Exploring the optimization of UV mutants of *Streptomyces clavuligerus* for clavulanic acid production

ABSTRACT

Streptomyces clavuligerus, initially described as a Cephamycin C producer, has been currently utilized to produce clavulanic acid (CA) which shows low antibiotic activity, being, however, a strong β-lactamase inhibitor, enzymes responsible for bacterial resistance to β–lactam antibiotics. Genetic improvement by physical and chemical agents is mandatory since, due to its genetic instability, industrial strains lose production capability making necessary a steady and frequent strain improvement and a screening procedure. The objective of this work was to obtain an increase in CA production in submerged cultures by utilizing mutants obtained by UV radiation of the wild strain *Streptomyces clavuligerus* ATCC 27064. Submerged cultures were performed, with the best mutants selected, in a 5L bench-scale bioreactor, batch-wise, utilizing a complex medium containing glycerol and a soybean protein isolate as the main components. The mutant 70 was able to produce a maximum CA concentration of 500 $mg.L^{-1}$, approximately 1.6 times higher than the wild strain. Another finding was a higher CA productivity of 29.5 $mg.h^{-1}.L^{-1}$ with this mutant, although a lower specific growth rate had been observed.

Keywords: *Streptomyces clavuligerus*; clavulanic acid production; UV radiation; UV mutants; process improvement.

1. INTRODUCTION

Streptomyces clavuligerus is able to produce a wide variety of antibiotics and other secondary metabolites including various β-lactam antibiotics. β-lactam antibiotics, such as penicillin and cephalosporin have been extensively used for the treatment of many infections caused by pathogenic bacteria over the past 60 years and their industrial process optimization has been the subject of many works [1,2].

CA is an important secondary metabolite commercially produced by *Streptomyces clavuligerus*. This compound is able to inhibit irreversibly many β-lactamases produced by microorganisms resistant to β-lactam antibiotics. Despite the great economic impact brought to the pharmaceutical industry by the genetic improvement of mutagenesis, little is known about the mutation mechanisms, DNA replication, and repair in *Streptomyces* strains applied to the industrial secondary metabolite production process. With regards to the CA production, there are only a few articles, available in the literature, which report the process improvement by mutant strains [3].

For many decades genetic improvement utilizing physical and chemical agents aiming at high yield mutants has been a necessary strategy and an important method in the effort toward improving the bioactive metabolites production process since industrial strains lose part of the production ability due to genetic instability, that affects different phenotypical properties, often pleiotropically, including morphological differentiation, production of secondary metabolites, such as pigments and antibiotics, antibiotic resistance, secretion of extracellular enzymes and sometimes genes for primary metabolism [4,5,6]. Recent works in the literature have also emphasized the importance of mutant isolation for the improvement of CA production by *S. clavuligerus* [7,8], as well as for the maintenance of genetic stability [9,10].

Mutagenesis induced by UV light radiation is not efficient enough to induce high antibiotic producers strains including a few representatives of the *Streptomyces* genera which present a high content of nitrogenous bases C+G in the genome. Only a few strains of *Streptomyces* show sensitivity to UV radiation due to the absence of a repair process known as photo reactivation. However, it is worth mentioning that UV radiation has been successfully applied for mutagenesis of *S. clavuligerus* and *S. coelicolor* since these species have proved sensitive to such type of radiation [10,11,12]. Therefore, the objective of this work was to improve the CA production process in a submerged culture by selecting UV radiation mutants of the wild strain *S. clavuligerus* ATCC 27064.

2. MATERIAL AND METHODS

2.1 Microorganism

Streptomyces clavuligerus ATCC 27064 (www.atcc.org) used throughout this work was stored as vegetative cells (5.0 $g.L^{-1}$ dry weight) at −70°C in cryotubes utilizing glycerol 20% w/v.

2.2 Mutagenic treatment

To obtain the CA productive mutants, the wild *S. clavuligerus* ATCC 27064 strain spores were initially irradiated with UV light for 200 seconds. The cells so obtained were stored and used in the present work as the starting strain. For the UV mutation, saline solution (0.9% NaCl) was poured into SB agar medium [13] with *S. clavuligerus* that was grown for 12 days at 28º C. The spores were then scraped off the agar surface using a sterile loop and 10 mL ($2.5x10^4$ spores. mL^{-1}) of the spore suspension was poured into a sterile glass plate. The resulting spore suspension was irradiated with UV light (15.2×10^{-6} $W.mm^{-2}$), at 60 s intervals until 300 s, at a distance of 60 cm. After serial dilution of the UV irradiated spore suspension, the sample was again spread over the SB medium to prevent it against photo reactivation for 10 days at 28º C.

2.3 Culture media and experimental conditions

Firstly, these selected strains by the mutagenesis were cultivated in an orbital shaker with a synthetic medium to determine colonies capable of producing CA. The production medium contained (in $g.L^{-1}$ distilled water): glycerol, 15.0; malt extract, 10.0; yeast extract, 1.0; Soytone 15; arginine 2.62; KH_2PO_4, 0.63; $MgSO_4.7H_2O$, 0.75; salt solution 1.0 mL ($MnCl_2.4H_2O$, 0.001; $FeSO_4.7H_2O$, 0.001; $ZnSO_4.7H_2O$, 0.001); and 3-(N-morpholino) propanesulfonic acid (MOPS) buffer, 21 (100 mM) and pH 6.8. The culture was grown for 72h, 250 rpm at 28ºC.

Three mutants (17a, 13a and 70) were selected for further experiments. Vegetative cells (in cryotubes) of the wild strain *S. clavuligerus* ATCC 27064 and of the three selected mutants were reactivated in the seed medium, based on the one used by Rosa et al. [14], containing (in $g.L^{-1}$ distilled water): glycerol, 15.0; bacto peptone, 10.0; malt extract, 10.0; yeast extract, 1.0; K_2HPO_4, 2.5; $MgSO_4.7H_2O$, 0.75; salt solution 1.0 $g.L^{-1}$ ($MnCl_2.4H_2O$, 0.001; $FeSO_4.7H_2O$,

0.001; $ZnSO_4.7H_2O$, 0.001); and 3-(N-morpholino) propanesulfonic acid (MOPS) buffer, 21 (100 mM) and pH 6.8.

The inoculum medium proposed by Teodoro et al. [15] contained (in $g.L^{-1}$ distilled water): glycerol, 15.0; isolate protein of soybean (containing about 15% w/v of total nitrogen), 20.0; K_2HPO_4, 0.8; $MgSO_4.7H_2O$, 0.75; salt solution 1.0 $g.L^{-1}$ ($MnCl_2.4H_2O$, 0.001; $FeSO_4.7H_2O$, 0.001; $ZnSO_4.7H_2O$, 0.001); MOPS buffer, 21 (100 mM) and pH 6.8. The production medium had the same composition as the inoculum medium, except that no MOPS buffer was used and silicone antifoam (0.1 $mL.L^{-1}$) and soybean oil (1.0 $g.L^{-1}$) were added.

The batch cultivations were performed in a bioreactor Bioflo III fermentor (New Brunswick Sci. Co. Inc., USA) with capacity for 5 L, and the total volume used was 4 L. All cultivations were conducted at 28°C, 800 rpm, 0.5 vvm, based on a 4 L working volume, and the pH was automatically controlled to stay at 6.8±0.1 by adding a 1 M HCl and/or 2 M NaOH solution. Cell suspensions from cryotubes (3.5 mL), having a concentration of 5 $g.L^{-1}$ dry weight, were inoculated into a 45-mL seed medium in a 500-mL Erlenmeyer flask and incubated in a rotary shaker (New Brunswick Scientific) at 28°C and 250 rpm for 24 hours. Next, Erlenmeyer flasks (500 mL) containing 45 mL of inoculum medium were inoculated with 5 mL of the cultivated seed broth and incubated in a rotary shaker at 28°C, 250 rpm for 24 hours. The contents of inoculum corresponding to 10% v/v were transferred to the fermenters resulting in initial fermentation volumes of 4.0 L for batch cultivation (5-L bioreactor).

2.4 Analytical methods

Cell growth was evaluated indirectly by measuring the broth rheological parameter K (consistency index) of the “power law” model using a Brookfield concentric-cylinders rheometer [16]. Literature has shown that the consistency index (K) is the most appropriate parameter to infer cell growth in broth containing insoluble particles, as is the case with complex fermentation broths. It can also be related to the morphology of filamentous microorganisms [15, 17].

The glycerol concentration (C_{Gly}) was determined by HPLC. Milli-Q water was used as the mobile phase. The equipment was operated at 80°C with a 1 $mL.min^{-1}$ flow rate. A Shodex KS 802 (Lonpak) column was utilized [18].

The CA concentration (Cp) was determined by HPLC, as described by Foulstone and Reading [19]. The CA from the Pharmaceutical product Clavulin (Glaxo-SmithKline Pharmaceutical, Rio de Janeiro, Brazil) was used as standard. The imidazole derivative of CA was injected into the HPLC unit. The C-18 μ-Bondapack (Waters) column was utilized as stationary phase and mobile phase was composed of 94% KH_2PO_4 0.1 M (pH 3.2) and 6% methanol, v/v. The derivative compost from CA was detected at 311 nm.

2.5 Kinetic parameters

Based on the values of the cell concentration (Cx) evaluated by the correlation described by Baumberg et al. [20], the maximum specific growth rate (μ_{max}) and the maximum CA specific productivity ($P_{CA/X}=C_{CA\text{-}max}/Cx$) for each strain were calculated. The CA maximum concentration ($C_{CA\text{-}max}$) was obtained as being the highest derivative of the CA concentration in relation to time, $(dC_{CA}/dt)_{max.}$

3. RESULTS AND DISCUSSION

3.1 Selection of mutants

Initially, the wild type strain was submitted to UV irradiation and only 1% of the mutant survived. About 180 strains were isolated based on their pigmentation (described above in mutagenic treatment). Afterward, the strains were tested based on the CA production through antimicrobial activity [21]. Phenotypic characterization including mycelium pigmentation (amy), sporulation ability (spo), and diffuse pigmentation (phs) were done (Table 1). Three mutant strains were selected by antimicrobial activity against the bacteria *Klebsiella pneumoniae*, determined by the size of inhibition halos in millimeters [21]. This mutants presenting higher CA concentration than the wild strain obtained in shake flasks after 72 h fermentation are described

(Table 1). These mutants namely 13a, 17a, and 70 were used for batch cultivations on a 5L bioreactor.

TABLE 1. Morphological characteristics and clavulanic acid production of UV radiation mutants compared with the wild strain *Streptomyces clavuligerus* ATCC 27064.

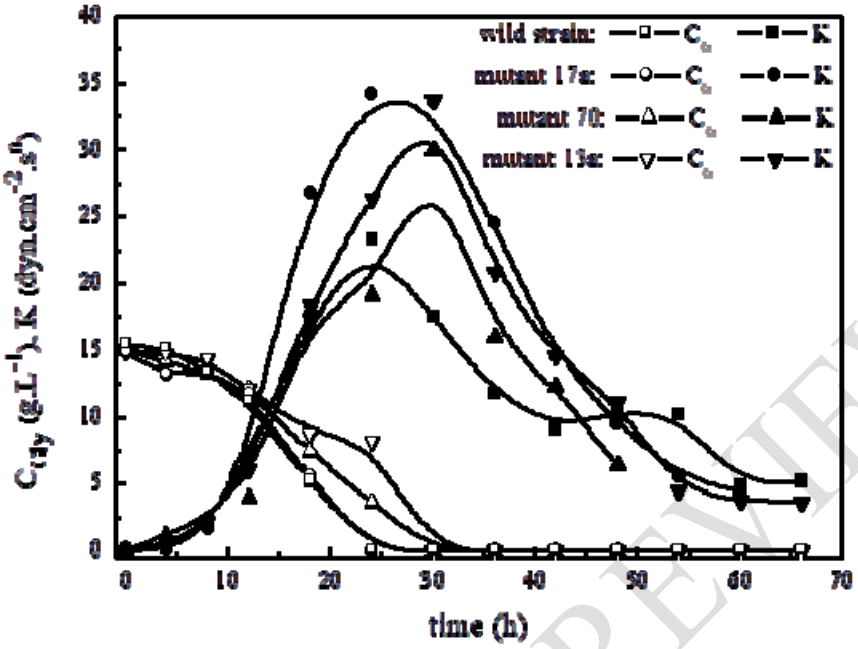
S. clavuligerus	**Morphological characteristics in medium SB**	**$C_{CA\text{-}max}$ in 72h ($mg.L^{-1}$)**
Wild strain	spore mass dark grayish green on media (phs -, amy^+, spo^+)*	CA^+ (85.0)
Mutant 13a	Substrate mycelia white, (phs -, amy -, spo^-)*	CA^+ (105.4)
Mutant 17a	spore mass green (phs -, amy +, spo^+)*	CA^{++} (113.3)
Mutant 70	Substrate mycelia white, (phs -, amy +, spo^-)*	CA^{++} (132.6)

* Diffusible pigments (phs^+), aerial mycelia (amy^-), abundant sporulation (spo^+), lack sporulation (spo^-), clavulanic acid production (CA^+), better CA production (CA^{++}).

3.2 Evaluation of CA production in batch cultivation

In order to verify the actual improvement in CA production by the mutants as compared with the wild strain, batch cultivations in 5 L bioreactor were performed at 28°C, 800 rpm and 0.5 vvm. This agitation of 800 rpm showed a higher productivity of CA in bioreactor with 4 L working volume [22,16]. The pH was kept as near to 6.8 as possible.

Figure 1 shows the time course of the consistency index (K) and glycerol concentration (C_{Gly}) along the cultivation of the mutants 13a, 17a, 70 and wild strain.



151

152 **Fig. 1.** Time course of the glycerol concentration (CGly) and the consistency index (K) for the
153 different strains cultivated in a 5L bioreactor with complex medium.
154

155 During all experiments, glycerol exhaustion occurred between 24 and 30 h and the
156 dissolved oxygen (DO) remained above 50% (data not shown) showing that the process was
157 not limited by oxygen and that glycerol as the main carbon and energy source can be
158 considered the limiting substrate. The K is the variable that depends on cell concentration and
159 cell morphology and the cell growth can be inferred by these values. It can be observed that the
160 mutant cell growth is higher, reaching K 34 (dina.cm^{-2}.s^{n}) in 24 h for mutant 17a, as compared
161 with the wild strain, reaching K 20 (dina.cm^{-2}.s^{n}) during the same period. The UV radiation
162 probably activated growth regulating genes or mutation provided better adaptation to the
163 production medium. Indeed, Aikawa et. al. [5] also observed the same effect with *Streptomyces*
164 *avermitilis* after mutagenesis with MMS, reporting a biomass increase up to 30 g.L^{-1} in 192 h
165 while with the wild strain only 20 g.L^{-1} in 167 h was achieved. In addition, the authors observed
166 a production of avermectin 8.2 times more with a spontaneous mutant compared with the wild-

type, and the mutant named IPT-85 obtained by MMS produced about 16 times more avermectin than the wild-type.

Figure 2 shows the CA production of the mutants and the wild strain studied. Although the mutant 17a presented the best performance regarding growth, the CA production was the lowest. This behavior was also observed by Teodoro et. al. [15] with cultivation of the wild strain in a different culture media. The authors reported that the high consistency index was associated with the low production of CA. Indeed, this phenomenon occurs frequently in secondary metabolite fermentations.

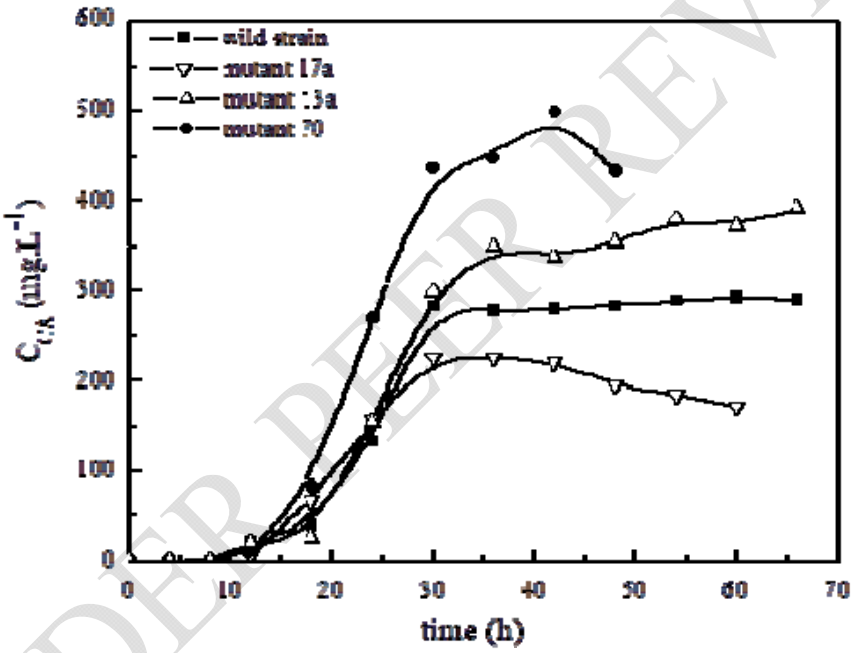


Fig. 2. Time course of the CA concentration (CCA), during cultivation of the mutants 13a, 17a, 70 and the wild strain, in a 5L bioreactor with complex médium.

Figure 3 shows the evolution of the CA volumetric productivity ($P_{CA}=C_{CA}/t$) of the experiments carried out. The P_{CA} shows how fast the process proceeds. It can be observed that with the mutant 70 not only the concentration presented is higher, but also the production rate in terms of productivity is much better than the wild strain. Lee et al. [7] studied a mutant strain obtained by *S. clavuligerus* ATCC 27064 treated with UV radiation; the authors performed a

submerged culture in 2.5 L bioreactor and reported the CA maximum productivity of 3.37 $mg.L^{-1}.h^{-1}$, lower than the observed in the present work of CA, 14 $mg.L^{-1}.h^{-1}$, as shown in Figure 3.

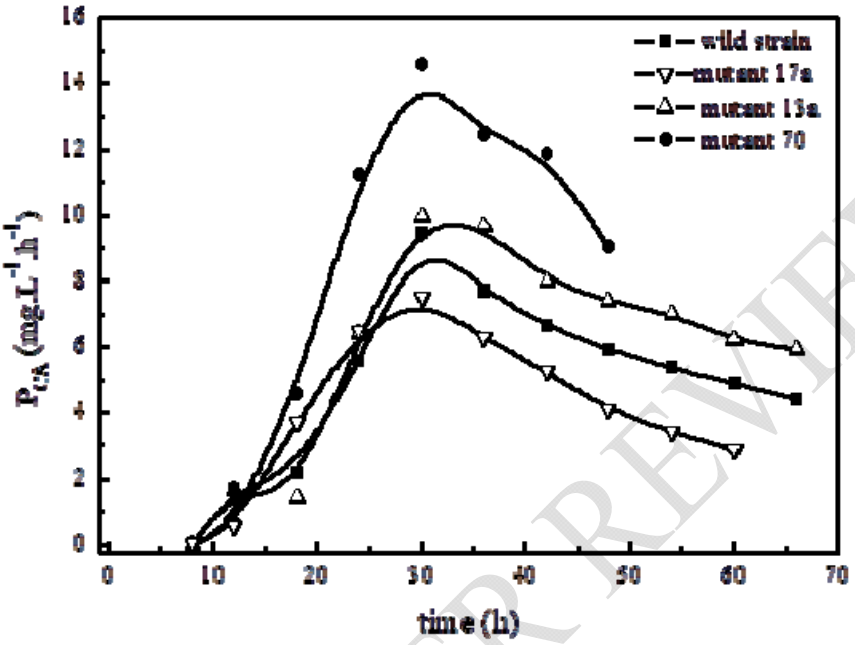


Fig. 3. Volumetric productivity in CA (PCA) for the mutants 13a, 17a, 70, and for the wild strain cultivated in a 5 L bioreactor with complex medium.

Table 2 presents the calculated values of these kinetic parameters. As it can be observed that μ_{max} of mutants 17a, 13a e 70 was lower than that of the wild strain. However, the mutant 70 although presenting the lowest μ_{max} showed to be the highest producer since its maximum productivity and maximum specific productivity were the highest among all strains. The maximum CA concentration ($Cp_{CA\text{-}max}$) was obtained for the mutant 70, around 29.5 $mg_{CA}.h^{-1}.L^{-1}$ and the highest concentration of 500 $mg_{CA}.L^{-1}$ was obtained at approximately 40 h cultivation. The increase in terms or product concentration and productivity achieved with the mutant 70 indicates that the methodology utilized for mutagenesis of *S. clavuligerus* is very promising to obtain high producing strains able to be used in industrial processes.

TABLE 2. Kinetic parameters for the different mutants and the wild strain of *S. clavuligerus* calculated from the results obtained in batch cultivation with complex medium.

S. clavuligerus	μ_{max} (h^{-1})	$C_{CA\text{-}max}$ ($mg_{CA}.h^{-1}.L^{-1}$)	P_{max} ($mg_{CA}.g_{cel}^{-1}.h^{-1}$)
Wild strain	0.163	20.4	1.72
Mutant 17 a	0.128	13.3	0.99
Mutant 70	0.110	29.5	2.67
Mutant 13 a	0.088	22.7	1.84

CA is an industrially important secondary metabolite due to its inhibitory action on β-lactamases. There are many examples of strain improvement strategies applied to *S. clavuligerus* in order to reach high levels of CA production with genetic engineering and random mutagenesis [23, 24]. In this study, we used UV mutagenesis and our results showed that the maximum CA production was obtained with the mutant 70, approximately 25% higher than the wild strain. It can be suggested that UV radiation produced mutagenesis that affected positively the ability of CA production genes whether acting on the regulatory or biosynthetic genes. It can also be observed that the production rate is much higher with this mutant as the slope of the CA production curve and that between 18 and 28h it is much steeper than those of the other mutants and wild strain. The following decrease in the production rate observed demonstrates that the concentration of glycerol, one of the precursors of CA strongly affects CA production as also observed by Mayer and Deckwer [25] and Chen et al. [26]. To obtain a further increase in the CA production, the addition of glycerol in the fed-batch process is advisable.

UV radiation enabled the obtaining of an improved mutant in terms of production ability as shown by higher production kinetic parameters, $C_{CA\text{-}max}$ and $P_{CA\text{-}max}$ of the mutant 70. Lower μ_{max} was observed for this mutant, showing that similarly to other secondary metabolites producing processes, production is frequently enhanced when growth conditions are unfavorable. This strain will be studied regarding its genotypic characteristics as well as its performance in different operating conditions (fed-batch and continuous cultures) and different medium in order to establish the technology for higher product concentration and production

rate. However, the Streptomyces chromosome is very unstable and undergoes very large deletions spontaneously at rates higher than 0.1% of spores. This genetic instability affects different production of secondary metabolites, such as pigments and antibiotics [27, 28]. Thus, more studies are necessary to know if the mutant strains maintain this higher production of CA in long-term.

4. CONCLUSION

The increased resistance to the currently recommended antibiotics is responsible for several human deaths and it has been a constant concern. The major cause of antibiotic resistance is the inappropriate use and excessive treatment of antibiotics thus, more guidance acts are necessary, as well as stricter regulation of antibiotics. Our findings clearly demonstrate that UV mutagenesis can lead to the improvement in CA production in *S. clavuligerus*. This work reinforces the importance of using random mutagenesis methods in *S. clavuligerus* for biotechnological research in productivity improvement.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Thykaer J, Nielson J. Metabolic engineering of beta-lactam production. Metab. Eng. 2003;5:56-69.
2. Ives PR, Bushell ME. Manipulation of the physiology of clavulanic acid production in *Streptomyces clavuligerus*. Microbiology. 1997;143:3573-579.
3. Kieser T, Chater KF, Bib MJ, Buttnrt MJ, Hpowood DA. Practical *Streptomyces* genetics, Norwich: The John Innes Foundation; 2000.
4. Baltz RH. Mutation *in Streptomyces* In: Antibiotic production Streptomyces. The Bacteria, New York: Academic Press; 1986.
5. Aikawa M, Lopes-Shikida SAR, Lemos M.F, Pradella JGC. Screening of spontaneous and induced mutants in *Streptomyces avermitilis* enhances avermectin production. Applied. Microbiol. Biotechnol. 1999;52:558-62.

6. Volff JN, Altenbuchner J. Genetic instability of the Streptomyces chromosome. Mol. Microbiol. 1998;27: 239-246

7. Lee SD, Park SW, Oh KK, Hong SI, Kim SW. Improvement for the production of clavulanic acid by mutant *Streptomyces clavuligerus*. Lett. Appl. Microbiol. 2002;34:370-75.

8. Kim IC, Kim CH, Hong SI, Kim SW. Fed-batch cultivation for the production of clavulanic acid by an immobilized *Streptomyces clavuligerus* mutant. World J. Microbiol. Biotechnol. 2001;17:869-72.

9. Fisher G, Decaris B, Leblond P. Occurrence of deletions, associated with genetic instability in *Streptomyces ambofaciens*, is independent of the linearity of the chromosomal DNA. J. Bacteriol. 1997;14:4553-558.

10. Baltz RH. Genetic methods and strategies for secondary metabolic yield improvement in actinomycetes. Antonie van Leeuwenhoek. 2001;79:251-59.

11. Singer CE, Ames BN. Sunlight ultraviolet and bacterial DNA base ratios. Science. 1970;170:822–25.

12. Qin R, Zhong C, Zong G, Fu J, Fu J, Pang X, Cao G. Improvement of clavulanic acid production in *Streptomyces clavuligerus* F613-1 by using a claR-neo reporter strategy. *Electron* J. Biotechnol. 2017;28:41-46.

13. Sánchez L, Braña AF. Cell density influences antibiotic biosynthesis in *Streptomyces clavuligerus.* Microbiology. 1996;142:1209-220.

14. Rosa JC, Baptista-Neto A, Hokka CO, Badino AC, Hokka CO. Influence of dissolved oxygen and shear conditions on clavulanic acid production by *Streptomyces clavuligerus*. Bioprocess Biosyst. Eng. 2005;27:99-104.

15. Teodoro JC, Baptista-Neto A, Cruz-Hernandez IL, Hokka CO, Badino AC. Influence of feeding conditions on clavulanic acid production in fed-batch cultivation with medion containg glycerol. Appl. Microbiol. Biotechnol. 2006;72:450-55.

16. Teodoro JC, Baptista-Neto A, Araujo MLGC, Hokka CO, Badino ACInfluence of glycerol and ornithine feeding on clavulanic acid production by *Streptomyces clavuligerus*. Braz. J. Chem. Eng. 2010;27:499-506.

17. Badino AC, Facciotti MCR, Schmidell W. Estimation of the rheology of glucoamylase fermentation broth from the biomass concentration and shear conditions. Biotechnol. Tech. 1999;13:723-26.

18. Adams CJ, Boult CH, Deadman BJ, Farr JM, Grainger MN, Manley-Harris M, Snow MJ. Isolation by HPLC and characterisation of the bioactive fraction of New Zealand manuka (*Leptospermum scoparium*) honey. Carbohydr. Res. 2008;17:651-659.

19. Foulstone M, Reading C. Assay of amoxicillin and clavulanic acid, components of augumentin, in biological fluids with performance líquid cromatography. Antimicrob. Agents. Chemother. 1982;22:753-62.

20. Baumberg S, Krügel H, Noack D. Genetics and product formation in Streptomyces, Berlim: Springer; 2012.

21. Ichikawa T, Date M, Ishikura T, Ozaki A. Improvement of kasugamycin-producing strain by the agar piece method and the prototroph method. Folia Microbiol. 1971;16:218-224.

22. Neto AB, Hirata DB, Cassiano Filho LCM, Bellão C, Badino Júnior AC, Hokka CO. A study on clavulanic acid production by Streptomyces clavuligerus in batch, fed-batch and continuous processes. Braz. J. Chem. Eng. 2005; 22:557-563.

23. Korbekandi H, Darkhal P, Hojati Z, Abedi D, Hamedi J, Pourhosein M. Overproduction of Clavulanic Acid by UV Mutagenesis of *Streptomyces clavuligerus*. Iran. J. Pharm. Res. 2010;9:177–81.

24. Kizildogan AK, Jaccard GV, Mutlu A, Sertdemir İ, Özcengiz G. Genetic engineering of an industrial strain of *Streptomyces clavuligerus* for further enhancement of clavulanic acid production. Turkish. J. Biol. 2017;41:342–53.

25. Mayer, AF, Deckwer WD. Simultaneous production and decomposition of clavulanic acid during *Streptomyces clavuligerus* cultivations. Appl. Micr. Biotech. 1996;45:41-46.

26. Chen K, Lin Y, Tsai C, Hsieh C, Houng J. Optimization of glycerol feeding for clavulanic acid production by *Streptomyces clavuligerus*. Biotechnol. Lett. 2002;24:455-58.

27. Hoff G, Bertrand C, Piotrowski E, Thibessard A, Leblond P. Genome plasticity is governed by double strand break DNA repair in Streptomyces. Sci. Rep. 2018;8:1-11.

28. Volff JN, Altenbuchner J. Genetic instability of the Streptomyces chromosome. Mol. Microbiol. 1998;27:239-246.