Percentage inhibition by ethyl acetate extracts
CFTR 1	70	45.58	68.16	58.8
CFTR 2	88.87	71.04	76.97	65.28
CFTR 3	87.419	49.1	77.15	58.9
CFTR 4	83.065	65.83	74.82	64.2
CFTR 5	83.38	63.66	74.46	62.05
CFTR 6	86.129	80.396	74.1	55.93
CFTR 7	77.419	73.56	69.6	51.97
CFTR 8	87.581	17.98	52.15	34.35
CFTR 9	87	67.44	64.02	66.18
CFTR 10	86.129	53.23	46.76	40.64
CFTR 11	88.065	73.2	63.129	48.7
CFTR 12	42.23	43.82	69.35	48.7
CFTR 13	73.819	32.2	67.213	32.58
CFTR 14	73.03	32.3	39.13	50.32
CFTR 15	65.5	42.32	19.3	18.5
CFTR 16	42.12	70.25	34.19	11.6
CFTR 17	62.79	76.92	18.5	43.8
CFTR 18	51.57	39.35	16.29	28.87
CFTR 19	67.126	30.41	9.6	48.22
CFTR 20	67.111	19.67	12.96	32.09
CFTR 21	64.96	20.57	9.6	6.4
CFTR 22	83.26	56.17	6.4	51.45
CFTR 23	59.05	31.6	9.6	31.6
CFTR 24	69.83	31.12	6	51.2
CFTR 25	93.18	54.2	10.1	21.6
CFTR 26	71.06	57.78	10.2	21.45
CFTR 27	61.22	58.85	6.2	19.35
CFTR 28	28.87	49.91	9	17.25
CFTR 29	56.57	86.92	8.27	64.62
CFTR 30	50.5	80.22	51.1	66.57
CFTR 31	78.23	80.3	72.07	64.45
CFTR 32	55.99	70.58	16.72	76.36
CFTR 33	57.74	49.34	42.2	48.46
CFTR 34	49.59	18.46	38.31	31.9
CFTR 35	31.16	34.15	47.24	13.9
CFTR 36	23.28	44.28	56.16	33.84
CFTR 37	13.97	48.52	33.44	44.72
CFTR 38	64.61	18.3	17.2	3.7
CFTR 39	40.74	33	38.31	0
CFTR 40	46.21	50.81	21.1	15.15
CFTR 41	11.64	51.14	36.68	45.4
CFTR 42	56.88	50.98	0	23.2

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Fig 1.Optimization of the growth media

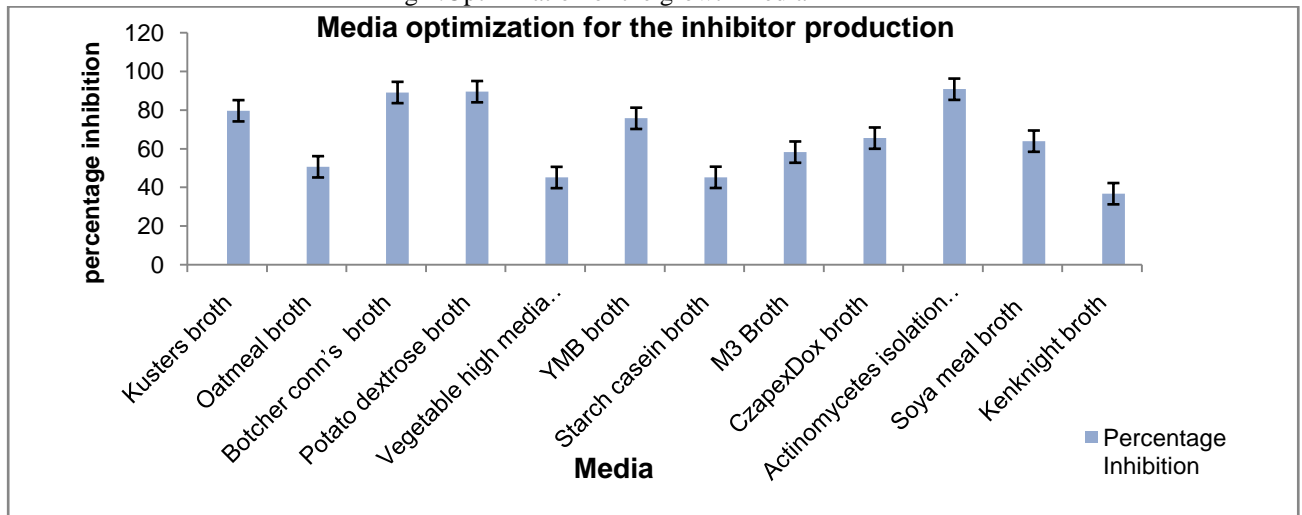
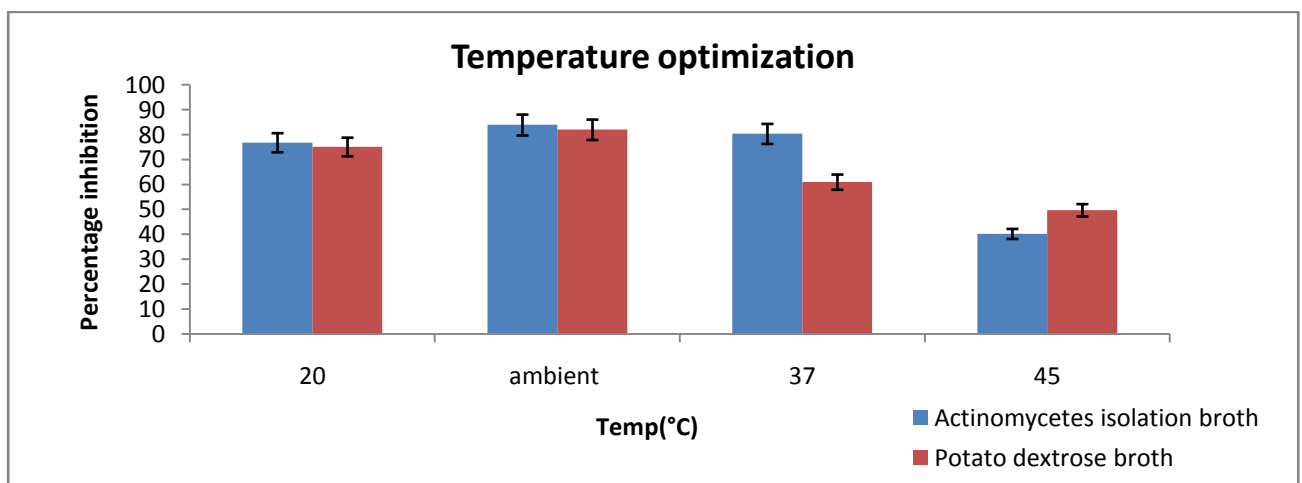


Fig 2. Growth Temperature optimization

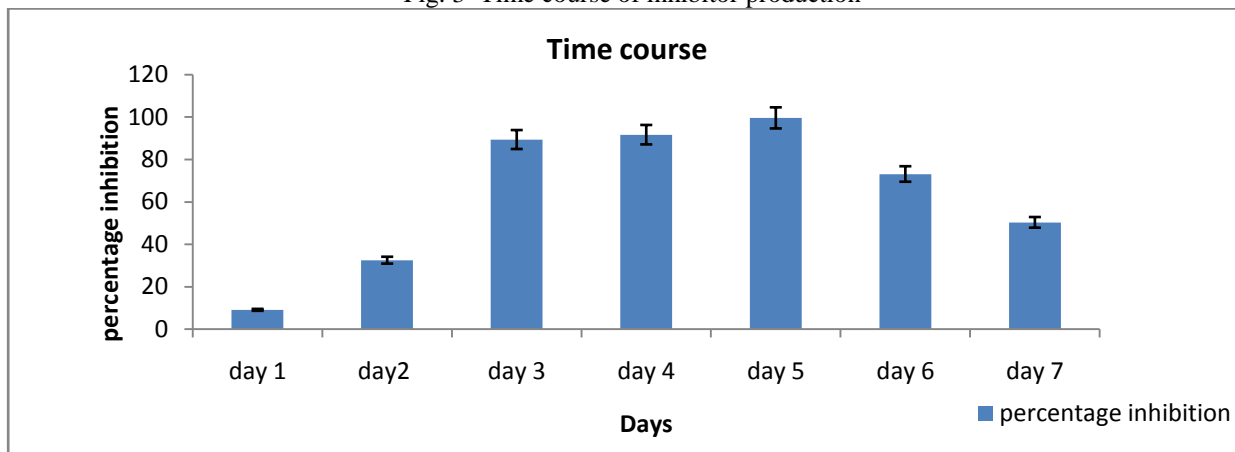


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Fig. 3 Time course of inhibitor production



### 3.3 Isolation of pure lipase inhibitor

Based on the optimized conditions, large scale trials were done at 8L (10L fermenter volume), and 35L (50L fermenter volume). In both the experiments, samples were analyzed daily for inhibitor production. The inhibitor production increased with time up to 5 days and decreased thereafter (data not shown). The biomass obtained was extracted in ethanol and further purified by preparative HPLC (mobile phase methanol and acetonitrile (82:18) and preparative TLC (mobile phase methanol and chloroform (10:90)). The purified inhibitor was 84% inhibitory towards pancreatic lipase (data not shown).

### 3.4 Structural elucidation of the purified lipase inhibitor

The isolated compound was subjected to various NMR experiments including  $^1\text{H}$ ,  $^{13}\text{C}$ , and DEPT-135 and HSQC followed by mass spectral analyses to confirm the structure. Mass spectral analyses revealed the peak pattern at  $m/z$  157.5963 and 179.5585 assigned to  $(\text{M}+\text{H})^+$  and to  $(\text{M}+\text{Na})^+$  wherein  $\text{M} = \text{C}_8\text{H}_{12}\text{O}_3$ . The  $^1\text{H}$  NMR spectra displayed several peaks and no peaks were seen in the aromatic region clearly indicating that no aromatic group was present. Many well defined multiplets were observed in region 3.5 to 3.70 ppm which were further confirmed utilizing DEPT-135 and HSQC to be consisting of two methyne group and one methylene group. Another methylene group was observed at 1.59 ppm which was further confirmed by DEPT-135 experiment. Two methyl groups were identified one at 1.29 ppm and another at 1.89 ppm. The methyl group at 1.29 ppm was observed to be buried in the peak due to grease in  $^1\text{H}$  NMR, however it showed clear and different peak in the regular  $^{13}\text{C}\{^1\text{H}\}$  NMR and further confirmation as methyl was done utilizing DEPT-135 experiment. Based on the above peaks confirmation and in combination of correlation established by  $^1\text{H}$ - $^{13}\text{C}$  HSQC experiment, we propose the structure as shown in the figure. The name of the isolated microbial lipase inhibitor is 6-ethyl-5-methyldihydro-2H-pyran-2,4(3H)-dione (Fig. 4) with molecular mass of 156.17908. 6-ethyl-5-methyldihydro-2H-pyran-2,4(3H)-dione was called by Shamstatin.

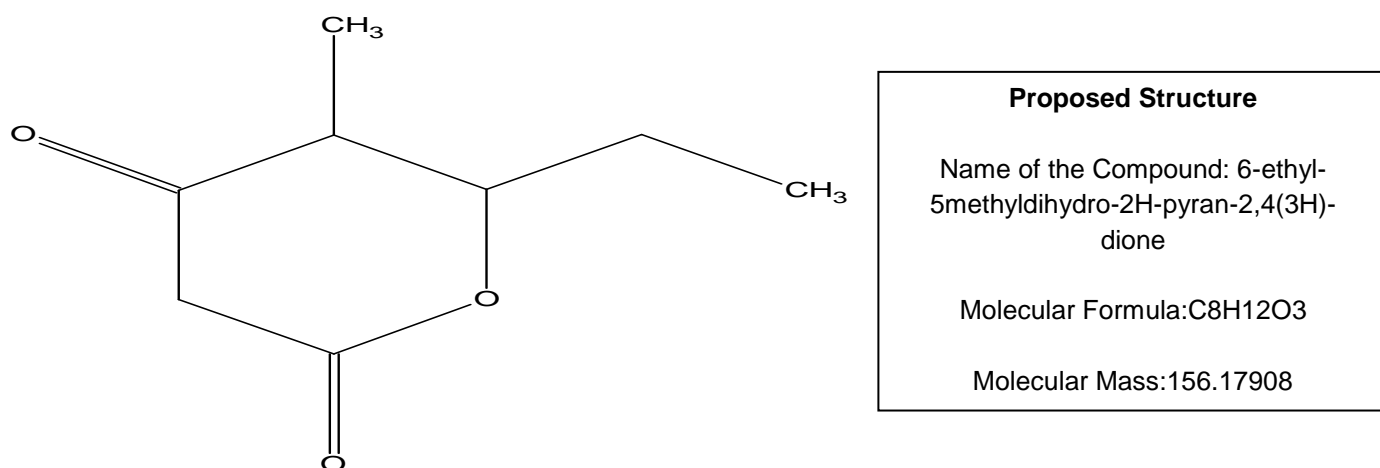


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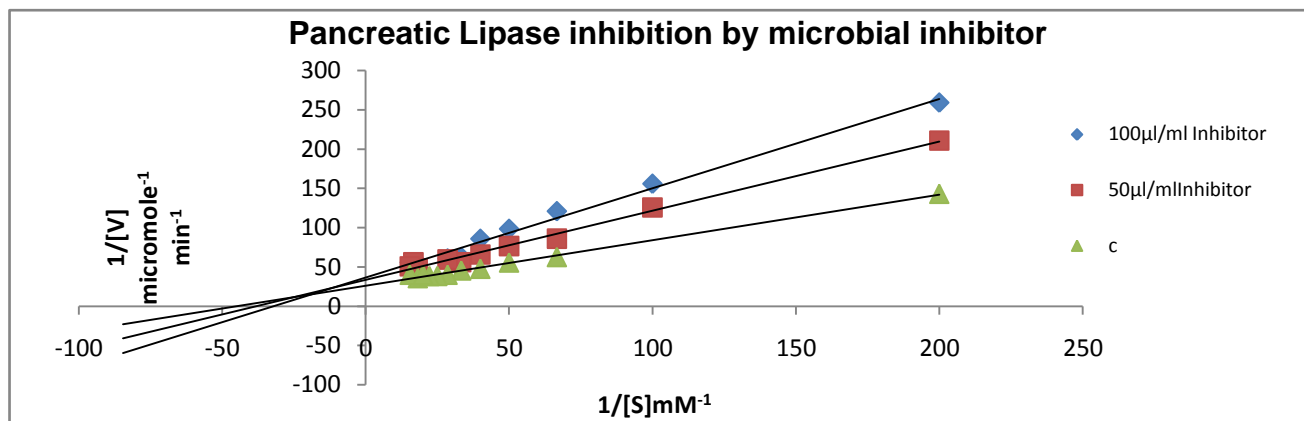
Fig. 4 The proposed structure of Shamstatin (6-ethyl-5methyldihydro-2H-pyran-2,4(3H)-dione).



## 3.5 Kinetics of inhibition

The kinetic properties of purified inhibitor was studied using different substrate concentrations (0-0.2mM). The microbial lipase inhibitor showed mixed type of inhibition. A mixed-inhibitor binds at a distinct site from the active site but it can bind to the enzyme or enzyme-substrate complex as well. It affects  $K_m$  and  $V_{max}$  of the reaction. The inhibitor decreased  $V_{max}$  and increased  $K_m$  (Fig 5). It simulated the activity of some of the properties of a competitive and a noncompetitive, or a noncompetitive and an uncompetitive, inhibitor. The experimental  $IC_{50}$  was found to be 23ng/ $\mu$ l.

Fig 5 Pancreatic Lipase inhibition by microbial inhibitor





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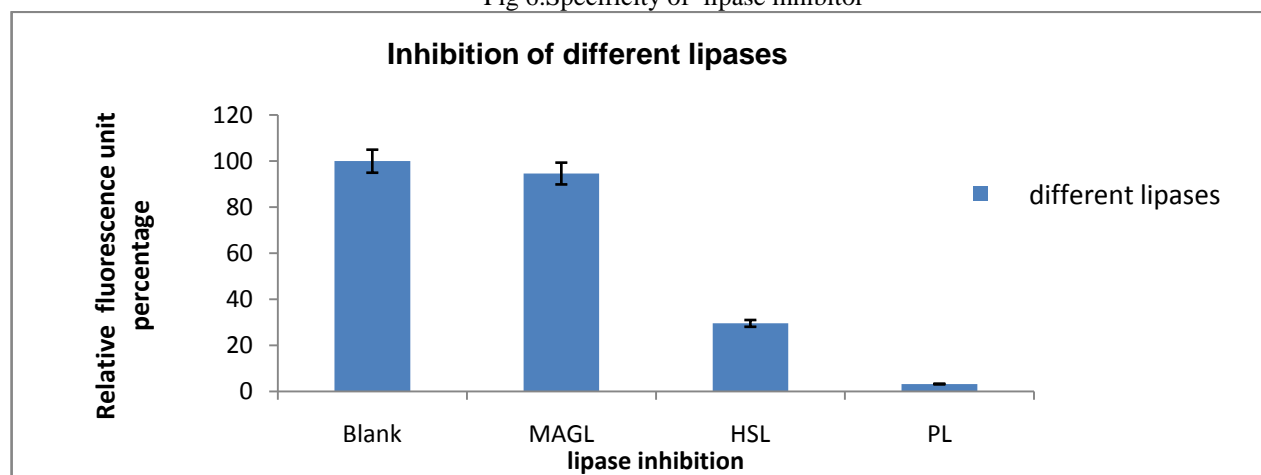
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## 3.6 Specificity of 6-ethyl-5-methyldihydro-2H-pyran-2,4(3H)-dione

The robust fluorimetric assay which is more sensitive than the colorimetric lipase inhibition assay was used to study the specificity of the isolated lipase inhibitor, 6-ethyl-5-methyldihydro-2H-pyran-2,4(3H)-dione. Three different lipases were used as targets for the inhibitor in three independent experiments. Among the three different lipases used, the lipase inhibitor showed higher inhibition with pancreatic lipase (PL) and hormone sensitive lipase (HSL). The inhibitor showed negligible inhibition towards monoacyl glycerol lipase (MAGL) (Fig 6).

Fig 6. Specificity of lipase inhibitor



Pancreatic lipase inhibition has been studied widely as an approach to combat obesity. Microbial lipase inhibitors studied so far have inhibition predominantly against pancreatic lipase. Orlistat is a commercially available lipase inhibitor [2]. A second microbial lipase inhibitor of 176 Daltons has also been studied extensively [5]. In our study, the microbial lipase inhibitor identified as 6-ethyl-5-methyldihydro-2H-pyran-2,4(3H)-dione has shown more than 80% inhibitory activity under optimized conditions. The purified lipase inhibitor from actinomycetes is a low molecular weight compound that had inhibition against pancreatic lipase and hormone sensitive lipase. Hence, obesity and its effects can be targeted by two different mechanisms, one by inhibiting gastrointestinal absorption of fats and the other by inhibiting the circulation of diglycerides and triglycerides in the living system. The identified compound has an internal dione which might be responsible for lipase inhibitory activity. Other microorganisms have also been identified possessing lipase inhibitory compounds [13,15]. However, the active inhibitory compound has not been structurally elucidated. Further work is required to confirm the mode of action of our isolated lipase inhibitor.

## IV. CONCLUSION

Here we have presented a new lipase inhibitor, 6-ethyl-5-methyldihydro-2H-pyran-2,4(3H)-dione, Shamstatin, isolated from soil *Streptomyces*. The lipase inhibitor had activity against pancreatic lipase and hormone sensitive lipase. The low molecular weight along with the low  $IC_{50}$  could make Shamstatin an advantageous lipase inhibitor. *In vivo* evaluation of the inhibitor is required to assess the applicability of the inhibitor for human consumption.

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