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ORIGINAL ARTICLE

Enhanced production of lipstatin from mutant of *Streptomyces toxytricini* and fed-batch strategies under submerged fermentation

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Abstract

Streptomyces toxytricini produces bioactive metabolite recognized as lipstatin and its intermediate orlistat. The main focus of this study is to enhance lipstatin production by strain improvement and precursor feeding. In this study, strain improvement to enhance the production of lipstatin was carried out by different doses (50, 100, 150, 200, and 250 Gy) of gamma radiation and precursors (Linoleic acid, Oleic acid, and L-Leucine). Screening showed that the highest yield of lipstatin (4.58 mg/g) was produced by mutant designated as SRN 7. The production of lipstatin (5.011 mg/g) increased significantly when the medium was supplemented with ratio 1:1.5 (linoleic acid + oleic acid). The addition of 1.5% L-Leucine leads to further increment in the production of lipstatin (5.765 mg/g). The addition of 10% soy flour in the culture medium resulted in the maximum production of lipstatin to 5.886 mg/g.

Keywords Antiobesity drug · Fatty acid precursors · Gamma radiation · Precursors · Lipstatin

Introduction

Obesity is one of the diseases which are raised by modern lifestyle such as lesser physical activity, more energy intake, overeating, stress, reduced metabolic rate, etc. It results in the accumulation of more body fat and associated with many other diseases such as type 2 diabetes, metabolic syndrome, cardiovascular diseases, hypertension, cancer etc. (Després and Lemieux 2006; Kumar and Dubey, 2015; Pischon and Nimptsch 2016). The obesity cases are increasing due to changes in the lifestyle of the people. According to the World Health Organization (WHO) updates, the number of obesity cases has tripled since 1975. According to WHO, 1.9 billion adults (18 years and above) were overweight in 2016 (WHO fact sheet 2018).

Lipids play an important role in maintaining the health of organisms, while excess lipid consumption results in obesity. Thus, control of consumption and digestion of lipids in the intestinal tract is recognized as an effective approach to check obesity (Kumar and Dubey 2015). Lipstatin is one of the most critical antiobesity compounds produced from *Streptomyces toxytricini* (*S. toxytricini*) and *S. virginiae* as a secondary metabolite. It is a potent and irreversible inhibitor of pancreatic lipase (Weibel et al. 1987). It contains one β-lactone ring, and two aliphatic side chains comprising 6 and 13 carbon atoms (Fig. 1). Sidechain carrying 13 carbon atoms also contains two isolated double bonds and one hydroxyl group, which is esterified to *N*-formyl L-Leucine (Hochuli et al. 1987; Kumar et al. 2012; Luthra and Dubey 2012; Kumar and Dubey 2015). Though, the exact biosynthetic mechanism of lipstatin biosynthesis is not well explored. Although, it is reported that the carbon skeleton of lipstatin molecule is biosynthesized via Claisen condensation of two fatty acid precursors, C8 (octanoic acid) and C14 (3-hydroxy-tetradeca-5,8-dienoic acid) (Goese et al. 2001; Kumar and Dubey 2015). These precursors are produced by fatty acid metabolism and on condensation create β-lactone moiety of lipstatin (Eisenreich et al. 1997; Goese et al. 2001).

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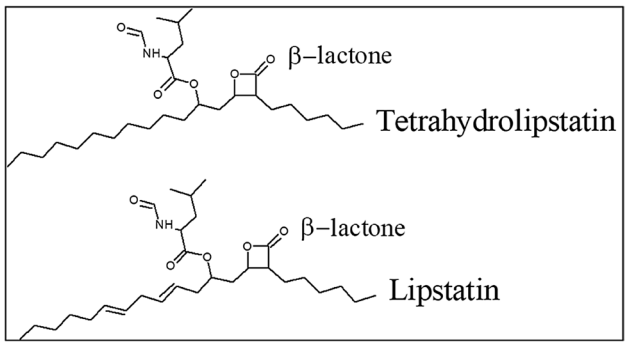

Fig. 1 Chemical structure of lipstatin and tetrahydrolipstatin (THL). Tetrahydrolipstatin (a hydrogenated form of lipstatin) is a pro-drug and active form of lipstatin

It was also demonstrated that the β-lactone ring of lipstatin maintains the chemical properties of lipstatin against pancreatic lipases (Weibel et al. 1987). The reduced form of Lipstatin, i.e., tetrahydrolipstatin (THL) is available in the market by the name of orlistat (Xenical and Alli) (Fig. 1). Orlistat controls the catalytic activity of pancreatic lipase enzymes, which are responsible for breaking down of ingested fat into lipids and these fats remain undigested rather than being absorbed. Orlistat has valuable therapeutic uses that are associated with the control of overweight or obesity such as control or prevention of hyperlipidemia, metabolic syndromes, atherosclerosis, arteriosclerosis, etc. (Kumar and Dubey 2015).

Streptomycetes have been known as authoritative sources of secondary metabolites used in the medical treatment of various diseases including obesity. Strain improvement strategies and optimized culture conditions play a very crucial role in enhancing the production of secondary metabolites in all the fermentation processes. Modification and improvement of the microbial strains are achieved through mutations by subjecting genetic material to a variety of physical or chemical agents called mutagens (Baltz 2001; Barrios-Gonzalez et al. 2003). After inducing a mutation, survivors are randomly picked and tested for their ability to produce metabolite of interest (Luthra et al. 2013a). Investigators have reported the enhanced production of lipstatin from mutated strains of *S. toxytricini* (Kumar and Dubey 2018; Luthra et al. 2013a; Zhu et al. 2014).

The aim of this study was to develop the mutant strains of *S. toxytricini* using *N*-methyl-*N'*-intro-*N*-nitrosoguanidine (NTG) and gamma irradiation to enhance the production of lipstatin. Including this, we also examined the effect of supplementation of fatty acid precursors (linoleic acid and oleic acid), L-Leucine and soy flour during submerged fermentation to further enhance the production of lipstatin.

Materials and methods

Microorganism

NTG-treated mutated strain of *S. toxytricini* that had been stored in glycerol at − 80 °C was resuscitated and used in the study. The cultures had been maintained in sterile Yeast Malt Glucose (YMG) agar slants (malt extract 10 g/L, yeast extract 4 g/L, glucose 4 g/L, $CaCO_3$ 2 g/L and agar 20 g/L) at pH 7.2 as described previously (Kumar and Dubey 2018; Luthra et al. 2013a).

Cultivation medium

S. toxytricini was inoculated in growth medium (malt extract 10 g/L, yeast extract 4 g/L, glucose 4 g/L, $CaCO_3$ 2 g/L) at 7.2 pH. The inoculum was transferred to the seed medium. The composition of seed culture medium was soy flour 10 g/L, glycerol 10 g/L, yeast extract 5 g/L. For the production of lipstatin, the inoculum prepared in seed medium was transferred into fermentation medium containing glycerol 22.5 g/L, soy oil 25 g/L, soy flour 35 g/L, soy lecithin 15 g/L, polypropylene glycol 0.5 g/L. The medium (lab, seed and production) was formulated in 250-mL conical shake flasks containing 30-mL medium (Luthra et al. 2013a). The inoculated culture medium was incubated at 28 °C and 220 r.p.m. The pH of the seed culture medium and fermentation medium was maintained at 6.8.

Mutagenesis using gamma irradiation

For mutagenesis, the spore/cells suspension of NTG mutants of *S. toxytricini* (giving the highest yield of lipstatin, Kumar and Dubey 2018) was prepared in 10^6/mL spores concentration and transferred into vials which were further exposed to gamma irradiator. Different doses of gamma radiation (25, 50, 100, 150, 200 and 250 Gy) from ^{60}Co source were given to the spore suspension of NTG mutant. The strain was irradiated at the Institute of Nuclear Medicine and Allied Sciences (INMAS), New Delhi, India. After mutagenesis, the irradiated spore suspension was serially diluted and plated on the YMG agar plate to get isolated colonies. The plates were incubated at 28 °C for 10 days and the number of colonies on each plate was counted after incubation. Colonies showing less than 1% survival rate (with respect to control) were selected for lipstatin production (Kumar et al. 2015; Kumar and Dubey 2018). The mutants were sub-cultured and maintained at 4 °C.

Screening and selection of hyper-producing mutants

After incubation, the plates were observed for the survivor of mutants. The mutant colonies were selected on the

basis of its morphological characteristics and screened by cultivating each colony into 30 mL of growth medium in 250-mL shake flasks. The shake flasks were incubated at 28 °C and 220 r.p.m. for 24 h. 3% (*v*/*v*) of the inoculum prepared in the growth medium was transferred into seed medium and incubated for 36 h at 28 °C and 220 r.p.m. and then 10% (*v*/*v*) of seed culture was inoculated into the fermentation medium. The fermentation medium was formulated in a 750-mL shake flask containing 80-mL medium. The flasks were run in a shaker at 28 °C and 220 r.p.m. for 11 days. All the experiments were performed in triplicate and the mean of three readings was documented.

Effect of feeding of precursor on lipstatin production

To improve the titer of lipstatin, it is necessary to adjust the feeding with medium components and relevant precursors during the fermentation. Among all the investigated mutants, the highest lipstatin producing mutant was selected and subjected for supplementation of precursors during idiophase for the enhancement in lipstatin production (Kumar and Dubey 2016).

Higher yield of the active metabolite (lipstatin) was optimized by the supplementation of linoleic acid, oleic acid in a different ratio, L-Leucine and soy flour by the following strategies:

Effect of feeding of linoleic acid and oleic acid

The combination of linoleic and oleic acid in various ratios viz. 1:1, 1:1.5, 1:2, 1:2.5 and 1:3, respectively, was fed in fermentation broth. Feeding was done with 0.5-mL solution mentioned above from 48 to 120 h at every 12-h interval during fermentation. The level of linoleic acid was kept in between 0.02 and 0.1 mg/g and oleic acid was maintained in the range of 0.02–0.15 mg/g in the fermentation broth.

Effect of feeding of L-Leucine on lipstatin production

To improve production, it is necessary to optimize the concentration of L-Leucine. Different concentrations of L-Leucine (1%, 1.25%, 1.5%, 1.75% and 2% *w*/*v*) were prepared and sterilized by membrane filtration. 2.0 mL of prepared solution of L-Leucine was added from 48 to 120 h at every 12-h interval. The residual concentration of L-Leucine during fermentation was estimated by HPLC. The addition of mix solution (linoleic acid + oleic acid) and L-Leucine in the fermentation broth was kept in a ratio 1:4.

Effect of nitrogen content feeding

To supply nitrogen content to the culture during fermentation, booster feeding was done with 5–10% defatted toasted soy flour. Soy flour slurry was prepared in demineralized water and sterilized by autoclaving. The fermentation broth was fed at 144 h and 192 h with 0.5-mL soy flour slurry.

Analysis of lipstatin activity in fermented broth

The concentration of lipstatin produced by selected mutants was analyzed at 11 days of cultivation. The concentration of lipstatin produced in the fermentation broth was assessed by HPLC using a C-18 column. Acetone was used to extract the fermented broth. 5-g sample was weighed and dissolved in 30-mL acetone and was sonicated. After 10 min of sonication, the volume was made 50 mL with acetonitrile. The resulting solution (20 µL) was filtered and injected into HPLC (Cecil) for the estimation of lipstatin. EC200/4.6 Nucleosil 100-5 C18 column was used to estimate the activity of lipstatin. The concentration of lipstatin present in the fermented broth was calculated by comparison of peak areas with that of lipstatin standard (Kumar and Dubey 2016; Luthra et al. 2013a).

Results

In the present study, the spore suspension of hyper-lipstatin-producing NTG mutant of *S. toxytricini* was subjected to different doses of gamma irradiation. Screening of highest lipstatin producing strain was done and it was subjected to precursor feeding for enhanced production of lipstatin. Culture plates showing less than 2% survival rate of spores were selected for the isolation of mutants. After inducing the mutation, well-growing ten mutants/survivors were selected on the basis of their morphological characteristics and analyzed for lipstatin production.

Production of lipstatin from selected mutants

10 selected mutants were analyzed for the production of lipstatin at 11 days of cultivation and it was found in the range of 3.52–4.58 mg/g (Fig. 2). Out of these mutants, SRN seven mutant showed the highest bioactive metabolite (lipstatin) production, i.e., 4.58 mg/g; whereas, SRN 3, SRN 4 and SRN 10 produced lipstatin activities at 4.26 mg/g, 4.18 mg/g, and 4.11 mg/g, respectively. Out of these mutants, the lowest amount of lipstatin was produced by SRN 9, i.e., 3.52 mg/g.

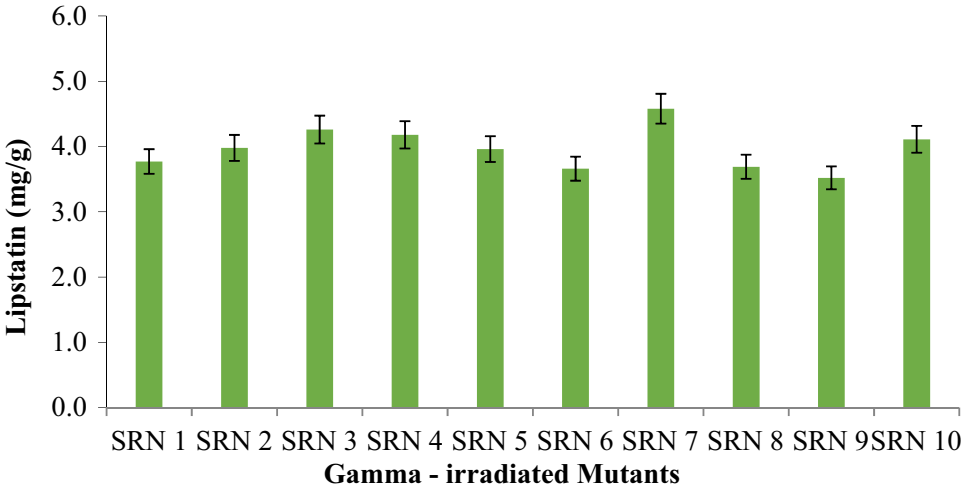

Fig. 2 Study of gamma irradiation on lipstatin yield. Ten colonies (SRN1–SRN10) were selected after exposing the culture to gamma rays and maximum lipstatin yield was obtained from SRN7 mutant

Morphological changes in mutants after gamma irradiation

Morphological changes after gamma irradiation were reported visually as well as microscopically, i.e., under the stereomicroscope. The obtained mutants were flat elevated, granular and had a white peripheral apron. It had many radial furrows and buff in color due to the development of the spores. The mutants were concentric and rugose in appearance. The γ-irradiated mutants were rapidly growing on medium and large in size as 20–28 mm in diameter on maturity as compared to UV- and NTG-treated mutants (Fig. 3).

Effect of feeding of precursors on lipstatin production

The secondary metabolite production is largely affected by adding precursor supplements during the fermentation process. The highest lipstatin-producing mutant (SRN 7) was further employed to investigate the effect of precursor supplementation and nitrogen component feeding during fermentation on lipstatin biosynthesis.

The lipstatin productivity is very much dependent on linoleic and oleic acid concentration as per the biochemical pathway. The biomass was measured in terms of the percentage of mycelial volume (PMV%) to know the concentration/volume of mycelia in the medium. Parameters like pH, PMV% and lipstatin production of the mutant (SRN 7) were estimated from 48 to 264 h. Interestingly, lipstatin production was studied from 72 to 264 h of the incubation period.

Effect of feeding a combination of linoleic acid and oleic acid

Figure 4 depicted the impact of feeding with a varying combination of linoleic acid and oleic acid (L + O) during fermentation on lipstatin yield. In this study, the concentration of oleic acid was extended to investigate its effect on productivity. A significant higher activity of lipstatin (5.011 mg/g)

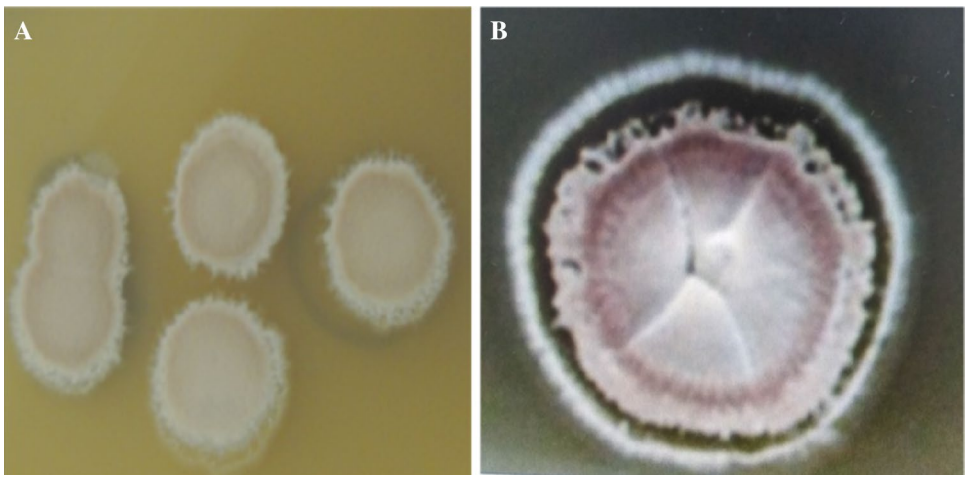

Fig. 3 Changes in mutant colony morphology of *S. toxytricini* (**a**) NTG mutant (**b**) γ-irradiated mutant on agar plates

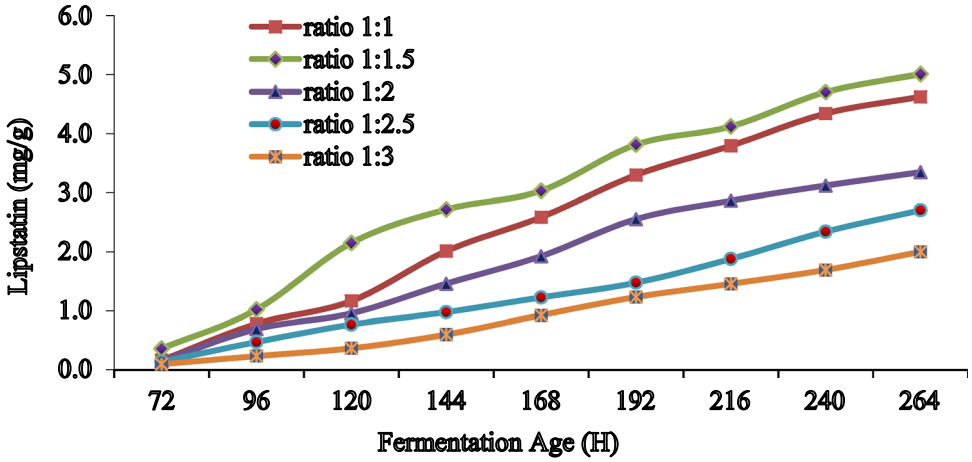

Fig. 4 Study of linoleic and oleic acid ratio on lipstatin yield at shake flask. Mutant SRN7 of *S. toxytricini* was grown till 264 h and get the highest yield of lipstatin at 1:1.1.5 ratio (L + O)

was achieved when supplemented with a ratio 1:1.5 (L + O) and it was followed by 1:1 (4.626 mg/g); whereas, the reduction in productivity of lipstatin was observed with ratios of 1:3 (2.001 mg/g), 1:2.5 (2.705 mg/g) and 1:2 (3.347 mg/g), respectively. On the basis of obtained yield, combination (L + O) of 1:1.5 was selected and employed for further investigation to study the effect of precursors to improve the lipstatin titer.

Effect of feeding of L-Leucine on lipstatin production

The result revealed an effective increment in the biosynthesis of lipstatin after supplementation of a range of L-Leucine in fermentation culture. Figure 5 showed that the effect of various concentrations of L-Leucine on lipstatin production. A remarkable increment in yield was recorded by supplementation of one more precursor in production broth. Out of five different concentrations, the maximum bioactive metabolite production (5.765 mg/g) was obtained when 1.5% L-Leucine was added in the fermentation medium. The supply of 1.75% L-Leucine resulted in comparatively less production of lipstatin (5.519 mg/g). Feeding of fermentation culture with 1.25% (5.406 mg/g), 2.0% (5.221 mg/g) and 1.0% L-Leucine (5.173 mg/g) assessed moderate concentration of lipstatin.

Effect of nitrogen content feeding

Additionally, as an attempt to boost the effect on the amount of lipstatin, soy flour slurry was used as a supplement of nitrogen. 5–10% soy flour slurry was fed to avoid nitrogen (ammonium) regulation. The provision of 5–10% soy flour during fermentation showed that the amount of lipstatin increment was proportional to the increase of soy flour. Maximum lipstatin production (5.885 mg/g) was obtained when the production medium was supplemented with 10% soy flour slurry (Fig. 6). The mycelial volume of the culture medium was effectively boosted after the supplementation of nitrogen content. The viscosity of the fermentation broth was increased with the age of culture.

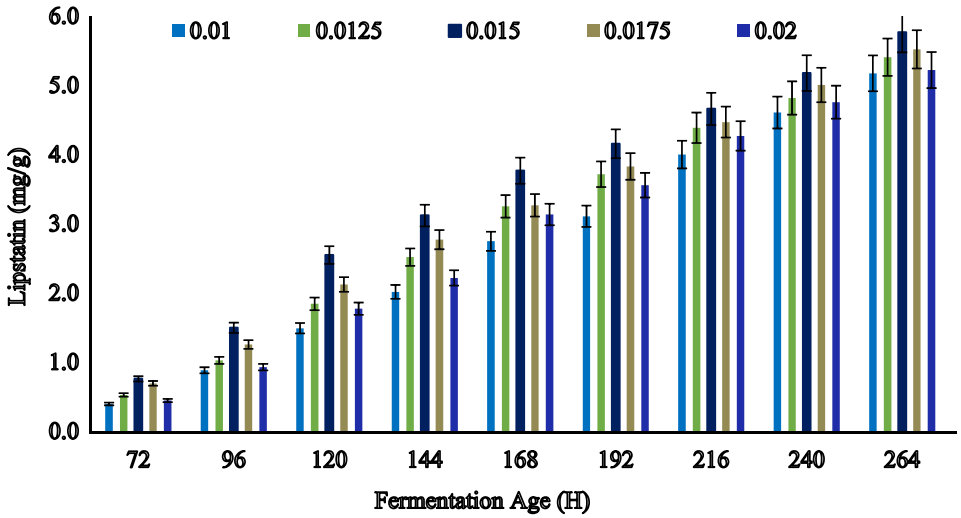

Fig. 5 Study of different concentrations (1, 1.25, 1.5, 1.75 and 2%) of L-Leucine on lipstatin yield at shake flask level. Highest lipstatin production 5.765 mg/g obtained when 1.5% L-Leucine fed into the medium

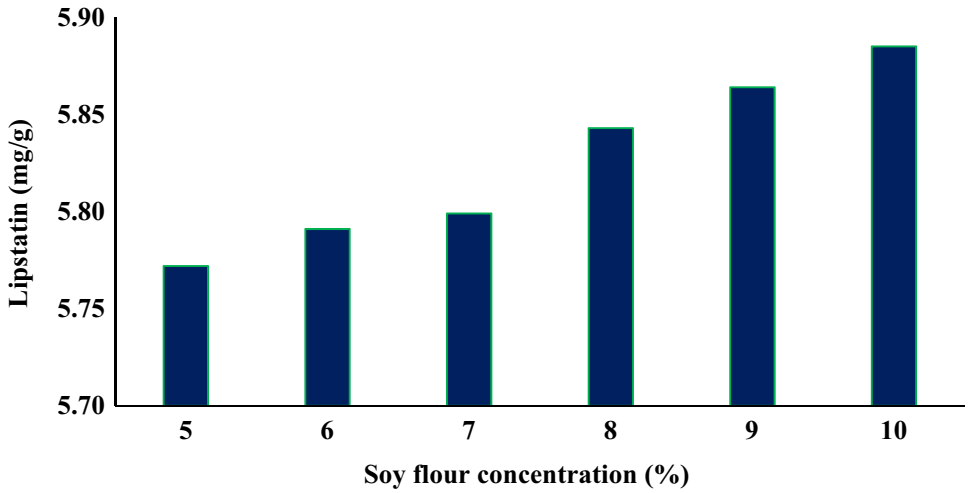

Fig. 6 Study of different concentrations (5, 6, 7, 8, 9 and 10%) of soy flour on lipstatin yield during the fed-batch mode. Maximum lipstatin yield 5.885 mg/g obtained at 10% of soy flour addition

These findings indicate that the addition of proper concentration of precursors and nitrogen supplementation in the fermentation medium is essential for an effective increment of lipstatin biosynthesis. Table 1 presented herein showed that the maximum concentration of lipstatin, 5.885 mg/g, was achieved at 264 h with pH 7.01 and PMV 86% after supplementation of precursors and provision of nutrient supplement in production medium.

Discussion

The metabolite-producing microbial strain can be considered as the heart of a fermentation industry and the over-production of the fermentation process is strongly affected by the strain improvement. The development of strain for over-production of metabolites needs the understanding of physiology, pathway regulation and the design of effective screening procedures (Parekh et al. 2000). The common approaches to enhance the production of secondary metabolites are strain improvement by genetic engineering, metabolic engineering, mutation, culture medium formulations, and optimization of culture conditions (Kumar and Dubey 2015). Mutagenesis is an important tool to improve the strain by a genetic modification that can be done by inducing mutations in the microorganisms and selecting natural variants. Industries have used a mutagenesis approach for strain and productivity improvement (Olano et al. 2008). To the best of our knowledge, the biosynthetic mechanism for the production of lipstatin is not well explored. Thus, in the absence of information about genes involved in the biosynthetic pathway, random mutagenesis using physical or chemical mutagens is a recommended approach to develop strains having high productivity (Barrios-Gonzalez et al. 2003).

Hopwood et al. (1985) suggested that 99.9% killing is best suited for strain improvement as the smaller number of survivors in the plate would have undergone repeated or multiple mutations which may lead to enhancement in the productivity of the culture. Including other investigators also have suggested screening of high productivity strains from

Table 1 Analysis of linoleic acid, oleic acid and L-Leucine in fermentation media with other process parameters

Age (h)	pH	PMV%	Linoleic acid (mg/g)	Oleic acid (mg/g)	L-Leucine (mg/g)	Lipstatin activity (mg/g)
48	6.99	40	0.354	0.468	0.037	–
72	7.05	45	0.358	0.408	0.047	0.757
96	6.98	52	0.367	0.415	0.058	1.496
120	7.02	58	0.345	0.444	0.033	2.547
144	6.98	63		–	–	3.262
168	6.96	68	–	–	–	3.844
192	7.06	68	–	–	–	4.587
216	7.06	73	–	–	–	4.968
240	6.92	77	–	–	–	5.439
264	7.01	86	–	–	–	5.885

low surviving culture plates which were exposed to mutagens (Kumar et al. 2015). Thus, in this study, the culture plates showing less than 2% of survival were screened for lipstatin production.

In our previous study, maximum lipstatin produced was 2.88 mg/g titer by *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (NTG)-mutated strain (Luthra et al. 2013a), that was further maximized to 3.29 mg/g by medium optimization using full factorial design (Luthra et al. 2013b). In the study presented herein, hyper-lipstatin-producing NTG mutant was subjected to gamma irradiation to enhance the product yield. Mutagenesis caused by gamma rays involves base substitutions (G:C–A:T transitions) during DNA replication which are caused by adenine mispairing with cytosine product deaminated by irradiation (Tindall et al. 1988). The combined exposure of NTG and γ-radiation had a positive effect on the titer of lipstatin. The screened mutant SRN 7 showed the enhanced production of lipstatin at 4.58 mg/g. After mutagenesis, it was found that the mutant colonies of *S. toxytricini* were produced more spores and attained comparatively large in size than NTG mutant. Similarly, the use of gamma radiation for strain improvement has been reported by many researchers for *Saccharomyces cerevisiae* to enhance the ethanol yield (Jang et al. 2014), *Aspergillus niger* to enhance production of enzyme (Awan et al. 2011), *Serratia marcescens* to enhance the production of prodigiosin (Elkenawy et al. 2017), production of chitinase by *Bacillus thuringiensis* (Gomaa and El-Mahdy 2018), etc.

The production of secondary metabolites from microorganisms is affected by various parameters such as availability of nutrients, oxygen supply, incubation temperature, pH of medium, etc. (Olano et al. 2008). Microorganisms grow and multiply in growth medium which supplies nutrients required by the organism. The enrichment of culture is most important for the proper growth of microorganisms. It is necessary to provide carbon sources, nitrogen sources, minerals for growth and multiplication of microorganisms in the culture medium. The production of specific metabolites in high titer could be possible by maintaining proper control and regulation at different levels via transport and metabolism of extracellular nutrients, precursor formation and accumulation of intermediates (Rokem et al. 2007).

As per the biosynthetic pathway, tetradeca-5,8-dienoyl CoA and hexylmalonic acid are formed by the metabolization of linoleic acid and octanoic acid which undergoes Claisen condensation and forms 3-oxo intermediate (Kumar and Dubey 2015). It goes through hydroxylation which reacts with *N*-formyl L-Leucine to synthesize lipstatin. In brief, linoleic acid and octanoic acid form the backbone of the final molecule; whereas, leucine is incorporated to synthesize lipstatin. Thus, supplementation of these compounds is associated with an enhancement in the production of lipstatin. In this study, it was found that the supplementation of precursors in the culture medium significantly enhanced the fermentation yield. An effective increment in the production of lipstatin was achieved by adding a combination of linoleic acid and oleic acid in a culture medium which was further maximized by the addition of L-Leucine during fermentation. It was observed that supplementation of linoleic acid and oleic acid (1:1.5) enhanced the production of lipstatin to 5.011 mg/g. The production of lipstatin was further enhanced to 5.765 mg/g by supplementation of 1.5% L-Leucine and it was enhanced to 5.885 mg/g by supplementation of 10% soy flour slurry. Thus, total enhancement in the production of lipstatin was from 3.29 (NTG Mutant) to 5.885 mg/g by gamma-ray mutagenesis and precursor feeding. The similar effects of precursor feeding are reported by previous investigators. European patent (EP 0,803,567) describes a fermentation process of lipstatin production with the use of precursors such as linoleic acid, caprylic acid, and *N*-formyl L-Leucine or Leucine. One more patent (US 8,501,444 B2) describes lipstatin production by combinatorial feeding of linoleic acid and omega-9 fatty acids, preferably oleic acid. In our previous study (Luthra and Dubey 2012), it had been reported that there was no significant impact of L-Leucine, linoleic acid and palmitic acid as a medium ingredient on lipstatin production with strain *S. toxytricini.*

It has been reported that oleic acid exhibited less toxicity than linoleic acid; hence, their proper combination provides the fatty acid precursors for growth of the microorganism (Tiwari et al. 2013). The yield of fermentation is improved by maintaining the residual concentration of free fatty acid. The provision of organic nitrogen sources such as soy flour exhibited a positive effect on lipstatin production. But supplementation of soy flour higher than 10% was not investigated due to viscosity of the slurry. It has been reported that organic nitrogen sources are rich in amino acids and peptides, which significantly affect carbon source uptake in actinomycetes (Hoskisson et al. 2003).

Furthermore, the pH of the culture is a profoundly environmental factor that plays a major role in product formation. It has been reported by Weibel et al. (1987) that the stability of lipstatin was affected by the pH of the culture medium. Lipstatin becomes inactive at alkaline pH due to the opening of the β-lactone ring. It may be suggested in this study that strain development by mutagenesis and supplementation of fatty acid precursors, L-Leucine, and maintenance of pH play a key role in lipstatin biosynthesis. This study might be more significant when random mutagenesis will be correlated with study at the molecular level to analyze the change in genes (DNA profile) associated with lipstatin biosynthesis by different approaches such as Inter-Simple Sequence Repeat (ISSR), Random Amplified Polymorphic DNA (RAPD), etc. (Gomaa and El-Mahdy 2018).

Conclusion

In this study, strain improvement of *S. toxytricini* was performed by mutagenesis. The effect of combined exposure of NTG and gamma radiation on bioactive metabolite formation was found positively related to NTG mutant. A mutant designated as SRN 7, with maximum lipstatin productivity 4.58 mg/g was obtained which produced 39% higher lipstatin than the NTG mutant. The isolation and screening of mutants were carried out on the basis of its survival and morphological characteristics. Further enhancement in lipstatin production was achieved by precursor supplement and nitrogen content addition. Supplementation of mix solution of linoleic acid and oleic acid in ratio 1:1.5 has effectively improved the product formation. A significant enhancement in lipstatin biosynthesis (5.886 mg/g) was obtained by the addition of 1.5% L-Leucine and 10% soy flour slurry in the culture medium. The pH of the fermentation broth was controlled to improve the culture condition and to obtain maximum mycelial volume. A total of 78.9% increment in lipstatin productivity was achieved by employing mutagenesis and precursor feeding along with nitrogen content supply.

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Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflicts of interest.

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