

Overproduction of Clavulanic Acid by UV Mutagenesis of *Streptomyces clavuligerus*

Hassan Korbekandi^a, Parisa Darkhal^b, Zohreh Hojati^c, Daryoush Abedi^b, Javad Hamedid^d and Meraj Pourhosein^a

^aGenetics and Molecular Biology Department, Isfahan University of Medical Sciences, Isfahan, Iran. ^bPharmaceutical Biotechnology Department, Isfahan University of Medical Sciences, Isfahan, Iran. ^cGenetics Department, Isfahan University, Isfahan, Iran. ^dBiology Department, Tehran University, Tehran, Iran.

Abstract

Clavulanic acid is produced industrially by fermentation of *Streptomyces clavuligerus* and researches have increased its production by strain improvement, recombinant DNA technology, and media composition and growth condition optimization. The main objective of this study was to increase the level of clavulanic acid production from *Streptomyces clavuligerus* (DSM 738), using UV irradiation. After incubation, the spores and aerial mycelia were scraped off the agar plate by a sterile loop. After passing through a cotton wool, the serially diluted spore suspension was spread on GYM- agar containing caffeine. The plates were irradiated with UV light, wrapped in aluminum foil and incubated. The colonies were sub-cultured again to express the mutations. An aliquot of the spore suspension prepared from the resulted culture was poured in GYM agar plates and incubated. The plates were overlaid with nutrient-agar containing penicillin G and *Klebsiella pneumoniae*, and incubated. The inhibition zone diameter was measured and compared with the wild type colony. Repeating this procedure, the overproducer mutants were selected. Concentration of clavulanic acid was determined by HPLC analysis. It was concluded that secondary metabolites, mainly antibiotics containing clavulanic acid, were produced about 6–7 days after the growth, and concentration of clavulanic acid was increased up to two-folds after UV mutagenesis.

Keywords: *Streptomyces clavuligerus*; Clavulanic acid; UV mutagenesis; Overproduction; Fermentation; UV irradiation.

Introduction

Streptomyces clavuligerus is an actinomycet, which produces more than 21 bioactive secondary metabolites (1-3). Between them, metabolites with β -lactam ring such as cephamycyn C, penicillin N and cephalosponins with antibacterial effect, and clavulanic acid, a β -lactamase inhibitor, are

more important (4). Clavulanic acid is a β -lactam antibiotic with weak antibacterial effect and strong β -lactamase inhibition (4-6). Augmentin[®], a brand name, containing a combination of amoxicillin and potassium clavulanate, is one of the best selling antibiotics (7-15). Clavulanic acid is produced industrially by fermentation of *Streptomyces clavuligerus* and researches have increased its concentration (up to ≈ 1.4 g L⁻¹) by strain improvement, recombinant DNA technology, and media composition and growth conditions

* Corresponding author:

E-mail: korbekandi@pharm.mui.ac.ir

Table 1. Productivity of four selected mutants, investigated in this study.

Mutant number	Clavulanic acid concentration (mg L ⁻¹)	Production time (h)	Productivity (mg L ⁻¹ h ⁻¹)
M2	293.11	72	4.07
M6	242.67	72	3.37
M60	343.87	72	4.77
M61	244.38	72	3.39

optimization (3, 7, 10, 15-24). UV irradiation is one of the strain improvement strategies through random mutation (7, 16). UV radiation, in the range of 200-300 nm, produces thymidine dimers and increases probability of deletion during the duplication process (25, 26). UV is a very convenient and relatively safe mutagen (27), however very few researchers (7, 16) have used this technique to overproduce clavulanic acid. The main objective of this study was to increase the level of clavulanic acid production by *S. clavuligerus*, using UV irradiation.

Experimental

Microorganism and cultivation

Streptomyces clavuligerus (DSM 738) was revitalized on GYM- broth and maintained on GYM-Agar. Sodium chloride solution (0.9%) containing tween-80 (0.2%) was poured onto GYM-Agar medium, on which *S. clavuligerus* was grown for 7 days at 28 °C. The spores and aerial mycelia were scraped off the agar surface, using a sterile loop. After filtering through cotton wool, the spore suspension was serially diluted to 10² CFU mL⁻¹ and one mL of this suspension was poured and spread onto each sterile glass plate containing GYM-Agar and caffeine (0.5 g L⁻¹) (28).

UV Irradiation

The plates were irradiated with UV light (200-300 nm, 1.7 W m⁻²) for 20 min, at a distance of 8 cm. After the irradiation, in order to protect against photoreactivation, the plates were wrapped with aluminum foil and incubated for 7 days. Finally, the colonies were sub-cultured (7 days, 28 °C) again to express and stabilize the mutants.

Bioassay

A radically modified bioassay method, based on the method developed by Lee et al (7), was used. Ten µl of the spore suspension (10² CFU

mL⁻¹), prepared from the resulted culture, was poured into each of the 3 wells (out of 4 wells) in GYM- agar plates. A spore suspension (10² CFU mL⁻¹) of the wild type micro-organism was poured into the 4th well. After an incubation period of 6 days at 28 °C, the plates were overlaid with nutrient-agar containing penicillin G (7 µg mL⁻¹) and *Klebsiella pneumoniae* (10⁵ CFU mL⁻¹), and at the end again incubated for 24 h at 37 °C. The inhibition zone diameter was measured and compared with the wild type colony.

Clavulanic acid production

The pH of the seed medium (20 mL) consisting of peptone (1%), malt (1%) and glycerol (2%) was adjusted to 7.0 ± 0.1. The medium was inoculated (5%) and incubated (28 °C, 24 h, 220 rpm) in a 100 mL shaking flask. The production medium (50 mL) containing malt extract (3%), glycerol (0.5%), soy flour (0.5%), FeSO₄ (0.0001%), and MnSO₄ (0.0001%) was inoculated (5%) with the seed culture and incubated (28 °C, 220 rpm) in a 250 mL shaking flask.

HPLC analysis

Clavulanic acid was quantified by HPLC, using a reversed-phase column (C-18). The mobile phase contained KH₂PO₄ (50 mM, 70%, 0.348 mL min⁻¹) and methanol (30%, 0.157 mL min⁻¹) and the pH was adjusted to 3.2. Samples were derivatized using imidazole. The derivatives were detected at 311 nm (29).

Statistical analysis

Statistical analyses were performed through the Mann-Whitney non-parametric test, using the statistical package SPSS for Windows version 13.0 software (SPSS Inc, Illinois).

Results

During the subculturing of *S. clavuligerus*



Figure 1. The developed bioassay plates used in this study.

on GYM agar for sporulation, it was observed that by using deep (≈ 8 mm) agar plates denser colonies appeared with more grey pigments. Pale yellow droplets were secreted 6-7 days after the growth, which disappeared after leaving holes on the surface of *S. clavuligerus* colonies for a period of 24 h. This procedure has not been described before.

It seems that a thicker layer of agar in the plates, resulting in a greater population of the microorganism, could cause a higher and faster production of secondary metabolites, possibly containing antibiotics and clavulanic acid. After a while, these droplets might have been diffused in the plates.

Development of a bioassay for clavulanic acid

In order to screen overproducing mutants, a cheap, quick and fairly reliable bioassay method considering *S. clavuligerus* as clavulanic acid producer, and *K. pneumonia* as the sensitive microorganism was needed. When the bioassay was performed about 4 days after the growth of *S. clavuligerus*, not only the inhibition zone of *K. pneumonia* was not observed, but also a significant overgrowth zone was seen. However, when this assay was performed after 6 days, the inhibition zone was clearly observed (Figure 1).

It might be interpreted that some primary metabolites stimulating the growth are secreted by *S. clavuligerus* colonies during 4 days, which

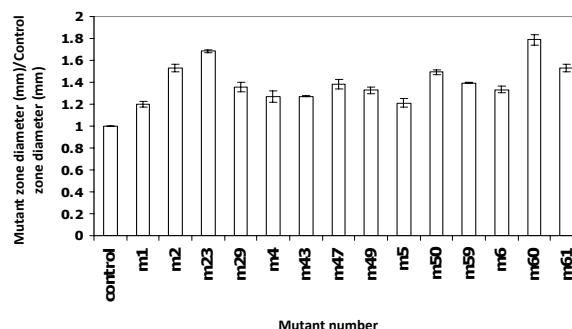


Figure 2. The ratio of mutants inhibition zone diameter over the wild type inhibition zone diameter in selected mutants- Data points have been expressed as mean \pm standard error ($n = 3$).

can lead to a faster growth of *K. pneumonia*. On the other hand, after 6 days, secondary metabolites (such as clavulanic acid), inhibiting the growth, might have been produced.

Screening of overproducing mutants

As mentioned earlier, a bioassay method was used to screen the overproducer mutants. Fourteen (out of 105) mutants showed inhibition zones more than the wild type control, with higher values for the mutants M60, M23 and M61 (Figure 2). Based on the Mann-Whitney statistical test, the differences between groups were found to be significant ($P < 0.001$).

Clavulanic acid determination by HPLC

The bioassay method used did not prove the production of clavulanic acid by the screened mutants. Therefore, we needed to confirm this and determine clavulanic acid concentration. Overproduction of clavulanic acid by the screened mutants was verified by the HPLC measurements (Figures 3 and 4). Intra-day and inter-day variations in precision (CV %) and accuracy (Error %) were found to be acceptable.

Comparing the concentrations of produced clavulanic acid with the biomasses of related mutants, clavulanic acid concentrations were found to be different among the various mutants. However, there was no considerable difference in the biomass concentrations (Figure 4). It might be interpreted that the overproduction of clavulanic acid, following the mutations, is not the result of overgrowth. This could be due to the changes in the metabolic pathway of the microorganism, resulting in the overproduction of clavulanic acid.

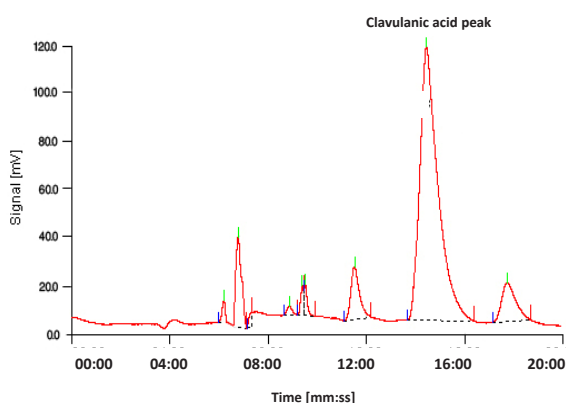


Figure 3. Chromatogram of the products, obtained using a reversed-phase column (C-18). The mobile phase contained KH_2PO_4 (50 mM, 70%, $0.348 \text{ mL min}^{-1}$) and methanol (30%, $0.157 \text{ mL min}^{-1}$) and the pH was adjusted to 3.2. Samples were derivatized using imidazole, and they were detected at 311 nm.

There was an analogy between the bioassay and HPLC method (Fig. 5), except in few cases, which might be due to the overproduction of other growth limiting factors.

Productivity of the selected mutants was also calculated (Table 1).

Discussion

The production of clavulanic acid was successfully increased 1.8 times by UV mutagenesis. It seems that the mutations occurred in the genes which were involved in the metabolic pathway of clavulanic acid production, but not in the primary metabolites responsible for growth. These mutants can be used industrially for production of clavulanic acid.

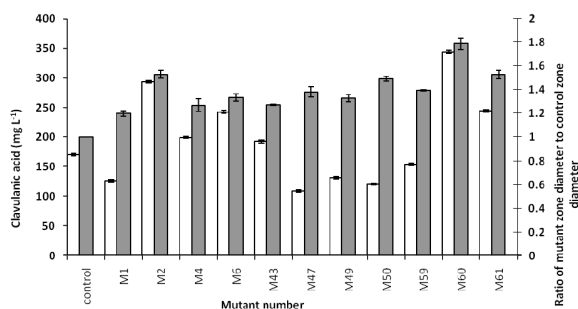


Figure 5. Clavulanic acid production versus the ratio of mutant zone diameter to control zone diameter. Clavulanic acid concentrations (white columns) were measured by HPLC, and the ratio of mutant zone diameter to control zone diameter (gray columns) was measured by Vernier caliper and division. Data points have been expressed as mean \pm standard error ($n = 3$).

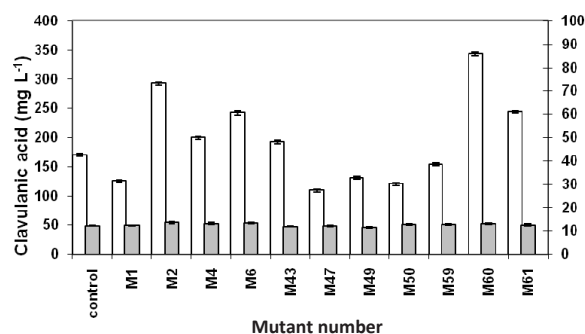


Figure 4. Clavulanic acid versus biomass production. Clavulanic acid concentrations (white columns) were measured by HPLC, and the wet biomass (gray columns) was measured by weighing. Data points have been expressed as mean \pm standard error ($n = 3$).

A group of researches (7) increased clavulanic acid production up to 8 folds by UV mutagenesis and medium optimization. We increased it up to 1.8 folds, using random mutagenesis, which can be continued more considering the fact that “mutation is an accidental event”. In another study (30), we also inserted *clar* gene in *E. coli* XLI blue, using *PZSclaR* plasmid, as a vector to increase clavulanic acid production. In the near future we will combine these methods, and by using the medium optimization, it is hoped to increase the productivity.

Acknowledgment

We wish to express our gratitude to the Isfahan University of Medical Sciences for financial support of this study.

References

- (1) Muth G, Brolle DF and Wohlleben W. Genetics of *Streptomyces*. In: Demain AL and Davais J. (eds.) *Manual of Industrial Microbiology and Bacteriology*. ASM Press, Washington DC (1998) 353-67.
- (2) Williams ST, Goodfellow M and Alderson G. Genus *Streptomyces*. In: Williams ST, Sharpe ME and Holt JG. (eds.) *Bergey's Manual of Systematic Bacteriology*. 4th ed. Springer, New York (1998) 2452-92.
- (3) Gouveia ER, Baptista-Neto A, Badino AC and Hokka CO. Optimization of medium composition for clavulanic acid production by *Streptomyces clavuligerus*. *Bacteriol. Lett.* (2001) 23: 157-61.
- (4) Liras P and Rodryiguez-Garcya A. Clavulanic acid, a beta lactamase inhibitor: biosynthesis and molecular genetics. *Appl. Microbiol. Biotechnol.* (2000) 54: 467-75.
- (5) Tahlan K, Anders C and Jensen SE. The paralogous pairs of genes involved in clavulanic acid and clavam metabolite biosynthesis are differently regulated in

- Streptomyces clavuligerus*. *J. Bacteriol.* (2004) 186: 6286-97.
- (6) Elson SW, Baggaley KH, Davison M, Foulston M, Nicholson NH and Risbridger GD. The identification of three new biosynthetic intermediates and one further biosynthetic enzyme in the clavulanic acid pathway. *J. Chem. Soc. Chem. Commun.* (1993) 1212-14.
 - (7) Lee SD, Park SW, Oh KK, Hong SI and Kim SW. Improvement for the production of clavulanic acid by mutant *Streptomyces clavuligerus*. *Lett. Appl. Microbiol.* (2002) 34: 370-5.
 - (8) Rolinson G. A review of the microbiology of amoxicillin/clavulanic acid over the 15 year period 1978-1993. *J. Chemother.* (1994) 6: 283-318.
 - (9) Richmond MH and Sykes R. The beta lactamase of gram negative bacteria and their possible physiological role. *Adv. Microb. Physiol.* (1973)9: 31-88.
 - (10) Lynch HC and Yang Y. Degradation products of clavulanic acid promote clavulanic acid production in cultures of *Streptomyces clavuligerus*. *Enzyme. Microbial. Tech.* (2004) 34: 48-54.
 - (11) Leflon-Guibout V, Speldooren V, Heym B and Nicholas-Chanoine M. Epidemiological survey of amoxicillin-clavulanate resistance and corresponding molecular mechanisms in *Escherichia coli* isolates in France: new genetic features of *bla* (TEM) genes. *Antimicrob. Agents Chemother.* (2000) 44: 2709-14.
 - (12) Janicka G, Wojciechowska D, Harenska K, Porada J and Klyszejko C. The resistance to beta lactam antibiotics of lactose-positive and lactose-negative strains of *Escherichia coli*. *Acta Microbiol. Pol.* (1997) 46: 399-403.
 - (13) Natsch S, Conrad C, Hartmeier C and Schmid B. Use of amoxicillin-clavulanate resistance in *Escherichia coli* over a 4-year period. *Infect. Control. Hosp. Epidemiol.* (1998) 19: 653-6.
 - (14) Vanhoof R, Carpentier M, Delannoy P, Fagnart O, Lontie M and Mans I. Study of the *in vitro* activity of amoxicillin-clavulanic acid and other beta-lactam antibiotics against *Escherichia coli* isolated urine specimens. *Acta Clin. Belg.* (2001) 56: 32-7.
 - (15) Rosa JC, Baptista-Neto A, Hokka CO and Badino AC. Influence of dissolved oxygen and shear conditions on clavulanic acid production by *Streptomyces clavuligerus*. *Bioprocess. Biosyst. Eng.* (2005) 27: 99-104.
 - (16) Hodgson JE, Fosberry AP, Rawlinson NS, Ross HN, Neal RJ, Arnell JC, Earl AJ and Lawlor EJ. Clavulanic acid biosynthesis in *Streptomyces clavuligerus*: gene cloning and characterization. *Gene* (1995) 166: 49-55.
 - (17) Cochet N and Demain AL. Effect of water activity on production of beta-lactam antibiotics by *Streptomyces clavuligerus* in submerged culture. *J. Appl. Bacteriol.* (1996) 80: 333-7.
 - (18) Sircar A, Sridhar P and Das PK. Optimization of solid state medium for the production of clavulanic acid by *Streptomyces clavuligerus*. *Process Biochem.* (1998) 33: 283-9.
 - (19) Kwon HJ and Kim SU. Enhanced biosynthesis of clavulanic acid in *Streptomyces clavuligerus* due to oxidative challenge by redox-cycling agents. *Appl. Microbiol. Biotechnol.* (1998) 49: 77-83.
 - (20) Large KP, Ison AP and Williams DJ. The effect of agitation rate on lipid utilization and clavulanic acid production in *Streptomyces clavuligerus*. *J. Biotechnol.* (1998) 63: 111-19.
 - (21) Chen KC, Lin YH, Wu JY and Hwang SCJ. Enhancement of clavulanic acid production in *Streptomyces clavuligerus* with ornithine feeding. *Enzyme. Microbial. Tech.* (2003) 32: 152-6.
 - (22) Wang YH, Yang B, Ren J, Dong ML, Liang D and Xu AL. Optimization of medium composition for the production of clavulanic acid by *Streptomyces clavuligerus*. *Process Biochem.* (2005) 40: 1161-6.
 - (23) Ortiz SCA, Hokka CO and Badino AC. Utilization of soybean derivatives on clavulanic acid production by *Streptomyces clavuligerus*. *Enzyme Microbial. Tech.* (2007) 40: 1071-77.
 - (24) Bushell ME, Kirk S, Zhao HJ and Avignone-Rossa CA. Manipulation of the physiology of clavulanic acid biosynthesis with the aid of metabolic flux analysis. *Enzyme Microbial. Tech.* (2006) 39: 149-57.
 - (25) Aneja KR. *Experiments in Microbiology, Plant Pathology and Biotechnology*. 4th ed. New Age International publications, New Delhi (2003) 131-136.
 - (26) Schaaper RM and Dunn RL. Spectra of spontaneous mutations in *Escherichia coli* strains defective in mismatch correction: the nature of *in vivo* DNA replication errors, *Proc. Acad. Sci. USA* (1987) 84: 6220-24.
 - (27) Kieser T, Bibb MJ, Butner MJ, Chatter KF and Hopwood DA. *Practical Streptomyces Genetics*. The John Innes Foundation, UK (2000) 99-106.
 - (28) Henrissat B, Driguez H, Veit C and Schulein W. Synergism of celluloses from *Trichoderma reesei* in the degradation of cellulose, *Biotechnol.* (1985) 3: 722-6.
 - (29) Foulstone M and Reading C. Assay of amoxicillin and clavulanic acid, the components of Augmentin, in biological fluids with high-performance liquid chromatography. *Antimicrob. Agents Chemother.* (1982) 22: 753-62.
 - (30) Salehi Z. *Detection and Isolation of *claR* regulatory gene from *Streptomyces clavuligerus* [dissertation]*. Isfahan, Isfahan University (2006) 109.

This article is available online at <http://www.ijpr-online.com>

Archive of SID

**Search full text articles ?
Visit <http://www.ijpr-online.com>**