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ORIGINAL ARTICLE

Implication of mutagenesis and precursor supplementation towards the enhancement of lipstatin (an antiobesity agent) biosynthesis through submerged fermentation using *Streptomyces toxytricini*

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Abstract
In the present study, lipstatin production was studied from different mutants of *Streptomyces toxytricini* which were developed using ultraviolet radiation (exposure time 30 s, 1, 2, 5 and 10 min), ethyl methane sulfonte, methyl methane sulfonate (MMS) and *N*-methyl-*N*′-intro-*N*-nitrosoguanidine (NTG) treatments (50, 100, 200, 500, 1000 µM, respectively). Highest yielding mutants were provided precursor supplementation of citric acid, thiamine and biotin (each 1 g/L) at idiophase for further enhancement in the production of lipstatin. Screened mutants produced biomass in the range of 5.8–7.16 g/L which were lesser than control. Screened mutants also exhibited pellet morphology in submerged culture. Out of these mutants, NTG8 mutant produced highest amount of lipstatin (1383.25 mg/L) with 9.606 mg/L/h productivity. Precursor supplementation to this mutant further increased the production to 2387.81 mg/L. Mutant was validated in 5 L bioreactor and lipstatin production was enhanced to 2519.34 mg/L.

Keywords Obesity · Lipstatin · Orlistat · *Streptomyces toxytricini* · Mutagenesis · Precursor supplementation

Introduction

Lipstatin is a proven inhibitor of catalytic activity of pancreatic lipases (Weibel et al. 1987; Kumar and Dubey 2015, 2016). It is hydrogenated into tetra-hydro-lipstatin (THL) which also possesses pancreatic lipase inhibitory properties. THL is used to synthesize Orlistat which is a FDA approved medicine for obesity treatment (Rodgers et al. 2012).

Natural producers of lipstatin are *Streptomyces toxytricini* (*S. toxytricini*) and *S. virginiae*, but only *S. toxytricini* is explored for the production of lipstatin at commercial scale. To enhance the lipstatin production from *S. toxytricini*, researchers performed optimization of culture conditions and medium modifications which enhanced production of lipstatin to 3290 mg/L (Luthra et al. 2013b), 4208 mg/L (Zhu et al. 2014) and 2262.63 mg/L (Kumar and Dubey 2016). Moreover, it was also suggested that further improvement in lipstatin production may be performed by metabolic engineering and strain improvement approaches (Kumar and Dubey 2015).

It is evident that biosynthesis of metabolites is under the control of genetic machinery thus strain development by alteration in expression of target gene might be promising approach to enhance the production of metabolites (Baltz 2001). Strain development by mutagenesis may be performed by two methods; classical (random) mutagenesis and targeted mutagenesis methods (Barrios-Gonzalez et al. 2003) depending on information available about genetic machinery and bottlenecks involved in the metabolite biosynthesis. In absence of this information, random mutagenesis is recommended for strain development (Barrios-Gonzalez et al. 2003) which is performed by physical and chemical mutagenesis through UV radiation, ethyl methanesulfonte (EMS), methyl methanesulfonate (MMS) and *N*-methyl-*N*′-intro-*N*-nitrosoguanidine (NTG), etc. (Miller 1983; Baltz and Stonesifer 1985; Stonesifer and Baltz 1985; Baltz 1998).

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It is also suggested that mutagenesis may possibly alter the growth conditions thus optimization of growth conditions for screened mutants must be performed (Luthra et al. 2013a).

Random mutagenesis of microorganisms has been commonly used to enhance the production of secondary metabolites e.g. endoglucanase and β-glucosidase from *S. griseoaurantiacus* through UV radiation (Kumar 2015), avermectin from *S. avermitilis* through UV mutation and NTG mutagenesis (Gao et al. 2010), mannitol production using UV, EMS and NTG mutagenesis from *Candida magnolia* (Savergave et al. 2013). In one of the literature work, random mutagenesis has improved the metabolite production by 11.63 fold. (Mo et al. 2013). To enhance the production of lipstatin by random mutagenesis of *S. toxytricini*; Luthra et al. (2013a) have reported the 2.34 and 2.88 mg/g production of lipstatin through UV and NTG mutagenesis, respectively. Including this, Zhu et al. (2014) have reported enhancement in lipstatin production through optimization of culture conditions and precursor supplementation to mutated strain of *S. toxytricini*.

Present study describes the development of mutant strain of *S. toxytricini* through UV, EMS, MMS and NTG induced mutagenesis and analysis for enhancement in lipstatin production. Including this, metabolic precursors were added at idiophase to further enhance the production of lipstatin at shake flask level. Moreover, the process was scaled up in 5 L bioreactor using mutant giving highest production of lipstatin.

Materials and methods

Streptomyces toxytricini was obtained from Agricultural Research Service (NRRL) were inoculated on semisolid growth medium (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 7.2 pH. All the experiments were conducted at shake flask level and validated on 5 L bioreactor (Sartorius, Germany). All the experiments were conducted in triplicate.

Mutagenesis experiments

Ultraviolet (UV) mutagenesis

In this experiment, 10^6 spores/ml of *S. toxytricini* were inoculated on Petri plate and kept for UV exposure (Luthra et al. 2013a; Kumar 2015). UV exposure (Philips; UV-C radiation, 4.9 W) was given at constant distance (30 cm) for 30 s, 1, 2, 5 and 10 min on different freshly inoculated Petri plates and control (without UV exposure). After UV exposure these plates were wrapped in aluminium foil and were kept in dark for incubation for overnight. Incubation of these plates was done at 27.5 °C.

Chemical mutagenesis

Stock solution of EMS, MMS and NTG (1 mM each) were prepared in 0.2 mM phosphate buffer (pH 7) and filtered, respectively. Spores/cells of *S. toxytricini* were harvested and diluted to 10^6/mL spores concentration in phosphate buffer. Different dilutions of EMS, MMS and NTG (50, 100, 200, 500, 1000 µM) and added to 1 mL of spore suspension and kept for 15 min incubation at 27 °C. To terminate the mutagenesis 0.16 M (1:10 ratio) sodium thiosulfate (Stonesifer and Baltz 1985; Malik et al. 2002) was mixed and cell suspension was centrifuged at 5000 rpm for 5 min. Pellet of cells was resuspended in phosphate buffer and again centrifuged 5000 rpm for 5 min. Resuspension of cells in phosphate buffer was repeated for three times and finally cells were resuspended 500 µL of sterilized double distilled water and transferred to Petri plates containing growth medium and kept for incubation at 27.5 °C till visible colonies were appeared on culture plates.

Screening of mutants

Survived bacterial colonies after UV exposure and chemical mutagenesis were analyzed for change in the production of lipstatin. The mutants were randomly selected on the basis of colony morphology from Petri plates showing least survival (less than 2%; Kumar et al. 2015) and sub-cultured on fresh Petri plates. The morphology and other parameters (size of colony structure of pellet, production of biomass and lipstatin) of screened mutants were studied. The mutants showing highest production of lipstatin were subjected to precursor supplementation study.

Culture medium

Process from growth of *S. toxytricini* to fermentation was conducted in different culture medium of varying chemical composition. There media were named as growth medium, preseed medium, seed medium and production (fermentation) medium.

Growth medium

Streptomyces toxytricini was grown and maintained on sterilized Petri plates containing growth medium. Cultures were inoculated into 250 mL Erlenmeyer flask containing 50 mL broth medium (malt extract 10 g/L, yeast extract 4 g/L, glucose 4 g/L, $CaCO_3$ 2 g/L) and incubated at 27.5 °C for 24 h in incubator shaker at 220 rpm.

Pre-seed medium and seed medium

Bacteria grown in growth medium were used for inoculation (2%) of 50 mL pre-seed (inoculum) medium (soy meal 10 g/L, glycerol 10 g/L and yeast extract 5 g/L at pH 7) and incubated in incubator shaker at 27.5 °C, 220 rpm for 24 h. Bacteria grown in pre-seed medium were used for inoculation (2%) of 50 mL modified seed medium (soy flour 25 g/L, corn starch 25 g/L, dextrin 30 g/L, $(NH_4)_2SO_4$ 2 g/L, CaCO3 6 g/L and soy oil 8 mL/L at pH 6.8) and incubated in incubator shaker at 27.5 °C, 220 rpm for 24 h.

Production (fermentation) medium

Bacteria grown in seed medium were used for the inoculation (2%) of 50 mL production medium (soy meal 40 g/L, glycerol 25 g/L, soy lecithin 17.5 g/L, Poly propyl glycol 0.25 g/L, soy oil 30 mL/L at pH 6.5). It was incubated for 144 h at 27.5 °C at 220 rpm. Bioreactor conditions were maintained as the pH at 6.8, fermentation period for 144 h, incubation temperature at 27.5 °C, dissolved oxygen level at 30–40%, rpm at 250 and airflow at 0.6 VVM.

In the above media, the pH of the growth medium, pre-seed medium and seed medium was adjusted at 7.2, 7.0 and 6.8, respectively. The alkaline pH causes inactivation of lipstatin thus pH of fermentation medium was adjusted at 6.5 and pH of different was decreased subsequently.

Supplementation of precursors to fermentation medium

Mutant strains of *S. toxytricini* showing highest production of lipstatin were used for precursor supplementation. Metabolic precursors such as citric acid (1 g/L), thiamine (1 g/L), and biotin (1 g/L) were supplemented after 96 h (idiophase) of incubation of fermentation medium (Zhu et al. 2014; Kumar and Dubey 2016). In this study, one precursor was supplemented at one time and change in lipstatin production was analyzed.

Analysis of lipstatin

High performance liquid chromatography (HPLC; Younglin, Korea) equipped with C-18 column was used for quantitative analysis of lipstatin. For this purpose 1 mL of broth was mixed with 9 mL acetone, sonicated and left overnight at room temperature. Extract was filtered using 0.22 µm Nylon filter. 20 µL of this filtered solution was injected into column. Flow rate of mobile phase comprising acetonitrile and (0.1% orthophosphoric acid) water in a ratio of 80:20 v/v was adjusted at 1.5 mL per min. Analysis was done by UV–Vis detector at 210 nm. Concentrations of lipstatin were calculated by comparison of peak area of standard lipstatin (Luthra et al. 2013a; Zhu et al. 2014; Kumar and Dubey 2016).

Results

Survival of bacterial colonies after mutagenesis

It was observed that increase in duration of UV radiation significantly decreased the number of viable bacterial colonies. Thus, UV exposure for 10 min resulted less than 1% of cell survival and only 20 bacterial colonies were visible on culture plate. Out of these, 10 colonies were separated from each other which were used for further analysis.

Similarly chemical mutagenesis by EMS, MMS and NTG also exhibited reduction in survival of bacterial cells. Mutants produced using 1000 µM concentration of each mutagen showed lowest cell survival (about 2%). These mutants were selected randomly and analyzed for the production of lipstatin. Ten mutants showing higher production of lipstatin from each mutagen were grown on Petri plates and investigated for morphology, biomass accumulation, pellet formation and precursor supplementation study.

Morphology of bacterial colonies on agar plate

In this experiment, the morphology of all the screened mutants was analyzed with respect to wild type (Table 1). It was observed that all wild type colonies of *S. toxytricini* were peach in color, elevated in centre and having smooth surface. Though morphology of all mutants produced by chemical mutagenesis was not changed from wild type but bacterial colonies of UV mutant were peach in color, flat and surface was not smooth. It was also noticed that UV3 and UV4 made some type chemical secretion into Petri plate.

Production of biomass

In this study, the production of biomass from each screened mutant was analyzed (Fig. 1). Out of screened UV mutants, production of biomass after overnight incubation was highest (7.16 g/L) in UV1 mutant which was 5.28% lesser than wild type (7.57 g/L). Lowest biomass (5.97 g/L) was produced by mutant UV4 and UV6 which was 21.15% lesser than wild type. Out of screened EMS mutants, highest biomass (6.93 g/L) was produced by mutant EMS6 which was 8.37% lesser than wild type and lowest biomass (6.06 g/L) was produced by mutant EMS2 which was 25.11% lesser than wild type. Out of MMS mutants, highest biomass (7.1 g/L) was produced by mutant MMS9 which was 6.18% lesser than wild type while lowest biomass (5.8 g/L) was produced by mutant MMS1 which was 23% lesser than wild type. Out of NTG mutants, highest biomass (7.06 g/L) was

Table 1 Color and morphology of different screened mutants produced by physical and chemical mutagenesis

Mutant	Colour and morphology of bacterial mutants			
	UV	EMS	MMS	NTG
1	Peach, circular, flat, smooth edges	Peach, circular, flat, edges not smooth	Peach, round, flat, edges are not smooth	Peach, round, flat, smooth edges,
2	Peach, round, edges not smooth,	Peach, round and cylindrical, flat, edges not smooth	Peach, circular, flat, smooth edges	Peach, round, flat, smooth edges
3	Peach circular, edges are not smooth	Peach, round, flat, smooth edges	Peach, round, flat, edges are not smooth	Peach, round, flat, smooth edges
4	Peach, circular, flat, cylindrical, edges are not smooth	Peach, circular, flat, edges are not smooth	Peach, circular, flat, smooth edges	Peach, circular, flat, edges are not smooth
5	Peach, round, smooth edges	Peach, circular, flat, edges are not smooth	Peach, circular, flat, edges are not smooth	Peach, circular, flat, smooth edges
6	Peach, flat, round and cylindrical, edges are not smooth	Peach, circular, cylindrical, flat, smooth edges	Peach, circular, flat, edges are not smooth	Peach, circular, flat, edges are not smooth
7	Peach, round, edges are not smooth	Peach, round, flat, edges are not smooth	Peach, round, flat, edges are not smooth	Peach, round, flat, edges are not smooth
8	Peach, circular, flat, smooth edges	Peach, round, flat, edges are not smooth	Peach, circular, flat, edges are not smooth	Peach, round, flat, edges are not smooth
9	Peach, circular, flat, edges are not smooth	Peach, round, flat, edges are not smooth	Peach, oval, flat, edges are not smooth	Peach, circular, flat, edges are not smooth
10	Peach, oval, flat, smooth edges	Peach, circular, flat, edges are not smooth	Peach, round, flat, edges are not smooth	Peach, round, flat, smooth edges

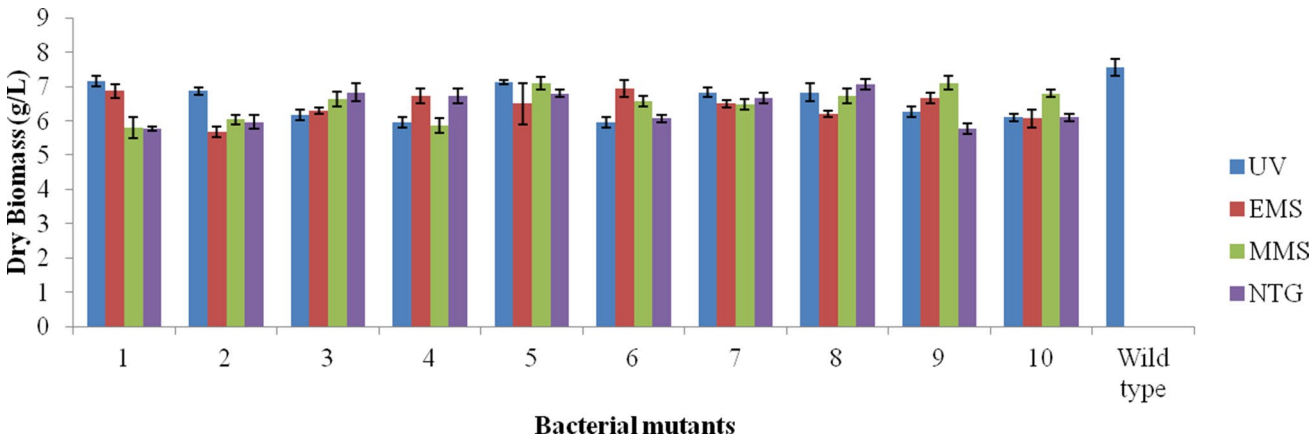

Fig. 1 Comparison of dry biomass produced from different mutants screened after UV, EMS, MMS and NTG treatment in growth medium. Biomass of isolated and screened mutant is compared with wild type. In each group of mutant, UV mutant (UV-1) produced 7.16 g/L biomass, EMS mutant (EMS-6) produced 6.93 g/L biomass, MMS mutant (MMS-9) produced 7.1 g/L biomass and NTG mutant (NTG-8) produced 7.06 g/L dry biomass while wild type strain produced 7.56 g/L biomass. Experiments were carried in triplicate and bars represent mean value ± SD

produced by mutant NTG8 which was 6.61% lesser than wild type while lowest biomass (5.76 g/L) was produced by mutant NTG9 which was 23.79% lesser than wild type. It was also noticed that biomass of all the screened mutants was between approximately 6–7 g/L.

Pellet formation in submerged growth

During submerged growth, *S. toxytricini* exhibited pellet morphology resulted by interwoven hyphae (Kumar and Dubey 2017). At microscopic level it was observed that pellet morphology was changed of all the mutants but pellet formation was not inhibited in any of the screened mutant. It was observed that pellets generated by wild type cells were having well grown hairy mycelia at surface, with pellet size

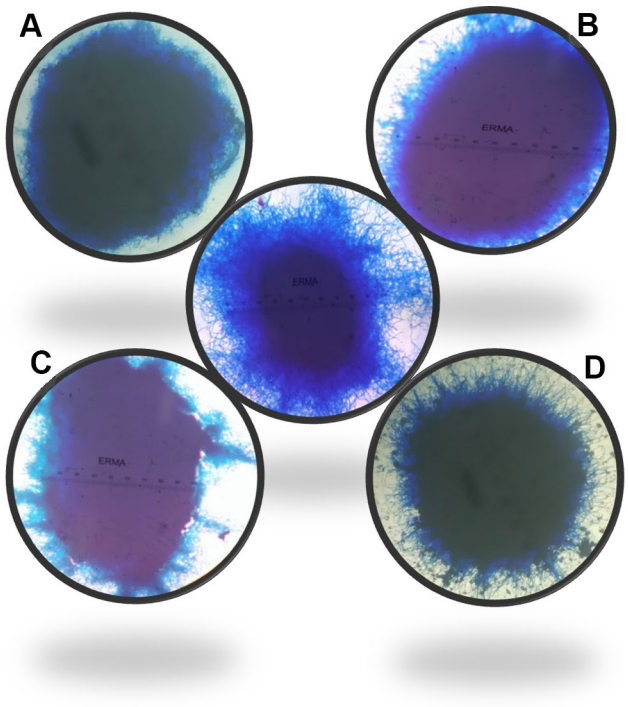

Fig. 2 Comparison of pellet morphology of different mutants. A-pellet of UV mutant, B-Pellet of EMS mutant, C-Pellet of MMS mutant, D-pellet of NTG mutant. Pellet of wild type is at centre. As compared to wild type, outer surface of pellet has less developed mycelia

in the range of 30 µM to 2 mm. While pellets generated by mutants were having less developed mycelia than wild type but their size was similar to wild type (Fig. 2).

Production of lipstatin

Screened mutants were analysed for production of lipstatin in fermentation medium and the production was compared with wild type (Fig. 3). It was observed that in optimized conditions wild type strain of *S. toxytricini* produced 971.34 mg/L of lipstatin. It was analysed that productivity of lipstatin was changed after mutagenesis. Out of screened UV mutants, highest amount of lipstatin production was 1309.92 mg/L (34.86% higher than wild type) from mutant UV8. Out of screened EMS mutants, highest amount of lipstatin production was 1333.32 mg/L (37.27% higher than wild type) from mutant EMS1. Out of screened MMS mutants, highest lipstatin production was 1202.76 mg/L (23.82% higher than wild type) by mutant MMS10. Out of screened NTG mutants highest production of lipstatin was 1383.25 (42.41% higher than wild type) in mutant NTG8. Thus, in this study it was observed that mutant produced by NTG mutagenesis produced highest amount of lipstatin.

Supplementation of precursors

Precursors (citric acid, biotin and thiamine) have been found associated with lipstatin biosynthesis (Kumar and Dubey 2016). Thus, in this experiment supplementation of citric acid, biotin and thiamine was done in optimized production medium to mutants (UV8, EMS1, MMS10, and NTG8) and enhancement in lipstatin production was analyzed.

Citric acid supplementation in UV8 mutant enhanced production of lipstatin to 1457.1 mg/L, which was 11.13%

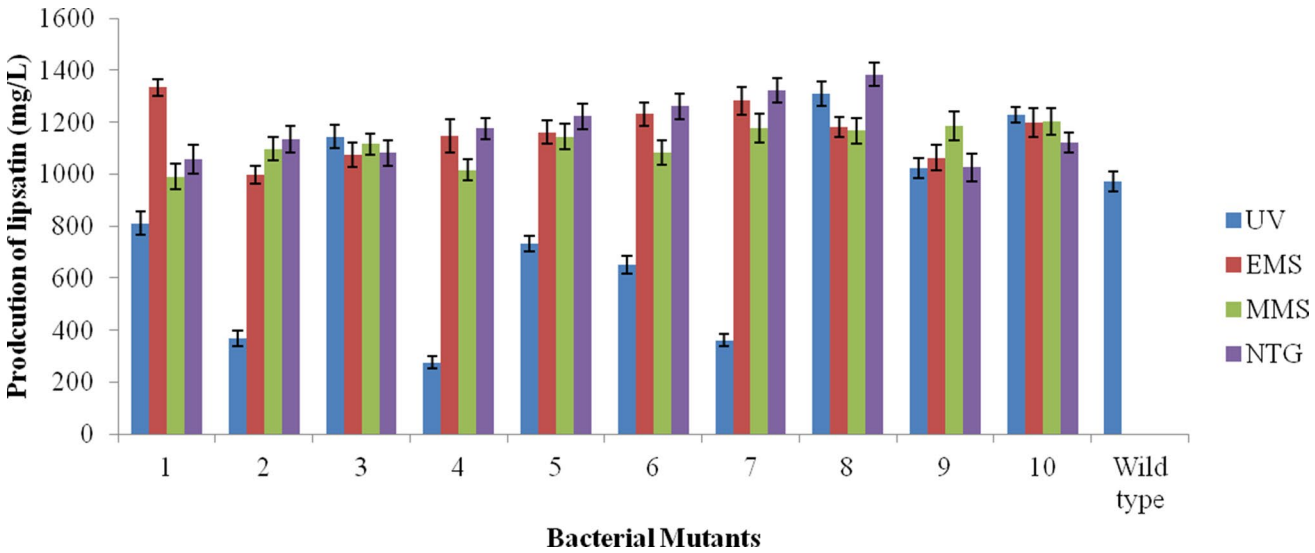

Fig. 3 Production of lipstatin by fermentation from different isolated mutants. Different mutant produced different amount of lipstatin. Mutant produced by UV radiation also caused reduction in the production of lipstatin. In mutants (produced by EMS, MMS and NTG) giving higher lipstatin titre than wild type were used in this study. UV mutant (UV-8) produced maximum lipstatin (1309.92 mg/L) in its group. EMS mutant (EMS-1) produced maximum lipstatin (1333.32 mg/L) in its group. MMS mutant (MMS-10) produced maximum lipstatin (1202.76 mg/L) in its group. NTG mutant (NTG-8) showed maximum lipstatin production (1383.25 mg/L) in its group. Experiments were carried in triplicate and bars represent mean value ± SD

higher from unsupplemented UV8 mutant. In EMS1 mutant, citric acid supplementation enhanced the production of lipstatin to 1571.43 mg/L, which was 17% higher than unsupplemented EMS1 mutant. In MMS10 mutant, citric acid supplementation enhanced the production of lipstatin to 1344.36 mg/L, which was 10.81% higher than unsupplemented MMS10 mutant. In NTG8 mutant, citric acid supplementation enhanced the production of lipstatin to 1630.98 mg/L, which was 18.09% higher from unsupplemented NTG8 mutant.

Thiamine supplementation in UV8 mutant enhanced the production of lipstatin by 14.58% (1502.26 mg/L), in EMS1 mutant it enhanced the production of lipstatin by 22.69% (1647.96 mg/L), in MMS10 mutant it enhanced the production of lipstatin by 15.76% (1404.45 mg/L), and in NTG8 mutants it enhanced the production of lipstatin by 25.90% (1741.52 mg/L).

Biotin supplementation in UV8 mutant enhanced the production of lipstatin by 17.28% (1537.69 mg/L), in EMS1 mutants it enhanced the production of lipstatin by 21.34% (1633.75 mg/L), in MMS10 mutants it enhanced the production of lipstatin by 19.79% (1453.35 mg/L), in NTG8 treated mutants it enhanced the production of lipstatin by 24% (1712.48 mg/L) (Fig. 4).

It was observed that NTG8 mutant showed highest enhancement in lipstatin by precursor supplementation. Further supplementation of citric acid, thiamine and biotin was made simultaneously to NTG8 mutant and production of lipstatin was enhanced to 2387.81 mg/L.

Scale up of process in bioreactor

All the experiments of lipstatin production by mutants and medium formulations were conducted at shake flask level. Lipstatin production was analyzed in 5 L bioreactor using NTG8 mutant (Fig. 5). It was observed that precursor supplementation and fermentation in bioreactor enhanced the production of lipstatin to 2519.34 mg/L which was 5.5% higher than shake flask level. In this study over all 2.59 fold enhancement in production of lipstatin was observed.

Discussion

It is estimated that market of antiobesity medicines will hike to $2.6 billion up to 2019 and orlistat will dominate the market (Kumar and Dubey 2015). Increasing occurrences of obesity and high cost treatment are exacerbating the situation.

Researchers have successfully reported the enhancement in the production of lipstatin by optimization of culture conditions and medium formulations (Luthra et al. 2013b; Zhu et al. 2014; Kumar and Dubey 2016) and other metabolites (Dhingra et al. 2014). Further improvement in yield requires to play with genetic machinery engaged in lipstatin biosynthesis. Moreover, due to little understanding about the biosynthetic pathway for lipstatin, metabolic engineering to enhance the yield is not feasible. Therefore, investigators are keen to develop high yielding strains through random mutagenesis using physical and chemical mutagens.

Different mechanisms are deduced for mutagenesis such as UV radiations cause thymine dimerization and GC to

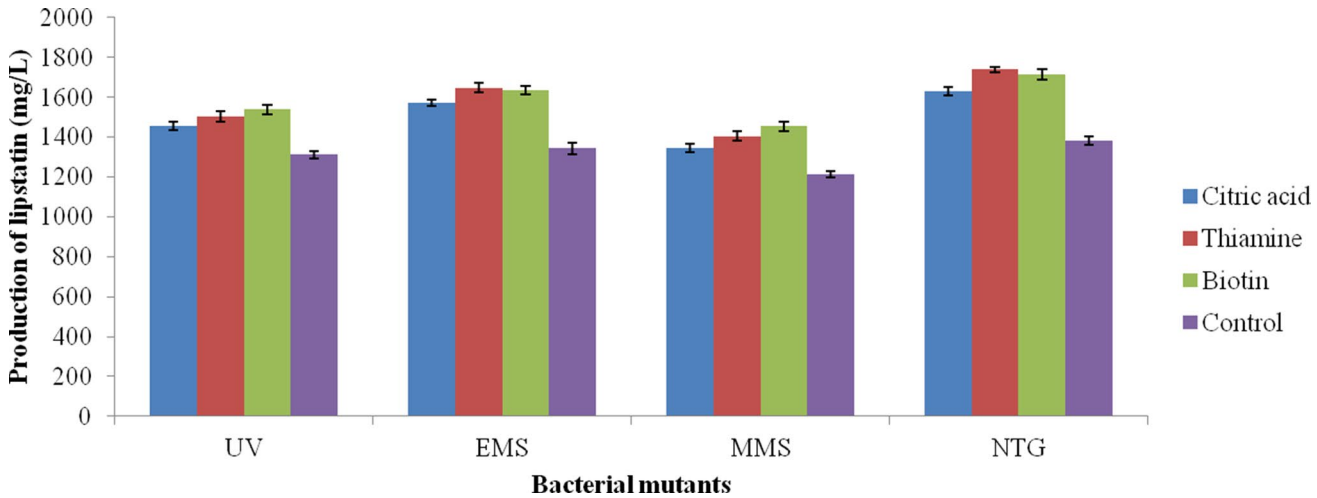

Fig. 4 Impact on lipstatin production by supplementation of precursors (citric acid, thiamine, and biotin) after 96 h of incubation is demonstrated. All the precursors showed positive impact on lipstatin production. Citric acid induced highest production of lipstatin in NTG mutant (1630.98 mg/L). Thiamine enhanced the highest production of lipstatin in NTG (1738.75 mg/L). Biotin enhanced the highest lipstatin production in NTG (1712.48 mg/L). Experiments were carried in triplicate and bars represent mean value ± SD

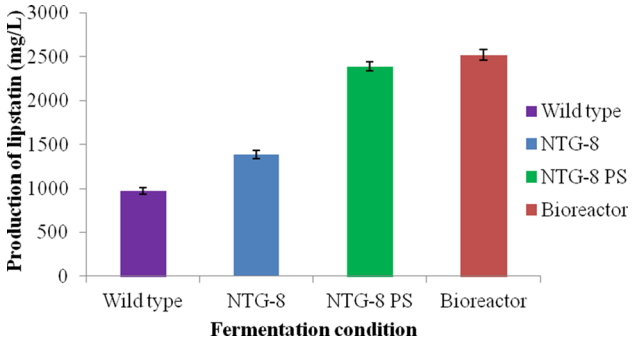

Fig. 5 Validation of shake flask level experiment at 5 L bioreactor level. At shake flask level NTG-8 mutant produced 1383.25 mg/L lipstatin and precursor supplemented (PS; citric acid + thiamine + biotin at 1 g/L each) NTG-8 produced 2387.81 mg/L lipstatin. Under controlled conditions at 5 L bioreactor, lipstatin production was further enhanced to 2519.34 mg/L. Experiments were carried in triplicate and bars represent mean value ± SD

AT transition (Ikehata and Ono 2011), chemical mutagens (EMS, MMS and NTG) cause alkylation, mispairing and GC to AT transition (Coulondre and Miller 1977, Miller 1983).

In this study, it was observed that UV induced mutants of *S. toxytricini* shown change in colony morphology. It was reported that morphology of bacterial cells is under the control of peptidoglycan biosynthesis and mutation in respective genes cause change in morphology (Shockman and Barrett 1983; Thibessard et al. 2002). To the best of our knowledge the impact of mutations on morphology *S. toxytricini* is not reported but it may be suggested that UV mutagenesis affected peptidoglycan biosynthesis which caused change in morphology.

Analysis of biomass revealed that there was reduction in biomass of all the screened mutants. Though molecular basis between mutagenesis and biomass produced from mutants are not available but scientists have reported the negative impact of UV mutagenesis on cell division (Hopkins et al. 2002), rate of respiration and bacterial biomass production (Hörtnagl et al. 2011). Similar effects may be assumed for mutants produced by chemical mutagenesis. Thus, it may be suggested that mutagenesis in *S. toxytricini* affected cell division and mycelial growth which caused reduced biomass generation from mutants.

During submerged growth, *S. toxytricini* exhibited pellet morphology produced by interwoven hyphae (Kumar and Dubey 2017). Mycelia are considered site for growth and branching (Celler et al. 2012) and mycelial development is under the control of genes (Koebsch et al. 2009; van Dissel et al. 2015). In this study, pellet formation took place in all the mutants with reduced mycelia at pellet surface. Thus, it may be suggested that mutagenesis perturbed mycelial growth. Moreover, reduction in mycelial growth possibly caused reduction in biomass produced from mutants.

It was analyzed that NTG8 mutant showed highest production of lipstatin (1383.25 mg/L i.e. 42.41% higher than wild type) but other research groups have reported higher production of lipstatin i.e. 2.34 mg/g lipstatin from UV induced mutants and 2.88 mg/g from NTG induced mutants of *S. toxytricini* (Luthra et al. 2013a). To the best of our knowledge the mutagenesis of *S. toxytricini* using EMS and MMS is not reported. Screened mutants were analyzed for 10 subculturing experiments and variations in lipstatin production were monitored. Similar results in all generations revealed that there was no significant change in lipstatin production which indicated that the mutations were stable (Kumar 2015).

In this study, precursors (thiamine, biotin and citric acid) were selected on the basis of their involvement in fatty acid biosynthesis. Lipstatin biosynthesis takes place by Claisen condensation between fatty acid metabolites produced in fatty acid metabolism (Eisenreich et al. 1997; Goese et al. 2001). Moreover, fatty acid biosynthesis and tricarboxylic acid (TCA) cycle are linked with each other and acetyl coenzyme A, citric acid and NADH promote fatty acid synthesis (Berg et al. 2002). The metabolites are incorporated in different manner into metabolic cycles. Thiamine is incorporated into thiamine pyrophosphate (catalytic cofactor of pyruvate dehydrogenase complex) and is involved in catalytic production of acetyl coenzymeA from pyruvate (Berg et al. 2002). Biotin is associated with fatty acid biosynthesis by involving in CO_2 transfer (Wakil et al. 1983). Presence of high citric acid, high acetyl coenzyme and high NADH triggers fatty acid biosynthesis (Berg et al. 2002). We suggested that regular supply of fatty acids during fermentation may promote lipstatin biosynthesis (Kumar and Dubey 2016). All the precursors were added to fermentation medium with mutant NTG8 and found that lipstatin production was enhanced to 2387.81 mg/L. Though these experiments were conducted at shake flask level hence we tried to scale up the process in bioreactor where physiochemical conditions were maintained and improvement of 5.5% in lipstatin production was achieved.

Overall production of lipstatin was obtained at 2519.34 mg/L which was 2.59 fold higher (259%) enhancement than wild type but this production was lesser than reported by some of the previous researchers such as 2.88 mg/g (Luthra et al. 2013a), 3290 mg/L (Luthra et al. 2013b) and 4208 mg/L (Zhu et al. 2014). Thus, further screening of high yielding strain is required.

Conclusion

In the present study, mutants of *S. toxytricini* were developed to enhance the production of lipstatin. It was find out that after mutagenesis there was reduction in the biomass

production of all the screened mutants. The pellet formation property was present in all the mutants but growth of mycelia at pellet surface was reduced than wild type. It was analyzed that wild type strain of *S. toxytricini* produced 971.33 mg/L of lipstatin and NTG8 mutant produced 1383.25 mg/L of lipstatin. Supplementation of precursors enhanced the production to 2387.81 mg/L which was further enhanced to 2519.34 mg/L in 5 L bioreactor.

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Compliance with ethical standards

Conflict of interest We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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