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Research Article

THE ROLE OF LINOLEIC ACID, PALMITIC ACID AND LEUCINE IN LIPSTATIN BIOSYNTHESIS BY *STREPTOMYCES TOXYTRICINI*

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ABSTRACT: Lipstatin, a new and very potent inhibitor of pancreatic lipase, was isolated from the *Streptomyces toxytricini*. Lipstatin contain a beta lactone structure that probably account for the irreversible lipase inhibition. As per the biosynthetic pathway the role of linoleic acid, palmitic acid and leucine in lipstatin biosynthesis by *Streptomyces toxytricini* was studied. Different oils were analyzed to measure the concentration of the linoleic acid and palmitic acid in the medium, and then the selected oil was used as media ingredients. While leucine was used as a media ingredient as well as feeding nutrient in the production medium. The lipstatin production depends on linoleic acid palmitic acid and leucine concentration as per biochemical pathway. But in present study it was observed that in control media the lipstatin activity was 1.108 mg/g while when we use linoleic acid and palmitic acid as media ingredients then the activity was 0.610 mg/g. Similarly in case of leucine was used as ingredient as well as feeding solution the maximum activity was observed 0.810 mg/g at 264 h. Thus, it may conclude that their is no significant impact of linoleic acid, palmitic acid and leucine as media ingredients and as a feeding solution on lipstatin production.

Key words: Lipstatin; *Streptomyces toxytricini* ATCC 19813, Linoleic acid, palmitic acid and leucine.

INTRODUCTION

Streptomyces toxytricini produces lipstatin during aerobically cultivating conditions. As per the biosynthetic pathway the basic building unit is β-lactam circle with two aliphatic rests of fatty acids with 6 – 14 carbon atoms. One of the side chains contain two double bonds and one hydroxyl group esterified as N-formyl-leucine. By catalytic hydrogenation lipstatin is able to get tetrahydrolipstatin (orlistat). Microorganism *Streptomyces toxytricini* is able to utilize fatty acids to acetyl-CoA. During fermentation biosynthesis of lipstatin occurs, condensation of C14 and C8 fatty acids is started which becomes from fats and long chain fatty acids. Linoleic acid contains the same number of double bonds as lipstatin chain and it is the basic building unit of lipstatin. Claisen condensation of 5, 8-tetradekadienoyl-CoA and oktanoyl-CoA is formed basic intermediate ester of 5-hydroxy-2-hexyl- 3-oxo-7, l0-hexadekadien acid. In the next step catalytic hydrogenation of ester group takes place, thereafter formation of β-lactone ring follows. Esterification of the side chain occurs in presence of leucine that result in the formation of Lipstatin {[(3-hexyl-4-oxo-oxetan-2yl) trideca-4,7-dien-2yl] – 2 formamido-4- methyl pentoate}. It has effect on the place where fats split up *e.g.* in stomach and small intestine. Its action pancreatic enzyme called lipase is stopped; inhibit absorption of fats from food till one third. This phenomenon is used in treatment of obesity, and can be used as prevention against hyperlipidemia and arteriosclerosis (U.S. Patent No. 4,598,089).

In the present study different oils were analyzed for the linoleic acid, palmitic acid concentration as a precursor. As per biosynthetic pathway lipstatin production depends on linoleic acid and palmitic acid concentration. Leucine concentration, was also analyzed as it was required during Esterification of β-lactone ring. Effect of Linoleic acid palmitic acid and leucine concentration was analyzed on lipstatin production.

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MATERIALS AND METHODS

Streptomyces strain, medium and culture characterization

The actinomycetes *Streptomyces toxytricini* (ATCC 19813) was used in the studies. The cells were maintained on yeast malt extract (YM) medium. The pH was adjusted to around 7.2 before autoclaving the medium at 121°C for 15 min. The cells were grown in 250 ml flask containing 30 ml of medium and incubated in orbital shaking incubator at 200 rpm and 28°C for 48±24 h. For inoculum preparation, the culture was grown in YMB medium (yeast extract 4.0g/l, malt extract 10.0g/l, D-glucose 4.0g/l) at 28°C and 200 rpm for 24 h. 2 % of lab inoculum at the age of 32 h was transferred into the seed media (soya flour 10.0g/l, glycerol 10.0g/l, yeast extract 5.0g/l) and flasks were incubated at 28°C at 180 rpm up to 48 ± 24 h or till growth appears. The production medium PM1 (glycerol 22.5g/l, soyabean flour 35.0g/l, soya lecithin 15.0g/l, soya oil 25.0g/l, PPG 0.50g/l) was inoculated with 5% (v/v) inoculum. Control feeding was done in production flask from log 48 to 120 h at 24 h interval. Samples were analyzed at different hours 144, 168,192, 216 and 240 h for pH, PMV and activity.

Lipstatin assay

Lipstatin activity in the culture broth was determined by HPLC. The culture broth of 5.0 gm was taken in 50 ml volumetric flask with 30 ml acetone and sonicates it for 10 minutes and make up the volume with acetonitrile. The resulting extracted solution was injected into the HPLC (Waters 2496) having C-18 column (Hypersil ODS, 5u C18 (150mm X 4.6 mm) for the estimation of lipstatin. Concentrations of lipstatin were calculated by comparison of peak areas with those standard lipstatin and subsequently lipstatin activity was calculated. Biomass was measured in terms of percentage mycelial volume (%) (Ferreira *et al.*, 2005).

Analysis of Linoleic acid content in different Oil

Different oils were analyzed for the Linoleic acid concentration through HPLC. Effect of different oils such as soyabean oil, sunflower oil, olive oil, ground oil, palm oil, soya oil, rice bran oil, mustard oil, til oil, coconut oil was studied in modified medium for better production of lipstatin. Linoleic acid was checked with all the oil.

HPLC Condition:

Column	:	Hypersil ODS C18 (150 x 4.6 x 5 µ)
Mobile phase	:	A) 0.1% Ortho-phosphoric acid in Water B) ACN
Column temperature	:	30° C
Ratio	:	25:75
Flow	:	2.0 ml / minute
Run time	:	30.0 min for sample
Injection volume	:	20 µl
λ max	:	200 nm
Retention Time of Linoleic acid	:	Approx 6.4 min

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Sample Preparation

1. **Standard Preparation**: Weigh 10mg of linoleic acid standard into a 10ml volumetric flask, add 30 ml of acetonitrile, sonicate to dissolve and make up the volume with acetonitrile. Dilute 5.0 ml of this solution to 25 ml in Acetonitrile. (200ppm).
2. **For oil Samples**: Weigh 1.0gm of oil containing Linoleic acid into a 25 ml volumetric flask, add 20 ml of acetone and sonicate for 5.0 min. Make up the volume with acetone and shake vigorously for 30 sec. Filter the solution through a whatman 41 filter paper. Now finally filter a portion of it through a 0.22µ Nylon filter paper and inject.

Analysis of Palmitic acid content in different Oil

Different oils were analyzed for the palmitic acid concentration through gas chromatography. Effect of different oils such as soyabean oil, sunflower oil, olive oil, ground oil, palm oil, soya oil, rice bran oil, mustard oil, til oil, coconut oil was studied in modified medium for better production of lipstatin. Palmitic acid was checked with all the oil.

Oven Temperature:

Rate (ºC/min)	Temperature (ºC)	Hold (min)
0.0	150	0
10	230	10

Chromatographic Parameters:

Gas Chromatograph	Agilent Technologies 7890 A
Auto Sampler	G4513A
Integrator	EZChrom Elite
Column	DB-FFAP (30m X 0.53mm X 0.5µm)
Detector	Flame Ionization Detector

Injector temperature	240ºC
Detector temperature	270ºC
Carrier gas	Nitrogen
Split ratio	20:1
Carrier gas flow	3.0 ml/min
Injection volume	1.0µL

Preparation of solutions

(i) Diluent: Toluene was used as diluents

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(ii) Preparation of Standard Solution: Weigh accurately 10.0 mg of linoleic acid, in 10.0 ml of volumetric flask containing about 2.0 ml of diluent, mix and dilute up to the mark with diluent.

(iii) Preparation of Test Solution: Weigh accurately 500.0 mg of sample in 5.0 ml volumetric flask containing 2.0 ml of diluent, which was mixed and diluted up to the mark with diluent (*i.e.* concentration is 100000 ppm).

Procedure: 1.0 µl of diluent was injected as blank and the chromatogram was obtained. Then 1.0 µl of standard solution (in duplicate) was injected. Then 1.0 µl of test solution was injected and chromatogram was obtained.

Retention and Relative Retention Time:

Component		**RT**	**RRT**
Palmitic acid	:	≈ 8.62 min	≈ 1.00

Calculations: Palmitic acid content was calculated by using the following formula

Content (%)

$$\frac{AT}{AS} \times \frac{WS}{10} \times \frac{5}{WT} \times 100$$

Where,

AT	=	Peak area response of (Palmitic acid) respectively from the Test solution
AS	=	Mean peak area response of standard solution
WS	=	Weight of (Palmitic acid) respectively in standard solution (in mg)
WT	=	Weight of test sample in mg

Lipstatin production was checked in linoleic acid and palmitic acid rich contents with different oil

Culture inoculum was prepared in YM broth and incubated at 28°C under shake conditions (200 rpm) for 48 h; subsequently 2% (v/v) inoculum was transferred to seed medium. The seed medium was incubated at 28°C under shake conditions (200 rpm) for 48±24 h. Subsequently two set (10 flask/set) of production media flask was inoculated and incubated at 28°C under shake conditions (220 rpm). One set with control media and another set with 1:1 of linoleic acid and palmitic acid concentration. Samples were analyzed after 24, 48, 72, 96, 144, 192 and 240 h for pH, PMV, lipstatin, linoleic and palmitic acid content.

Lipstatin production was checked with Leucine as production ingredient as well as feeding solution

Culture inoculum was prepared in YM broth and incubated at 28°C under shake conditions (200 rpm) for 48 h; subsequently 2% (v/v) inoculum was transferred to seed medium. The seed medium was incubated at 28°C under shake conditions (200 rpm) for 48±24 h. Subsequently three set (10flask/set) of production media flask was inoculated and incubated at 28°C under shake conditions (200 rpm). Two set with control media and one set with 2.5g/l leucine concentration. Control feeding was done in all flask and one set of control flask 30 mg leucine was feeded. Samples were analyzed after 24, 48, 72, 96, 144, 192 and 240 h for pH, PMV, leucine content, and lipstatin titer value by HPLC.

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HPLC condition for leucine analysis

Column	:	ZIC Hillic (150 x 4.6 x 5 µ)
Mobile phase	:	A) 25mM Phosphate Buffer pH 6.0 B) ACN
Column temperature	:	30° C
Ratio	:	30:70
Flow	:	0.3 ml / min
Run time	:	20.0 min for sample
Injection volume	:	20 µl
λ max	:	200nm
Retention Time of Leucine.	:	Approx 10.2 min

Sample Preparation

1) **Standard Preparation**: 150ppm in Acetonitrile and water 30:70

2) **For oil Samples**: Weigh 5.0gm of broth containing Leucine into a 25 ml volumetric flask, add 20 ml of acetone and sonicate for 15.0 min. Make up the volume with acetone and shake vigorously for 30 sec. Filter the solution through a whatman 41 filter paper. Now finally filter a portion of it through a 0.22µ Nylon filter paper and inject.

RESULTS AND DISCUSSION

Different oils were analyzed to measure the concentration of the linoleic acid and palmitic acid in the medium. The lipstatin production depends on linoleic acid and palmitic acid concentration as per biochemical pathway. Linoleic acid and palmitic acid concentration in different oils was recorded as shown in Table 1. Linoleic acid content in sunflower oil, soya oil, tin oil, rice bran oil, palm oil, groundnut oil was 0.17%, 0.05%, 0.52%, 0.05%, 0.10%, 0.60%, respectively. While Palmitic acid content in sunflower oil, soya oil, tin oil rice bran oil, palm oil, groundnut oil was 0.02%, 0.01%, 0.13%, 0.02%, 0.19%, 0.29%, respectively as shown in Table 1. Maximum linoleic acid and palmitic acid was found in ground nut oil while minimum linoleic acid was found in Rice bran oil and Palmitic acid in soya oil as shown in Table 1.

Table 1: Analysis of Linoleic and Palmitic acid content in various oil.

Sample name	Linoleic acid	Palmitic acid
Sunflower Oil	0.17%	0.02%
Soya Oil	0.05%	0.01%
Tin Oil /Gingely Oil	0.52%	0.13%
Rice Bran Oil	0.05%	0.02%
Palm Oil	0.10%	0.19%
Groundnut Oil	0.60%	0.29%

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Culture performance in medium containing Palm and groundnut oil instead of soya oil in ratio of 1:1:

S. toxytricini was inoculated in selected lab inoculum medium, 2% of 32 h grown lab inoculum was transferred into seed medium and 10 % of matured seed was transferred into the production medium. Control and P1 experiment contained palm oil and groundnut oil instead of soya oil in 1:1 ratio. Linoleic acid, Palmitic acid and lipstatin were analyzed at different hours 24, 48, 72, 96, 144, 192, 240 and 264. As linoleic acid and palmitic are important precursors in their metabolic pathway hence, in process parameters (such as pH, PMV, oil %), activity, linoleic acid and palmitic acid was measured as shown in Table 2.

Control and P1 production medium were checked at every hour 24, 48, 72, 96, 144, 192, 240 and 264 with following process parameters such as pH, PMV, oil, linoleic acid, palmitic acid content and lipstatin activity. The lipstatin activity started with 72 h in experiment as well as control production flask. In control flask maximum activity reached the 1.108 mg/g, whereas experiment flask showed the highest activity of 0.610 mg/g. The pH was start rising in experiment flask from 72 h onward and controlled by optimized feeding of glycerol. Packed mass volume gradually went up with the pH. Oil percentage throughout process was maintained. Palmitic acid linoleic acid content was started with 0.324 mg/g and 0.556 mg/g in experiment production flask. Gradually both the content got decreased with the incubation and declined to minimum by 96 h. Palmitic acid linoleic acid content was 0.056 mg/g and 0.231 mg/g respectively at end of production flask as shown in Table 2.

Different oils were analyzed for the linoleic acid and palmitic acid concentration, because the lipstatin production is depend on linoleic acid and palmitic acid concentration. Linoleic acid and palmitic acid concentration in different oils was measured. Soya oil was selected for further study. The oil may be any oil from synthetic or natural origin or a mixture, of which compositions and preparation are known to the skilled person. Examples for suitable oils are soya oil, palm oil, sunflower oil, flax oil, rape seed oil and corn germ oil. Preferably, more than 0.3 % w/w and not more than 10 % w/w linoleic acid were included. Independently from the amount of linoleic acid, the amount of the oil may be varied. Preferably, more than 1 % w/w and not more than 5 % w/w of the oil are included (EP 1 860 194 A1).

Table 2: Analysis of Palmitic and Linoleic acid as an ingredient in production media P1 with other process parameters analysis.

Control media

Time (h)	pH	PMV (%)	Oil (%)	Linoleic acid (mg/g)	Activity (mg/g)
0	6.68	10	8	1.011	-
24	5.37	10	9	0.321	-
48	5.82	12	8	0.083	-
72	6.12	20	8	0.082	0.042
96	6.58	26	6	0.096	0.097
120	6.77	28	7	0.132	0.115
144	6.66	30	5	0.198	0.285
168	7.13	35	4	0.113	0.410
192	7.23	38	5	0.234	0.512
216	7.56	41	4	0.211	0.756
240	7.12	47	3	0.219	0.923
264	7.45	56	3	0.231	1.108

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Palmitic and Linoleic acid as media ingredient

Time (h)	pH	PMV (%)	Oil (%)	Linoleic acid (mg/g)	Palmitic acid (mg/g)	Activity (mg/g)
0	6.62	9	8	0.556	0.324	-
24	5.36	10	9	0.321	0.225	-
48	5.61	12	8	0.083	0.156	-
72	6.07	20	8	0.082	0.067	0.057
96	6.22	26	6	0.096	0.053	0.094
120	6.42	31	6	0.156	0.055	0.142
144	6.83	30	5	0.198	0.078	0.297
168	7.25	36	5	0.223	0.071	0.325
192	7.65	38	5	0.234	0.110	0.421
216	7.43	43	4	0.285	0.085	0.466
240	7.63	47	3	0.219	0.063	0.556
264	7.52	53	3	0.231	0.056	0.610

Analysis of Leucine as an ingredient and feeding source in optimized production media

2% lab inoculum of *S. toxytricini* was inoculated into the seed medium. Then 10 % of seed inoculum was transferred into the production medium. Leucine was used as an ingredient as well as feeding nutrient in the production medium. Analysis was performed for improved lipstatin yield through feeding of Leucine at 96, 144 and 168 h as an important precursor in their metabolic pathway. In process parameters such as pH, PMV, oil %, activity, Linoleic acid and Leucine was measured as shown in Table 3.

In P1 production media (control) after 24, 48, 72, 96, 144, 168, 192, 240, 264 h of incubation pH, PMV, oil, linoleic acid, leucine content and activity was analyzed. It was observed that at 7.07 pH with 12 % PMV and 9 % oil there is no activity, leucine was found after 48 h. After 72 h of incubation pH was increased to 7.28 with 20 % PMV and 7 % oil that shows 0.137 mg/g activity with 0.165 mg/g linoleic acid. As the incubation time increases in process parameters such as pH, PMV, oil, linoleic acid content and activity also increases i.e. at 96 it was 6.51, 36 %, 6 %, 0.137 mg/g, 0.339 mg/g activity. At an age of 144 h it was 5.27, 38 %, 5 %, 0.157 mg/g, 0.445 mg/g activity. After 168 h it was 5.88, 55 %, 7 %, 0.153 mg/g, 0.513 mg/g activity. After 192 h it was 5.68, 80 %, 5 %, 0.128 mg/g, 0.740 mg/g activity. After 240 h it was 5.82, 70 %, 4 %, 0.110 mg/g, 0.845 mg/g activity. After 264 h of incubation maximum activity was obtained 1.037 mg/g with 0.104 mg/g linoleic acid, 5.63 pH, 65 % PMV and 4 % oil as shown in Table 3.

When leucine was used as media ingredient in Production media. It was observed that at 5.73 pH with 15 % PMV and 9 % oil there is no activity but 0.265 mg/g leucine was found after 48 h. After 72 h of incubation pH was increased to 8.0 with 22 % PMV and 7 % oil that shows 0.167 mg/g activity with 0.085 mg/g linoleic acid and 0.123 mg/g leucine. As the incubation time increases in process parameters such as pH, PMV, oil, linoleic acid content and activity also increases but leucine content decreases i.e. at 96 it was 7.75, 30 %, 6 %, 0.002 mg/g, 0.230 mg/g activity, 0.103 mg/g leucine. At an age of 144 h it was 6.89, 24 %, 5 %, 0.257 mg/g, 0.245 mg/g activity, 0.045 mg/g leucine. After 168 h it was 5.82, 28 %, 7 %, 0.285 mg/g, 0.390 mg/g activity, 0.001 mg/g leucine. After 192 h it was 5.43, 22 %, 5 %, 0.608 mg/g, 0.476 mg/g activity with zero leucine. After 240 h it was 5.29, 27 %, 3 %, 0.811 mg/g, 0.560 mg/g activity. After 264 h of incubation maximum activity was obtained 0.610 mg/g with 0.212 mg/g linoleic acid, 5.08 pH, 30 % PMV and 4 % oil as shown in Table 3.

When leucine was used as feeding solution after 96, 144, 168 h in P1 Production media. It was observed that at 7.07 pH with 15 % PMV and 8 % oil there is no activity after 48 h. After 72 h of incubation pH was increased to 7.78 with 22 % PMV and 8 % oil that shows 0.157 mg/g activity with 0.022 mg/g linoleic acid. As the incubation time increases in process parameters such as pH, PMV, oil, linoleic acid content and activity also increases i.e. at 96 it was 7.16, 31 %, 7 %, 0.060 mg/g, 0.276 mg/g activity, 0.321 mg/g leucine.

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At an age of 144 h it was 6.17, 48 %, 6 %, 0.119 mg/g, 0.356 mg/g activity, 0.410 mg/g leucine. After 168 h it was 5.92, 55 %, 8 %, 0.137 mg/g, 0.441 mg/g activity, 0.3201 mg/g leucine. After 192 h it was 5.84, 70 %, 5 %, 0.191 mg/g, 0.506 mg/g activity with 0.123 mg/g leucine. After 240 h it was 5.70, 75 %, 6 %, 0.362 mg/g, 0.783 mg/g activity with 0.083 mg/g leucine. After 264 h of incubation maximum activity was obtained 0.810 mg/g with 0.280 mg/g linoleic acid, 0.052 mg/g leucine, 5.43pH, 76 % PMV and 7 % oil as shown in Table 3.

Table 3: Analysis of Leucine as an ingredient and feeding source in optimized production media P1

Control

Age (h)	pH	PMV (%)	Oil (%)	Linoleic acid (mg/g)	Activity (mg/g)	Leucine (mg/g)
24	6.8	9	8	0.144	-	0
48	7.07	12	9	0.174	-	0
72	7.28	20	7	0.165	0.137	0
96	6.51	36	6	0.137	0.339	0
144	5.27	38	5	0.157	0.445	0
168	5.88	55	7	0.153	0.513	0
192	5.68	80	5	0.128	0.740	0
240	5.82	70	4	0.110	0.845	0
264	5.63	65	4	0.104	1.037	0

Leucine as media ingredient

Age (h)	pH	PMV (%)	Oil (%)	Linoleic acid (mg/g)	Activity (mg/g)	Leucine (mg/g)
24	5.16	9	10	0.021	-	0.304
48	5.73	15	9	0.083	-	0.265
72	8.00	22	7	0.085	0.167	0.123
96	7.75	30	6	0.002	0.230	0.103
144	6.89	24	5	0.257	0.245	0.045
168	5.82	28	7	0.285	0.390	0.001
192	5.43	22	5	0.608	0.476	0
240	5.29	27	3	0.811	0.560	0
264	5.08	30	4	0.212	0.610	0

Leucine as feeding solution

Age (h)	pH	PMV (%)	Oil (%)	Linoleic acid (mg/g)	Activity (mg/g)	Leucine (mg/g)
24	5.32	9	9	0.821	-	-
48	7.07	15	8	0.083	-	-
72	7.78	22	8	0.022	0.157	-
96	7.16	31	7	0.060	0.276	0.321
144	6.17	48	6	0.119	0.356	0.410
168	5.92	55	8	0.137	0.441	0.320
192	5.84	70	5	0.191	0.506	0.123
240	5.70	75	6	0.362	0.783	0.083
264	5.43	76	7	0.280	0.810	0.052

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EP 0 803 576 (Roche) disclosed a process wherein selected lipstatin precursors, namely linoleic acid, caprylic acid and N-formyl-L-leucine or preferably L-leucine, were inoculated into the fermentation. The yield of the fermentation was low because of the toxicity of the fatty acids and the amounts of feed solutions were very small.
The feeding of the linoleic acid and caprylic acid and/or their salts was preferably conducted so that their concentration in the broth remained inferior to 1,000 mg per liter. This fermentation process uses a medium that is substantially free from fat and oil because they resulted in uncontrolled fatty acid liberation during the fermentation and high residue at the end of the fermentation.

Varga *et al.* (2007) describes fermentation process of lipstatin preparation under help of precursors such as linoleic acid, caprylic acid and N-formyl-L-leucine or L-Leucine. The yield of fermentation is low because of toxicity of the both acids. In comparison with U.S. Patent 6,844,174 production medium contains free fatty acids and natural oils in fermentation process, synthetic oils or their mixture. Consequently fatty acids are liberated from oils during fermentation process depending on pH of production media and high residual concentration of oil at the end of fermentation. The problem is question of emulsification of production media with suitable emulsifier such as lecithin, Triton X-100, Brij 35 S. Disadvantage is the increase viscosity of production media with increasing of emulsifier and worst transfer of oxygen into the fermentation broth which has negative influence on lipstatin production.

US 4,598,089 and EP 129,748 describes the cultivation and fermentation of particular *S. toxytricini* strain (*i.e. Streptomyces toxytricini* NRRL15443) producing lipstatin. Patent applications Teva (WO 03/048335), Biotika (WO 2007/078263), Biocon (WO 2004/003212) and Krka (WO 2007/134836), as well as the early Roche Patent application US 4,598,089 and EP 129 748, further disclose the various fermentation processes for lipstatin production. All are based on a fermentation process using natural triglycerides or fatty acids, particularly linoleic and caprylic acids, as a main source of carbon and source of fatty acid precursors used for lipstatin biosynthesis. Optionally, feeding of leucine or N-formyl-L-leucine during the fermentation process is disclosed (US 4,598,089 and EP 129 748) and is identified to be particularly useful in order to increase the final yield of lipstatin. Lipstatin isolated from *Streptomyces toxytricini* is a potent irreversible inhibitor of pancreatic lipase (Weibel *et al.,* 1987; Hochuli *et al.,* 1987). The data provided from the feeding experiments and tracer studies indicated that the carbon skeleton of lipstatin molecule is biosynthesized *via* Claisen condensation of two fatty acid precursors, 8-carbon atoms (octanoic acid), and 14-carbon atoms (tetradeca-5,8- dienoic acid; Fig. 1; Eisenreich *et al*., 1997; Goese *et al*., 2000, 2001; Schuhr *et al.,* 2002; Eisenreich *et al.*, 2003). The reaction producing an ultimate 3-oxo or hydroxyl intermediate before the β–lactone formation in lipstatin (Goese *et al.* 2001) resembles the biosynthesis of a mycolic acid (Portevin *et al*. 2005).

Without wishing to be bound by any theory it is presently assumed that the concurrent use of oil and linoleic acid has a combinatorial effect eventually resulting in a reduced toxicity of linoleic acid for the strain used to produce lipstatin. This allows conducting the main cultivation step in presence of amounts of linoleic acid otherwise being toxic. The lipstatin precursor linoleic acid will in turn be partially metabolized by the production strain to lipstatin leading to higher yields of said lipase inhibitor. According to an embodiment of the present invention, a process for the production of lipstatin is provided. Said process comprises of the steps (i) providing a medium having not less than 0.3 % w/w a linoleic acid or its ester(s) or salt(s) and more than 20 % w/w of oil, (ii) adding a seed culture comprising a lipstatin producing microorganism, (iii) cultivating, and (iv) optionally isolating lipstatin. The present process may be conducted in any kind of fermentation vessel such as a sterile flask or fermenter (EP 1 860 194 A1).

Thus, it may be concluded that no significant affect will be seen in the Lipstatin production when we use linoleic acid, palmitic acid and leucine as media ingredients as well as feeding solution.

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